The Spectroscopic Characterization of Mitochondrial Porin in Membrane Mimetic Systems

by

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Thesis Abstract

Voltage-dependent anion-selective channels (VDAC), or mitochondrial porins, regulate the flow of metabolites across the mitochondrial outer membrane. They presumably span the membrane as β-barrels, but the residues forming the individual β-strands are unknown. This information is essential for understanding the structure and function of the protein. Using *Neurospora* VDAC as a template, published data were reassessed to delineate a unified model for porin structure Bay and Court 2002, which was subsequently refined in collaboration with Greg Runke Runke *et al.* 2006.

The focus of this work was the development and analysis of systems for maintaining high levels of folded porin for the acquisition of high resolution data needed for model testing. The conformation of hexahistidinyl-tagged *Neurospora* porin in detergent was probed by fluorescence, near-UV circular dichroism and ultraviolet absorption spectroscopy. Derivatives of tryptophan and tyrosine were also examined by fluorescence spectroscopy and UV absorbance spectroscopy to model the interactions between the detergents and the amino acid side chains in the protein. Detergent-specific levels of β-strand and tyrosine exposure were observed. In all cases, the two tryptophan residues reside in weakly asymmetric, hydrophobic environments, suggesting transient tertiary interactions. Porin solubilized in these detergents forms functional channels in liposomes and membrane insertion is accompanied by increased levels of β-strand and loss of protease sensitivity.

These data were used to develop mixed detergent folding systems. A mixture of SDS and dodecyl-β-D-maltopyranoside (DDM) supports a β-strand rich conformation at high protein concentrations. The tertiary contacts and protease resistance of the SDS/DDM-

solubilized porin are very similar to those of the protein following reconstitution into liposomes.

Finally, the role of sterols in porin folding was examined, as the addition of sterols to detergent-solubilized VDAC is required for channel formation in artificial membranes. Sterols do not alter the secondary structure of VDAC, and subtle alterations to tertiary interactions were detected, suggesting that sterols do not promote an insertion-competent structure, but rather facilitate insertion into artificial bilayers. In summary, this analysis of the folded states of detergent-solubilized porin has revealed a system that maintains high concentrations of mitochondrial porin in a state that is very promising for structural studies.

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Runke, G., E. Maier, et al. 2006. Biophysical Journal 90 (9): 3155-64 with permission from the Biophysical Journal

List of Abbreviations

Å	angstrom
α	tyrosine exposure
αNcPor-N	rabbit antibodies directed against residues 7- 20 of *N. crassa* mitochondrial porin
$A_{280\ nm}$	absorbance at 280 nm
$A_{400\ nm}$	absorbance at 400 nm
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATR-FTIR	attenuated total reflection-Fourier transform infrared spectroscopy
BAK	Bcl-2-antagonist/killer protein
BAX	Bcl-2-associated protein X
Bcl-2	B-cell lymphoma protein 2
Bcl-XL	B-cell lymphoma protein XL
Bid	BH3 interacting domain protein
BLB	black or planar lipid bilayers
BOC-Y-OMe	N-t-butyloxycarbonyl-L-D-tyrosine-methyl ester
Cα	alpha carbon
CCA	convex constraint algorithm
CD	circular dichroism spectropolarimetry
CDL40	tumor necrosis factor (ligand) superfamily member 5
cDNA	reverse transcribed messenger ribonucleic acid into deoxyribonucleic acid
CMC	critical micelle concentration
cm	centimetre
CT	chymotrypsin
C-terminus	carboxyl terminus
CTXIII	cardiolipotoxin analogue III protein
DEC	dielectric constant
deg	degree
DDM	dodecyl-β-D-maltopyranoside
DNA	deoxyribonucleic acid
dmol	decimole
DPC	dodecyl phosphocholine
ε	extinction co-efficient
EDTA	ethylenediamine-tetraacetic acid
EG	ethylene glycol
FhuA	ferrichrome-iron uptake porin A
FT	freeze-thaw
g	gram
g	gravity
GLK	glycine-leucine-lysine
GFP	green fluorescent protein
H^1-NMR	proton nuclear magnetic resonance spectroscopy

His$_6$-porin	hexahistidinyl tagged recombinant *Neurospora crassa* mitochondrial porin
HsVDAC	*Homo sapien* voltage-dependant anion channel
HT	high tension
IM	inner membrane
I$_{M1}$	OmpA first membrane bound folding intermediate
I$_{M2}$	OmpA second membrane bound folding intermediate
I$_{M3}$	OmpA third membrane bound folding intermediate
IMS	innermembrane space
INS	insertion
Int$_{max}$	fluorescence intensity at λ_{max}
I$_w$	OmpA water-soluble intermediate
kb	kilobase
kDa	kiloDalton
L	litre
LamB	lactam activated maltoporin B
LDAO	lauryl dimethylamine oxide
λ	wavelength
λ_{ex}	excitation wavelength
λ_{max}	wavelength at which fluorescence emission or ellipticity is at maximum value
λ_{max}Tyr	wavelength at which tyrosine-derived fluorescence emission is at maximum value
λ_{max}Trp	wavelength at which tryptophan-derived fluorescence emission is at maximum value
λ_{min}	wavelength at which ellipticity is at minimum value
LPS	lipopolysacharide
LUV	large unilamellar vesicle
M	molecular weight
mdeg	millidegree
min	minute
μg	microgram
mg	milligram
μL	microlitre
mL	millilitre
MLV	multilamellar vesicles
μM	micromolar
mM	millimolar
MRE	mean residue ellipticity
M	molar
mV	millivolt
N	OmpA native folded state
NADH	nicotinamide adenine dinucleotide
NAc-Y-NH$_2$	N-acetyl-tyrosine amide
NAc-W-NH$_2$	N-acetyl-tryptophan amide
NAc-W-OEth	N-acetyl-L-tryptophan ethyl ester

NcVDAC	*Neurospora crassa* voltage-dependant anion selective channel
Ni-NTA	nickel nitrolotriacetic acid
nm	nanometre
nM	nanomolar
NMR	nuclear magnetic resonance spectroscopy
nS	nanoSeimens
N-terminus	amino terminus
OG	*n*-octyl-β-D-glucopyranoside
OM	outer membrane
Omp85	protein import channel outer membrane porin 85
OmpA	outermembrane porin A of *Escherichia coli*
OmpF	outermembrane porin F of *Escherichia coli*
OmpX	outermembrane porin X of *Escherichia coli*
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidyl choline
PhoE	phoshoporin E of *Escherichia coli*
PMSF	Phenyl methylsulphonyl fluoride
PorA	*Neisserial meningitidis* porin A
PorB	*Neisserial meningitidis* porin B
pS	picoSiemen
PTP	permeability transition pore
θ	ellipticity
r	ratio of the depths of the tryptophan-derived fluorescence minima near 340 nm and 359 nm in SDF plots
RMSD	root mean square deviation
s	second
ScVDAC	*Saccharomyces cerevisiae* voltage-dependant anion channel
SDUV	second derivative of UV absorption spectrum
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDS-UREA-PAGE	sodium dodecyl sulphate-urea-polyacrylamide gel electrophoresis
Sec	bacterial protein secretory complex in inner membrane
SEM	sorbitol, ethylenediamine-tetraacetic acid, MOPS buffer
SON	sonication
SUV	small unilamellar vesicle
T	trypsin
TBS	tris buffered saline
TM	transmembrane
TNF-α	tumor necrosis factor portein-α
TOM	translocase of the outer membrane
Tob	Topology of the outer membrane β-barrel protein
TROSY-NMR	transverse relaxation optimized spectroscopy-nuclear magnetic resonance spectroscopy
Trp	tryptophan
Tyr	tyrosine
U	OmpA unfolded state

UV	ultraviolet
VDAC	voltage-dependant anion selective channel
VDAC1	voltage-dependant anion selective channel isoform 1
VDAC2	voltage-dependant anion selective channel isoform 2
VDAC3	voltage-dependant anion selective channel isoform 3

CHAPTER 1 Literature review

Some of the material presented in this literature review has been published previously, and has been updated for the purpose of this review. In particular, sections 1.1 to 1.6.0, Table 1.1, and Figures 1.2-1.4 are taken from (Bay, D. C. and D. A. Court. 2002. Biochemistry and Cell Biology. 80 (5): 551-562) with permission from the NRC Press.

Sections 1.6.1 to 1.6.7 and Figure 1.4 panel D also contain material published in (Runke, G. Maier, E. Summers, W. A. Bay, D. C. Benz, R. Court, D. A. 2006. Biophysical Journal. 90 (9): 3155-3164), with permission from the Biophysical Journal. My contributions to this work were the construction of the genes encoding three porin variants studied, work on circular dichroism and fluorescence assays with W.A.T. Summers, and participation in figure generation and manuscript writing.

1.1 Mitochondrial porin function

The mitochondrial outer membrane (OM) is a semi-permeable barrier between the mitochondrion and the cytosol that allows the intracellular exchange of molecules, including products and substrates for the electron transport chain and oxidative phosphorylation, cofactors for mitochondrial enzymes, nucleotides for RNA and DNA synthesis, and cytoplasmically synthesized proteins. While mitochondrial protein transport across the OM depends on the TOM complex (translocase of the outer membrane; reviewed in Paschen and Neupert 2001), the remaining molecules traverse the membrane through water-filled channels formed by members of a family of small (around 30 kDa) pore-forming molecules. These proteins have been labelled voltage-dependent anion-selective channels (VDAC) (Colombini 1980), or mitochondrial porins (Freitag *et al.* 1982), by analogy with the pore-forming proteins in bacterial OMs.

Pore-forming proteins were first isolated from the mitochondrial OM of *Paramecium aurelia* by Schein and colleagues (1976). These proteins were introduced into "black lipid bilayers", planar artificial membrane systems in which the conductive properties and ion selectivity of transmembrane (TM) channels can be determined. The resulting pores displayed what are now recognized as the classical features of mitochondrial porin. First, insertion of each channel into the membrane created a distinct increase in conductivity across the lipid layer. The conductivity of the channels could be decreased upon the application of voltage of at least 20 mV across the membrane. This voltage-dependent gating converted the pores from an open state of about 4 nanoSiemens (nS) conductivity to a partially closed state of less than 2 nS. In the open state, the pores were slightly anion selective, while in the closed state, they were more cation selective.

Multiple isoforms of porin have been detected in many organisms, particularly in fungi (for example Blachly-Dyson *et al.* 1997), insects (for example, Aiello *et al.* 2004, Graham and Craigen 2005) vertebrates (for example, Blachly-Dyson *et al.* 1993, Blachly-Dyson *et al.* 1994, Sampson *et al.* 1996, Sampson *et al.* 1997, Xu *et al.* 1999, Messina *et al.* 1999, Cesar Mde and Wilson 2004) and plants (for example, Heins *et al.* 1994, Elkeles *et al.* 1995, Abrecht *et al.* 2000, Al Bitar *et al.* 2003, Wandrey *et al.* 2004, also see Appendix 1 (Young, Bay, Hausner and Court, submitted)). Where channel forming activity has been assayed, at least one isoform forms pores with the features characteristic of classical VDACs. These isoforms generally are referred to as VDAC1 or porin (Blachly-Dyson *et al.* 1993), and unless otherwise indicated, these VDAC1s are the focus of the following discussion.

VDAC interacts with various substrates, such as ATP (Rostovtseva and Colombini 1996) and NADH (Zizi *et al.* 1994), and soluble and membrane-bound polypeptides. Proteins

such as hexokinase and creatine kinase have enhanced access to substrates by complexing with VDAC (Brdiczka *et al.* 1990; Brdiczka 1990, Brdiczka *et al.* 1994). VDAC and the adenosine nucleotide transporter form a complex at the contact sites between inner and outer membranes (Beutner *et al.* 1996) and with the addition of an 18-kDa ligand binding protein form the peripheral benzodiazepine receptor (McEnery 1992 see Fig. 1.1).

One of the most intriguing roles of porin is its proposed participation in the initiation of apoptosis. VDAC, the ADP/ATP carrier of the inner membrane, and cyclophilin D comprise the large permeability transition pore (PTP, Crompton *et al.* 1998, Woodfield *et al.* 1998). Interactions of VDAC with pro and anti-apoptotic members of the Bcl-2 family, appear to regulate the PTP, but the mechanism is currently unresolved. Shimizu and colleagues (Shimizu *et al.* 1999, Shimizu *et al.* 2000, Shimizu *et al.* 2000) propose that the pro-apoptotic protein Bax interacts with VDAC to form the PTP through which cytochrome *c* can be released, thereby triggering apoptosis. This interaction has not been replicated by Rostovstesa *et al.* (Rostovtseva *et al.* 2004) who have detected pore closure induced by the pro-apoptotic protein Bid, leading to the hypothesis that release of cytochrome *c* results from rupture of the mitochondrial membrane. Alternatively, Vander Heiden *et al.* (Vander Heiden *et al.* 2001) have evidence supporting an interaction between Bcl-XL and VDAC that promotes increased permeability of the outer membrane. Furthermore, interactions between an isoform of mammalian porin, VDAC2, and BAK, but not BAX, may regulate apoptosis by increasing

Figure 1.1 Cartoon diagram summarizing mitochondrial porin interactions with various compounds and proteins. Mitochondrial porin (blue cylinder) within the outer membrane is shown to interact with a variety of proteins and compounds from the cytosol, intermembrane space, inner membrane and matrix of the mitochondrion. Interacting proteins shown are as follows, hexokinase (purple circle) Bid (light pink rectangle), Bax (green oval), mitochondrial benzodiazepine receptor (green oval), ATP/ADP nucleotide carrier (red rectangle), cytochrome c (black circles), and VDAC modulator (light blue oval) and compounds ATP (pink oval), NADH (brown oval) and cyclophilin D (light blue oval). Small black and white filled circles represent small anionic and cationic species that diffuse through porin.
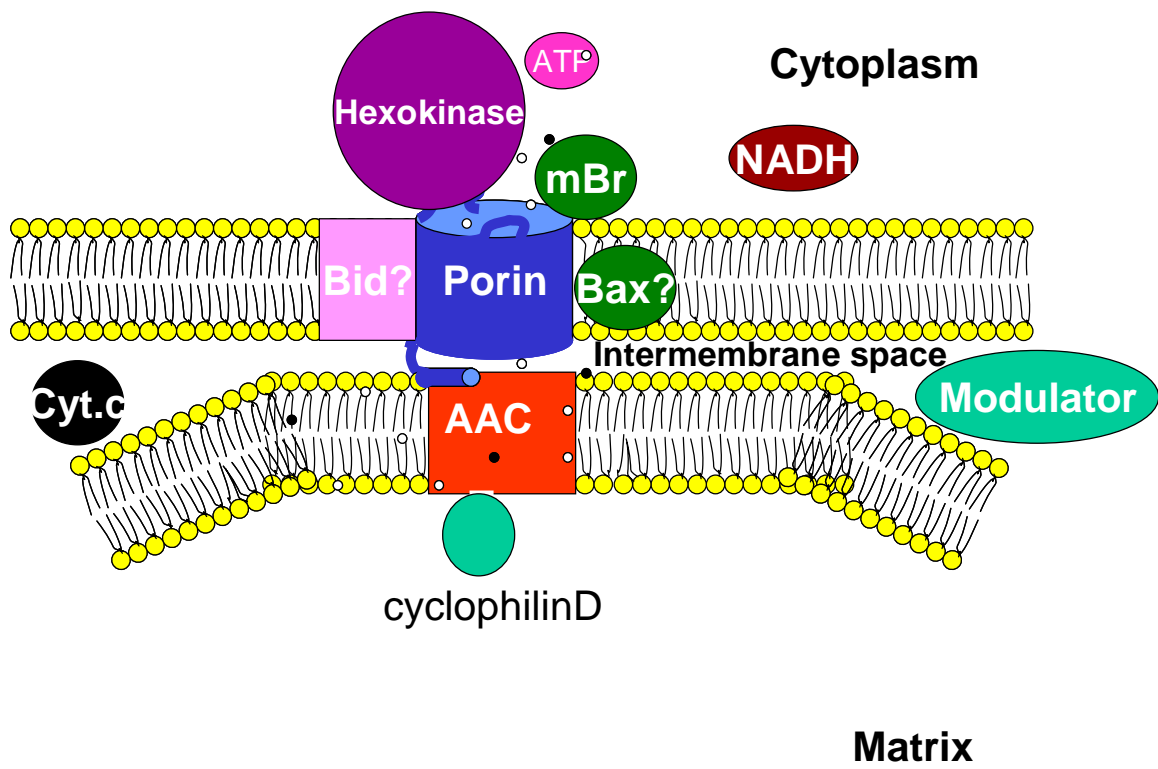
the susceptibility of BAK to proteolysis, thereby downregulating apoptosis (Cheng *et al.* 2003). Finally, cytochrome *c* release from porin-containing vesicles lacking Bcl-family proteins has been demonstrated, suggesting that porin oligomerization is responsible for PTP formation (Zalk *et al.* 2005). VDAC interactions with other compounds such as reactive oxygen species (Madesh and Hajnoczky 2001), may play an important role in regulating apoptosis by controlling the release of cytochrome *c* from the inter membrane space and may also act as a global regulator for the cell (as reviewed by Kim *et al.* 2006, Shoshan-Barmatz *et al.* 2006, Lemasters and Holmuhamedov 2006 see Fig.1.1).

From the first reports of the primary sequence of VDAC (Mihara and Sato 1985, Kleene *et al.* 1987), there has been great interest in the topology of the protein across the membrane. An understanding of the structure of the pore is essential for further investigation of its roles in protein complexes and of the mechanisms by which it gates and generates the characteristic anion selectivity. A variety of predictive and experimental methods have led to the conclusion that VDAC forms a β-barrel in the OM. A large body of data has been obtained to elucidate the precise structure of the barrel; experiments have used VDAC from several species, in environments ranging from detergent micelles to intact mitochondria. In this review, I will apply these results to VDAC from *Neurospora crassa* in an attempt to generate a unified model.

This review will also examine mitochondrial and bacterial porin folding both *in vivo* and *in vitro*, which is essential information for preparing the mitochondrial protein in forms amenable to structural analysis.

1.2 Bacterial porins: conceptual models for VDAC

Bacterial porins have served as references for pore forming mitochondrial proteins that display high β-strand content, including the OM protein import pore (TOM40; Künkele *et al.* 1998) and VDAC, as will be discussed herein. Bacterial porins are useful for comparison due to the availability of a large number of high-resolution crystal (for example Weiss *et al.* 1991, Cowan *et al.* 1992, Cowan *et al.* 1995, Ferguson *et al.* 1998, Pautsch and Schulz 1998, Forst *et al.* 1998, Przybylski *et al.* 1996) and NMR (for example Fernández *et al.* 2001, Fernández *et al.* 2001, Arora *et al.* 2001, Tamm *et al.* 2003, Fernández *et al.* 2004) structures from members of each of the six bacterial porin families. Bacterial porin structure will be summarized briefly; for in-depth reviews of these structures, see Jap and Walian (1996) and Koebnik *et al*. (2000).

X-ray diffraction of bacterial porins reveals β-barrel channels (see Figs. 1.2A and 1.2B) that are composed of antiparallel amphipathic TM β-strands tilted at angles between 35 and 60° (Jap and Walian 1996, Koebnik *et al.* 2000). The β-barrels are composed of 8–24 TM β-strands that are present in multiples of two to satisfy hydrogen bonding requirements and twisting stresses (Murzin *et al.* 1994, Jap and Walian 1996, Koebnik *et al.* 2000). The typical bacterial porin surface diameter is 3.4 nm (*Escherichia coli* OmpF and PhoE and *Rhodobacter capsulatus* porin), but the pore diameter decreases at various points throughout the channel due to the presence of loops within the barrel (Fig. 1.2B), restricting transport to molecules of 600 Da or less (Jap and Walian 1996). Some bacterial β-barrel proteins are substrate specific, including LamB, a maltose

Figure 1.2 The β-barrel of OmpF, as determined by X-ray diffraction (Cowan *et al.* 1992).

A) Side and **B**) top views were obtained from files S3D00196 and S3D00195, respectively, from SWISS-3DIMAGE, accession No. P02931. In Fig. 1.1**A**, the extracellular face is on the top of the figure. In these diagrams, β-strands are indicated by thick ribbons and loops and turns by narrow cords. In Fig. 1.1**B**, note latching loop 2 extending away from the "left"side of the barrel and loop 3, which rests inside the lumen.

C) Sample CD spectrum, generated by D. Bay, of recombinant NcVDAC solubilized in 1% Genapol X-80. The spectrum was obtained as described in Runke *et al.* (2000) and deconvoluted using the convex constraint algorithm (CCA; Perczel *et al.* 1992) and the membrane protein data set of Park *et al.* (1992). The experimental data (black curve) and the deconvoluted data representing α-helix (red curve), β-strand (green curve), and random coil (blue curve) components are presented. Note that the experimental and β-strand curves are very similar in the diagnostic regions of 190–200 and 215–220 nm, indicative of the high β-strand content of NcVDAC. **D**) Schematic representation of two-dimensional NcVDAC arrays. This model is based on the data presented by Guo and Mannella (1993). Numbered open circles indicate low-density lumens of channels arranged in a six-member array; each array is identified by numbers of the same colour. Electron-dense protein arms extending between channels in adjacent arrays are indicated by pairs of yellow arrowheads aligned along the length of the arm or by single arrowheads directed away from the central array. Reproduced from Bay and Court (2002), with permission from NRC press.

A   Matrix Porin (*E. coli*)

B   Matrix Porin (*E. coli*)

C

D
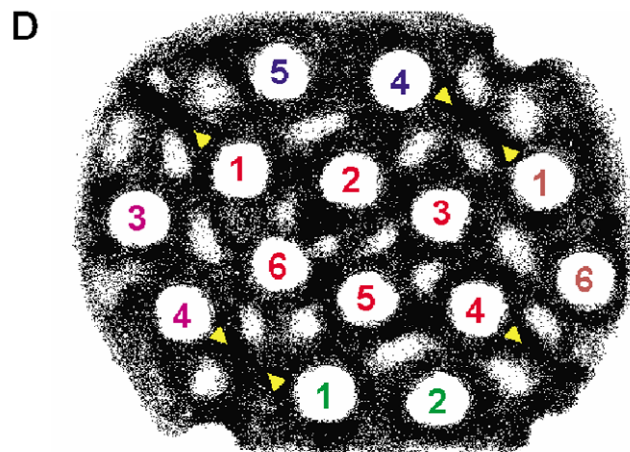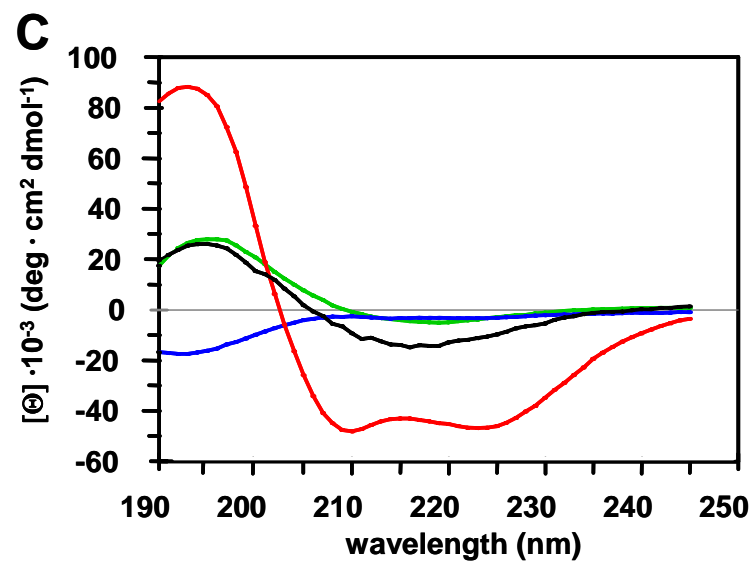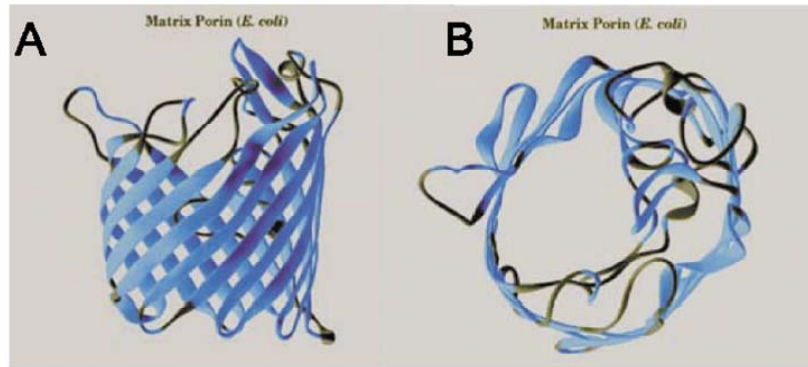
and maltodextrin uptake porin, and FhuA, an iron–siderophore complex uptake channel. In contrast, OmpF is a general diffusion pore (Koebnik *et al.* 2000).

The β-strands in bacterial porins are connected by either short turns or long extracellular loops (Fig. 1.2), with each loop playing a specific role in channel gating, ion selectivity, size restriction, or structural support. Loop 3, for example, folds into the porin lumen in OmpF (Fig. 1.2B), creating a network of salt bridges and hydrogen bonding contacts to the barrel. In this constriction zone, charged residues contribute to the ion selectivity of the channel. Alterations to salt bridges and hydrogen bonding regions in loop 3 have been speculated to cause channel gating, making this loop a crucial structure for porin function (Koebnik *et al.* 2000). Loop 2, the shorter "latching" loop in OmpF, plays a more structural role by linking adjacent porin monomers together through hydrogen bonding and hydrophobic interactions leading to the formation of a trimer (Jap and Walian 1996, Koebnik *et al.* 2000).

Highly stable homotrimers, such as those of LamB and OmpF, are the characteristic quaternary structures of bacterial porins, making the OM resilient to changes within a harsh environment. Experimentally, porin trimers are resistant to denaturants such as 5 M guanidium hydrochloride and 2% sodium dodecyl sulfate (SDS) at 70°C. In addition, monomeric β-barrel proteins, such as OmpA, OmpX, and FhuA, and homodimers, such as phospholipase A (PldA), have also been identified (Koebnik *et al.* 2000). Mitochondrial porin homo-oligomers have been demonstrated by SDS-polyacrylamide gel electrophoresis (PAGE) of "water soluble" porin (Pfaller *et al.* 1985) and by chemical cross-linking of porin in artificial bilayers (Zalk *et al.* 2005). However, its native oligomeric state within the OM

has not been identified, and it has been suggested to vary depending on cellular conditions (as reviewed by Shoshan-Barmatz *et al.* 2006).

Critical differences exist between mitochondrial and bacterial porins. Mitochondrial porins are more readily denatured by urea (Popp *et al.* 1997), heat, and detergents like SDS (Shao *et al.* 1996). In addition, the environments of the bacterial and mitochondrial OMs are very different, perhaps placing different constraints on pore structure. Finally, as discussed in this review, the presence of a short amino-terminal α-helix is unique to the mitochondrial porins. In spite of these differences, bacterial β-barrel proteins do provide a conceptual framework for the analysis of mitochondrial porin structure.

1.3 Circular dichroism spectropolarimetry of native and recombinant VDAC

Circular dichroism (CD) spectropolarimetry studies of native VDAC (isolated directly from mitochondrial OM) or of recombinant VDAC (prepared from *E. coli* over-expressing VDAC cDNA) support the hypothesis that VDAC spans the membrane as a series of β-strands (Mihara and Sato 1985, Kleene *et al.* 1987, Forte *et al.* 1987) (see Fig. 1.2C and Table 1.1 and references therein). VDAC from *N. crassa* and *Saccharomyces cerevisiae* have been examined in detail using this method. Native VDAC resuspended in a variety of detergents and reconstituted into artificial phospholiposomes generates CD spectra indicative of high β-strand content in the range of those of bacterial porins (30–70%; Table 1.1). Within the limits of this method, the β-strand content of recombinant "His$_6$-tagged" porin is the same as that of the native protein, demonstrating that these forms are comparable with those isolated from mitochondria and therefore will be very useful experimental tools (Popp *et al.* 1996, Koppel *et al.* 1998).

Table 1.1 Secondary structure content of bacterial porins and VDAC estimated from circular dichroism, X-ray diffraction and ATF-FTIR. All CD data presented were collected in the far-UV region.

Porin/VDAC	Secondary Structure (%)			References
	β-strand	α-helix[¶]	other[*]	
N. crassa				
VDAC[(native)]	$62^{a\S}$, $63^{b\S}$,$45^{b\#}$	$30^{a\S}$, $7^{b\S}$,$12^{b\#}$	$8^{a\S}$,$31^{b\S}$,$37^{b\#}$	Shao *et al.* 1996
				Koppel *et al.* 1998
pH 4-4.5	$38^{a\S}$,$30^{b\#}$	$28^{a\S}$,$27^{b\#}$	$34^{a\S}$,$45^{b\#}$	Shao *et al.* 1996
				Koppel *et al.* 1998
$65^{o}C$ ^{a§}	0	30	70	Shao *et al.* 1996
His$_6$VDAC	$45^{b\#}$,$47^{c¥}$,$22^{b¤}$	$12^{b\#}$,$31^{c¥}$,$28^{b¤}$	$37^{b\#}$,$22^{c¥}$,$51^{b¤}$	Koppel *et al.* 1998
				Runke et al. (2000)
residues 1-20	--	$40\text{-}50^{£}$,$30^{Þ}$, $0^{ϵ}$	--	Guo *et al.* 1995
S. cerevisiae				
VDAC1[(native)] $^{b\#}$	30	27	37	Koppel *et al.* 1998
His$_6$VDAC1$^{b\#}$	35	20	45	Koppel *et al.* 1998
Pea root plastid				
His$_6$porin^{b}	$70^{¥}$,$10^{¤}$,$25^{ϵ}$	$20^{¥}$,$40^{¤}$,$35^{ϵ}$	$10^{¥}$,$50^{¤}$,$40^{ϵ}$	Popp *et al.* 1996
Phaseolus vulgaris - ATR-FTIR				
VDAC31$^{¥}$	50	14	39	Abrecht *et al.* 2000
VDAC32$^{¥}$	53	9	46	
Rb. capsulatus porin				
CD	70^{a},29^{b},30^{c}	18.5^{a},12^{b},27^{c}	11.5^{a},40^{b},44^{c}	Park *et al.* 1992
X-ray diffraction	57	6	37	Weiss *et al.* 1991

[*]Includes values for β-turn secondary structures
[¶]Includes values for α_τ-helix secondary structures.
CD spectral data deconvoluted by:
  [a]Yang's method (Chang *et al.* 1978, Yang *et al.* 1986)
  [b]Variable Selection method (Manavalan and Johnson 1987, Johnson 1990),
  [c]Convex Constraint Algorithm method (Perczel *et al.* 1992).
CD spectra collected in:
 [§]2% octyl-β-glucoside (β-OG) pH 7,
 [#]1% lauryl dimethylamine oxide (LDAO) pH 8,
 [¥]1-2% Genapol X-80 pH 7,
 [£]trifluoroethanol,
 [Þ]methanol,
 [ϵ] water,
 [¤]1% sodium dodecyl sulfate (SDS)
Reproduced from Bay and Court 2002 with permission from the NRC press.

In contrast with the high overall β-strand character of porin, a synthetic peptide encoding the first 20 residues of *N. crassa* VDAC (NcVDAC) displays a high α-helical content (Guo *et al.* 1995; Table 1.1), as predicted (Mihara and Sato 1985, Kleene *et al.* 1987, Forte *et al.* 1987). In this respect, VDAC differs significantly from some bacterial porins, in which the N-terminus forms part of a β-strand (reviewed in Jap and Walian 1996).

Unfortunately, the CD data are not of sufficient precision to be used alone in creating or testing secondary structural models. There are several factors that contribute to variability in the percentage of β-strand detected. First, there are differences that result from the choice of detergent used to solubilize these membrane proteins in aqueous solution. For example, the structure of native VDAC from *Neurospora* is estimated to contain 63% β-strand when in OG but only 45% when the protein is in lauryl dimethylamine oxide (LDAO) (Table 1.1). This observation highlights the uncertainty regarding the ability of different detergents to mimic conditions in the OM. In addition, many detergents tend to interfere with data collection in the far-UV region, limiting the precision of the method. A more biologically-relevant system involves VDAC reconstitution into phospholiposomes, where protein function and structural composition can be determined (Shao *et al.* 1996). However, as with detergents, liposomes are not suitable for secondary structure estimations from CD spectra, due to light scattering at wavelengths below 200 nm and the low amounts of protein that can be incorporated within the membranes (Shao *et al.* 1996, Bathori *et al.* 1993).

Predicted secondary structure compositions also vary with the deconvolution methods and reference data set used. For example, the α-helical content of NcVDAC folded in OG varies from 7 to 30%, depending on the deconvolution algorithm (Table 1). Furthermore, as demonstrated for experiments involving *R. capsulatus*, the β-strand content determined by

CD analysis (30–70%; Park *et al.* 1992) does not precisely reflect the data obtained by X-ray diffraction (57%; Weiss *et al.* 1991).

In spite of these difficulties, CD is extremely valuable for comparing porins with respect to structural stability under different conditions (Shao *et al.* 1996, Koppel *et al.* 1998, this thesis). For example, experiments involving fungal VDAC reconstituted in OG or liposomes demonstrated a reversible loss of β-strand content and an increase in α-helical content at low pH (Table 1.1). In addition, this approach may provide information about conformational changes that can occur within the channel that may contribute to gating.

In summary, although CD spectropolarimetry is a valuable technique for obtaining general structural information, it fails to provide the precise data needed to construct an accurate model of VDAC. However, it is an excellent technique for examining conformational changes in VDAC under various conditions.

1.4 Electron microscopy of two-dimensional VDAC arrays

Two-dimensional VDAC arrays suitable for electron cryomicroscopy can be produced by phospholipase A2 treatment of isolated OM fractions (Mannella and Frank 1984, Mannella 1989). Electron microscopic analysis of NcVDAC arrays at 1–3 nm resolution shows unit cells composed of six VDAC, with protein extensions between channels in adjacent unit cells (Guo and Mannella 1993) (see Fig. 1.2D). Distinct pore-like structures are observed in which circular, stain-filled lumens of low density are surrounded by high-density protein walls (Mannella and Frank 1984, Mannella 1989). These analyses did not reveal the structural details of the channels but did show an irregular topology of the pore wall at the membrane surface, including slight extensions past the barrel wall (Mannella 1989, Thomas *et al.* 1991). Modelling of the NcVDAC Cα backbone predicts a lumen diameter of 2.8 ± 0.1

nm with a ring thickness between 0.3 and 0.5 nm, which is in agreement with the diameters

of 3–4 nm estimated by liposome swelling assays and 2 nm as determined by single channel

conductance experiments in artificial bilayers (Colombini 1980, Freitag *et al.* 1982). It should

be noted that the latter values are approximations, as they were calculated with the

assumption of a perfectly cylindrical pore.

Subsequent attempts to refine the two-dimensional arrays of NcVDAC in the presence

of amphipathic polyanions resulted in the loss of the protein "extensions" due to protein

lattice contractions in the membrane crystals suggesting that the proteins adopt a compact

conformation (Guo and Mannella 1993). It remains unclear whether the extensions are the

N-termini involved in protein–protein contacts between adjacent channels or regions of a

single pore that undergo conformational changes due to tighter channel packing (Mannella *et al.* 1992, Guo and Mannella 1993, Guo *et al.* 1995).

Three-dimensional analysis of the NcVDAC structure by applying Fourier

reconstruction methods to aurothioglucose-embedded crystal arrays again predicted grooves

and regions of uneven height and pore thickness and major protrusions of the pore above the

surface of the phospholipid membrane (Guo *et al.* 1995). An irregular pore surface has also

been visualized in phospholipid crystal arrays of human VDAC (HsVDAC). The pores in

these arrays are 5.1–5.8 nm in diameter, between 4.0 and 4.6 nm in height, with 0.3–0.4 nm

ring thickness (Dolder *et al.* 1999). These dimensions are slightly larger than those measured

in fungal VDAC arrays but were obtained at lower resolution (5 nm) (Guo *et al.* 1995, Dolder

et al. 1999). The use of phospholipids to construct the arrays led to membrane layer stacking

effects that resulted in altered orientations of channels within the (x, y) plane of the array,

making it difficult to resolve a higher quality structure of the channel (Dolder *et al.* 1999).

Refinements in crystallization techniques and the analytical approaches to the data for VDAC are being developed and are expected to result in higher resolution of the existing arrays (Verschoor *et al.* 2001). However, caution must be taken in drawing conclusions from these results, since VDAC was crystallized in alternating orientations and in an artificial membrane, not in mitochondrial OM fractions.

Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) was used to investigate the structure of *Phaseolus vulgaris* VDAC (Abrecht *et al.* 2000). In addition to providing independent estimates of β-strand content (Table 1.1), this method was able to demonstrate that the β-strands in the membrane are tilted at a 45° angle with respect to the axis of the barrel.

To date, X-ray diffraction (Cowan *et al.* 1992), Raman spectroscopy (Vogel and Jahnig 1986), and more recently solution state three-dimensional transverse relaxation optimized spectroscopy TROSY-NMR (Arora *et al.* 2001, Fernández *et al.* 2001, Fernández *et al.* 2004) of bacterial porins have been the best methods to precisely identify residues within TM regions and therefore should represent the next step in the structural analysis of VDAC.

1.5 What do predictive and experimental data tell us about the VDAC β-barrel?

Given the limitations of CD analysis and electron microscopy of VDAC arrays, other techniques such as primary sequence analysis, comparative algorithm-based computer analysis, immunolocalization methods, site-directed mutagenesis, and biotinylation experiments have been used to probe VDAC TM structure (see references in the following discussion).

There are a large number of deduced primary sequences for channels that display the characteristic properties (voltage dependency, anion selectivity) of VDAC (Mihara and Sato 1985, Kleene *et al.* 1987, Kayser *et al.* 1989, Troll *et al.* 1992, Ha *et al.* 1993, Blachly-Dyson *et al.* 1993, Blachly-Dyson *et al.* 1994, Heins *et al.* 1994, Fischer *et al.* 1994, Elkeles *et al.* 1995, Elkeles *et al.* 1997, Xu *et al.* 1999, Abrecht *et al.* 2000). These sequences show regions of alternating hydrophobic and hydrophilic residues, as expected for TM β-strands in a wall that surrounds an aqueous lumen (Benz 1994) (Fig. 1.3A). However, assignment of TM β-strands based solely on amphipathic sequences can be unreliable, as was demonstrated for bacterial porins (see Cowan *et al.* 1992).

Computer-based structural prediction algorithms have been developed and are based on comparisons of bacterial porin sequences with the corresponding resolved structures. Alignment of bacterial and eukaryotic porins by their hydropathy profiles has been one method of assigning TM regions in VDAC (Rauch and Moran 1994) (Fig. 1.3A, boxed residues, and Fig. 1.4A). By applying Kyte–Doolittle hydropathy index values in a moving window of residues, TM regions are predicted using a set of rules in which β-strands can be broken by residues with high potential for turn conformation (P, G, N, D, W) and the influence of strong turning residues is considered (see Rauch and Moran 1994, Fasman 1989, Kyte and Doolittle 1982). Using this approach, human, fungal (*S. cerevisiae*, *N. crassa*), and slime mold (*Dictyostelium discoideum*) VDAC were predicted to contain 16 TM β-strands (Fig. 1.4A) (Rauch and Moran 1994), reminiscent of OmpF (Figs. 1.2A and 1.2B) (Cowan *et al.* 1992).

A multiple alignment algorithm, the Gibbs sampler, has also been applied to VDAC primary sequences (Mannella *et al.* 1996). This technique determines residue frequencies in

motifs characteristic of TM β-strands in bacterial porin sequences of known structure and

then uses the derived bacterial motifs to screen mitochondrial membrane proteins. The

bacterial TM β-motif determined using the Gibbs sampler is an 11-residue sequence

comprising alternating polar and nonpolar residues at the lipid–water interface of the lumen

of the barrel. In this motif, charged residues are predicted to occur at high frequency at

positions 1 and 10, aromatic groups occur at positions 11 and, less frequently, 3 and 9, and

glycines are commonly found within central regions of the motif (positions 6 and 8)

(Mannella *et al.* 1996). Position 11 is notable, as these residues contribute to the "girdle" of

aromatic amino acids found at the interface between the outer leaflet of the membrane and

the aqueous surroundings (discussed in Koebnik *et al.* 2000). Significant matches were made

with bacterial motifs in three regions of yeast VDAC (ScVDAC); the corresponding regions

of NcVDAC are indicated in Fig. 1.3A. This method could not exclude the possibility that

more β-strands exist outside these regions, since it typically discovered fewer than half of the

β-strand sequences in bacterial porins of known structure (Mannella *et al.* 1996).

Another forecasting technique utilizes a Neural Network, trained on bacterial OM

proteins, to predict regions facing the periplasm and extracellular space as well as TM strands

(Diederichs *et al.* 1998). The Neural Network calculates z-coordinates of Cα backbone atoms,

in the (x, y, z) plane, from the true membrane protein sequence. Periplasmic turns and loops

have z values between 0.2 and 0.4 and extracellular loops have z values between 0.6 and 1.0

(Diederichs *et al.* 1998). We have used this program to analyze the NcVDAC primary

sequence, assuming that the cytosolic and intermembrane space (IMS) segments are

equivalent to extracellular and periplasmic regions in bacterial OM proteins. Excluding

Figure 1.3 A) *Neurospora crassa* VDAC primary sequence identifying experimentally examined regions of the protein and predicted TM β-strands. Boxed residues indicate β-strands predicted using the algorithm developed by Rauch and Moran (1994). Underlined amino acids highlight β-strands predicted by the hydropathy plot of (Benz 1994). Blue lines indicate β-strands estimated by the Gibbs sampler (Mannella *et al.* 1996). Red lines indicate β-strands predicted by the transFold web server (Waldispuhl *et al.* 2006). Pink lines define regions of VDAC bound by antibodies on the cytosolic side (cytosol+) or after OM lysis (cytosol–) from experiments performed by Stanley *et al*. (1995). Residues used in dual biotinylation studies (Song *et al.* 1998, Song *et al.* 1998) are labelled in red with the residue number of the second amino acid and its relative position (*cis/trans*) indicated in green below. **B**) Neural Network plot (Diederichs *et al.* 1998) predicting *N. crassa* VDAC secondary structure from its protein sequence. Peaks between *z* values of 1 and 0.6 indicate residues involved in β-turns or loops that face the cytosol, *z* values within 0.6–0.4 suggest regions that compose TM β-strands, and *z* values below 0.4 predict turns or loops facing the IMS. Residues at the apex of each strong peak or valley are labelled. **C**) Alignment of NcVDAC and ScVDAC primary sequences. Conserved residues are underlined in the yeast sequence. Residues indicated in red and green were replaced with amino acids of opposite charge (see text for details); red indicates replacements that did alter ion selectivity, while those shown in green did not (Blachly-Dyson *et al.* 1990, Peng *et al.* 1992, Thomas *et al.* 1993). Residue numbers are given above and below the NcVDAC and ScVDAC sequences, respectively. Reproduced from Bay and Court (2002), with permission from NRC press.

A

27   35   46

MAVPAFS_7DIAKSANDLLNKDFYH_{23}LAA GTIEVKSNT PNN_{38} VAFKVTGK STHDKVT_{53}

Cytosol -   cisT53   transT69
cisH23

56   63   68   74   80   87   90   97 100

SG ALEGKFTD KPNGL T_{69}VTQT WNTANA_{79} LETKVEMA DN LAKGLKAE GI FSFLPATN

cisS190   cisT53
transT53

108   116 119   126 130   143   152   160

ARGAKFNLH FKQSNFHGR AFFDL LKGP T_{135}ANIDAIVG HEGFLAGASAGYD_{156}VQKA

cisT53   cisT53

164   178   184   192 195   209   215

AITGYSAAVGYHAPTYS AAITATD NLSVFS_{190}ASYY HKVNSQVEA GS KAT WNSKTGN

cisT69   Cytosol -

216   229   238   241   249   257

TVGLEVATKYRIDP VSFVKGKIN DR_{240}GVAAIAYN VLLREGVTLGVGASFD_{264}TQKLD

cisT53   Cytosol +   cisT53

274   283

QATHKVGTSFTFES

Cytosol -

B

N. crassa VDAC

C
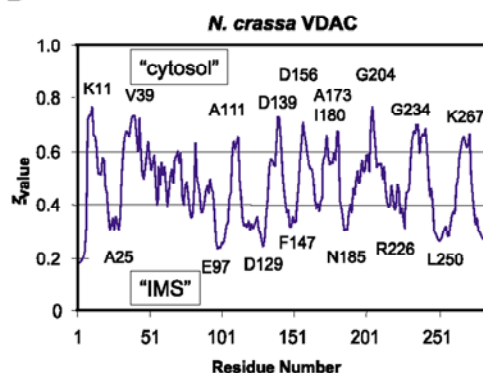
```
      1            11           21           31          41          51
Nc  MAVPAFSDIAKSANDLLN KDFY HLAAGT I E VKSNT P NNVAFKV TGK S –THDKVT SG

Sc  MS PPVYSDIS RNIN DLL N KDFY HAT PAA F DVQTT TANG I KFS LKA KQPVK DGP LST
      1            11           21          31          41          51          111

      61           71           81          91         101         111
Nc  ALEGKFT DKP NGLTVTQTWNTANALETKVEMADNLAKGLKAEGI FSFLPATNARGAK

Sc  NVEA KLNDK QTGLGL TQGWSNTNNLQT KLE F A N– L TP GL KNEL I TSLTPGV–A KSAV
      61           71           81          91         101         111

      121          131          141         151         161
Nc  FNLHFKQSNFHGRA F FDL– LKGPTANI DAI VG HEGFLAGA SAGYDVQKAA ITGYS A

Sc  LNTTFT E PF F TAR GAF DLCL K SPTF VGDLTMAHEG IVGGAEF GYD I SA GS ISRYAM
      121          131          141         151         161

      171          181          191         201         211
Nc  AVGYHAP T YSAA I TATDNL SVFS AS YYHKVN SQVEAG S KA T –WNSKTGN –TV GL

Sc  AL SYF AKD YS LGATLN–NEQ ITTV DF FQN VNAF LQVA – KA TMMNCKLPN SNVN I
      171          181          191         201         211

      221          231          241         251         261         271
Nc  EVATKYR IDPVSF VKGK INDRGVAAI AYNVLLRE GVTLGVGASFDTQKLD QATHKV

Sc  EF ATR YLPDASSQV KAKVSD SGI VTLAY KQLLR PGV TLGVGS SFDAL KLS E PVHKL
      221          231          241         251         261         271

      281
Nc  GT SFT FE S
Sc  GWSLS FDA
```

20

Figure 1.4 Models of NcVDAC. The models proposed by **A**) Rauch and Moran (1994) and **B**) Song *et al.* (Song *et al.* 1998) have been simplified to indicate the locations of residues discussed in the text. **C**) A proposed model of Bay and Court (2002) based on predictive and experimental evidence of *N. crassa* VDAC topology within the OM. The data were insufficient to predict structures for sections between T135-D156, S190-S211, and D264-S283; therefore, two possible conformations are presented and are indicated by question marks. **D**) The model of Runke *et al.* (2006) refining the predictions for the C-terminus region of NcVDAC. Deletions created by Runke *et al.* (2006) and by Popp *et al.* (1996) are shown by grey shaded regions. In all panels, cylinders indicate amino acids that compose the N-terminal α-helix. Arrows represent turns or loops between the indicated residues and TM β strands represented by rectangles. Red or circled residues indicate amino acids determined to be on the cytosolic side of the membrane to blue or squared residues using biotinylation assays (Song *et al.* 1998, Song *et al.* 1998) (see Fig. 1.3A). Small solid and open circles indicate residues shown by Blachly-Dyson *et al.* (1990) that either influence or do not influence the ion selectivity of the pore. Panels A-C were reproduced from Bay and Court (2002), with permission from NRC press and panel D was reproduced from Runke *et al.* (2006) with permission from the Biophysical Journal.

A

CYTOSOL

INTERMEMBRANE SPACE

B

CYTOSOL

INTERMEMBRANE SPACE

C

CYTOSOL

INTERMEMBRANE SPACE

D

CYTOSOL

INTERMEMBRANE SPACE
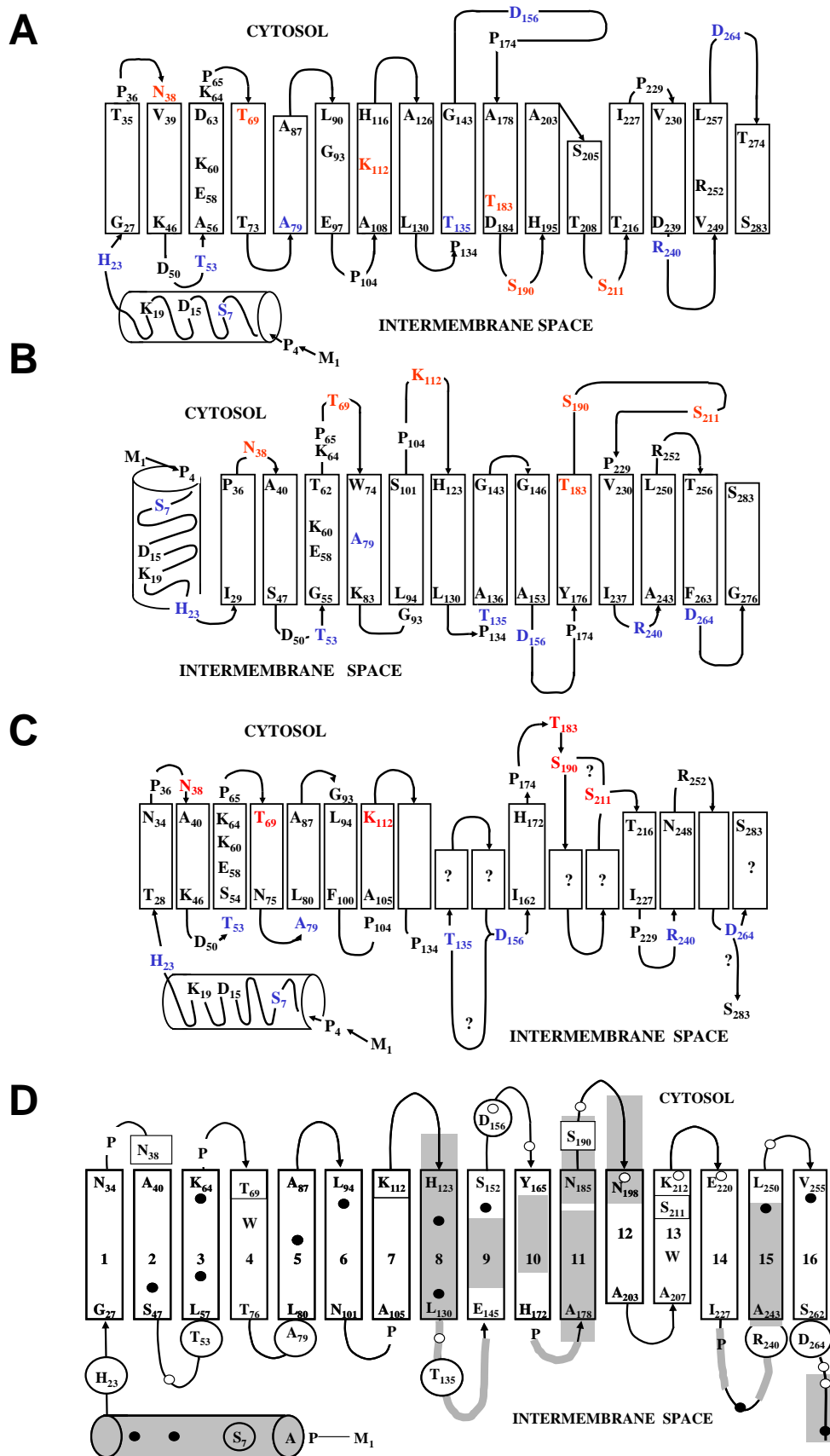
the C-terminus, where overlapping windows of sequence for analysis obviously do not exist, eight cytoplasmic and seven IMS loops are easily identified (Fig. 1.3B). This would suggest a β-barrel composed of at least 14 β-strands. This method of prediction may be better suited for identifying residues involved in turns or loops, since it cannot delineate individual β-strands in a long stretch of hydrophobic or amphipathic sequence, for example, residues 30–90 in NcVDAC (see Figs. 1.3A and 1.3B).

Another program referred to as transFold, has recently been developed to predict TM arrangement of β-barrel proteins and the interstrand residue interactions directly from primary sequence (Waldispuhl *et al.* 2006, Waldispuhl *et al.* 2006). Since integral β-barrel membrane protein structures are limited to bacterial outer membrane porins, this program uses pairwise interstrand residue statistical potentials derived from globular or cytoplasmic proteins to predict the secondary structure of TM β-barrel proteins (Waldispuhl *et al.* 2006). This approach differs from other available structure prediction programs because it does not require a training phase on known TM β-barrel structures, is the first to explicitly capture and predict long-range interactions, and can resolve smaller membrane proteins (≤ 200 residues) along with larger proteins. The web-version of transFold (Waldispuhl *et al.* 2006) was used to predict a β-strand TM topology of NcVDAC (Fig. 1.3A). Interestingly, this prediction algorithm identified a total of 18 potential TM β-strands, failed to identify the α-helical N-terminus, and instead predicted a β-strand in this region. In many cases this program also predicted β-turns of 2 residues, which are in poor agreement with bacterial porin β-turns that consist of generally three or more residues. 17 of the predicted 18 β-strands overlap with TM regions predicted using a simple hydropathy plot (Benz 1994), a feature that is part of the transFold algorithm (Waldispuhl *et al.* 2006, Waldispuhl *et al.* 2006). Thus, this new program

appears unable to predict a reasonable secondary structure arrangement for mitochondrial porins.

All of these prediction methods have three problems: (*i*) statistically, the prediction is only as good as the number of solved structures used for comparison, (*ii*) eukaryotic porins differ from most bacterial β-barrel proteins due to the presence of an α-helical N-terminus (Guo *et al.* 1995) and the lack of detectable loop regions within channel lumen (Mannella *et al.* 1992, Guo and Mannella 1993), and (*iii*) the predictions do not agree completely with experimentally obtained data determining the locations of residues in the OM, as discussed below.

1.6 Experimental contributions to the elucidation of VDAC structure

Experimentally obtained data from channel modifications, porin variants, or immunolocalization studies are critical to support or contradict secondary structure estimations for VDAC proteins. *Neurospora crassa* VDAC has been examined in the most detail (Stanley *et al.* 1995, Song *et al.* 1998, Song *et al.* 1998, Runke *et al.* 2006) due to the large amounts of intact mitochondria that can be easily isolated from this organism, facilitating isolation of the protein and *in organello* analysis. On the other hand, genetic tools for use in *S. cerevisiae* have been available for more than 10 years, allowing the *in vivo* expression of mutant VDAC genes that were generated by site-directed mutagenesis (Blachly-Dyson *et al.* 1990, Thomas *et al.* 1991, Peng *et al.* 1992, Zizi *et al.* 1995, Wandrey 2004). In the following sections, these methods will be briefly introduced, and a model of VDAC structure will be developed, based on the data accumulated for NcVDAC. Data obtained from yeast VDAC mutants will be discussed in relation to the *Neurospora* sequence, as most of the residues investigated in the yeast system are conserved in the *Neurospora*

protein (Fig. 1.3C). Caution must be taken when directly applying data obtained from the yeast system to *Neurospora* because not all residues are conserved and they may not share similar functions.

Site-directed mutagenesis has been used to generate yeast VDAC variants to identify residues involved in barrel or loop regions (Blachly-Dyson *et al.* 1990, Peng *et al.* 1992, Thomas *et al.* 1993). The underlying assumption in these studies is that replacing charged residues in the barrel, but not in exposed loops or turns, with ones of opposite charge should alter the ion selectivity of the channel.

Another approach to localizing particular residues utilizes cysteines in VDAC, either native residues in ScVDAC (Zizi *et al.* 1995) or those engineered into NcVDAC (Song *et al.* 1998, Song *et al.* 1998), which normally does not contain this amino acid. These cysteines can be covalently linked to biotin and the purified protein inserted into artificial bilayers. The effect of adding streptavidin, a biotin-binding moiety, to either the *cis* or *trans* side of the membrane, with respect to the side of porin addition, is then determined (Zizi *et al.* 1995). Assuming that most VDAC molecules assemble into the bilayer in the same orientation, the binding of the relatively bulky 60-kDa streptavidin molecule can only occur when the biotinylated cysteine residue is exposed to the side of the membrane to which streptavidin is added. Residues in the interior of the membrane can only be labelled if they are exposed to the aqueous phase and are physically accessible to streptavidin. The orientation of VDAC in the membrane has not been correlated with that in intact mitochondria, so residues can only be localized with respect to each other. This approach led to the development of the model shown in Fig. 1.4B.

CD has also proven to be effective in combination with fluorescence spectrophotometry and BLB experiments for characterizing the secondary structure of detergent solubilized His-tagged NcVDAC variants which harbour small deletions within potential membrane spanning segments (Runke *et al.* 2006). By combining calculated secondary structure composition, information concerning Trp residue environments in detergent-solubilized VDAC, and channel forming ability in BLBs, three classes of porin variants were determined. The first class produced large, stable pores, indicating deletions likely outside of β-strands. The members of the second class of porin variants had minimal pore-forming ability, indicating disruptions in key β-strands or β-turns and the third class of porins formed small unstable pores with a variety of gating and ion-selectivity properties (Runke *et al.* 2006).

By combining information determined from the model proposed in Bay and Court (2002) (Bay and Court 2002), and the data described in Runke *et al.* (2006) (Runke *et al.* 2006), TM arrangements within central and C-terminal portions of the protein have been refined and are discussed below (see Fig. 1.4 for models). The Runke *et al.* (2006) porin model was refined based on three assumptions: first, the variants with limited pore-forming ability lack at least part of a region normally in a β-strand or β-turn conformation that contributes to channel formation. Second, the assignment of strands based on the effects of single residue replacements on ion selectivity of yeast VDAC1 (Blachly-Dyson *et al.* 1990) uses the assumption that residues contributing to ion selectivity line the barrel, but residues in exposed loops or turns could also influence ion selectivity. Finally, the last consideration in model building was that the five proline residues in porin could not be included in β-strands because these residues disrupt secondary structure (Rauch and Moran 1994).

1.6.1 N-terminus

The location of the N-terminus (residues 1–20) of VDAC has been a source of debate. Initially, it was argued that it folds into an amphipathic α-helix that forms part of the barrel (Mihara and Sato 1985, Kleene *et al.* 1987, Forte *et al.* 1987). However, as described below, more recent evidence supports an IMS location for this part of the protein. Electrophysiological characterization of ScVDAC mutants has been used to investigate the location of aspartate 15 and lysine 19 of ScVDAC, which are conserved in the *Neurospora* protein (see Fig. 1.3C). Site-directed mutagenesis of the yeast gene to convert D15 to a lysine (D15K) and K19 to glutamate (K19E) led to the production of channels with altered selectivity (Blachly-Dyson *et al.* 1990). The interpretation of these results is that the N-terminus is within the membrane and participates, along with a mobile "voltage sensor", in the ion selectivity of the channel (Peng *et al.* 1992). It is important to note that residues outside the β-barrel could also contribute to ion selectivity, and therefore, the results do not necessarily dictate a membranous location for the N-terminus.

Immunolabelling experiments have also been used to address the orientation of the N-terminus. Extensive labelling of rat heart (Konstantinova *et al.* 1994, Konstantinova *et al.* 1995) and *Neurospora* (Stanley *et al.* 1995) mitochondria with antibodies against the N-terminal part of the corresponding VDAC only occurred after the OM had been ruptured, supporting an IMS location for the N-terminus.

Biotinylation experiments involving residue S7C of NcVDAC also indicate that the N-terminus is not embedded in the membrane. When biotinylated, this residue could be bound by streptavidin from either side of an artificial bilayer, suggesting that the N-terminus is flexible and may participate in gating activity (Song *et al.* 1998). Deletion of this segment

does not prevent pore formation in artificial bilayers (Popp *et al.* 1996, Koppel *et al.* 1998). Further, the addition of an N-terminal His-tag to NcVDAC (Court *et al.* 1996) or a green fluorescence protein to the N-terminus of mouse and human VDAC (Zaid *et al.* 2005, Schwarzer *et al.* 2002) does not prevent the assembly of these proteins into the mitochondrial OM. Taken together, these studies suggest that the N-terminus is likely external to the OM and exposed to the IMS (Figs. 1.4C and 1.4D).

1.6.2 β-Barrel

TM β-strand assignment is much more complicated than that of the N-terminus, due to the lack of consistent experimental evidence for central portions of the sequence. To facilitate the following discussion, it is much simpler to address sections of residues as opposed to identifying each TM β-strand individually, since there are several possible assignments that fit the experimental data. The following discussion refers to the models in Figs. 1.4C and 1.4D.

1.6.3 Residues 21–46

The putative α-helical region of the N-terminus extends to residue H23. This leaves a three- or four-residue turn, resulting in the first β-strand spanning residues T28 to N34 in NcVDAC (Rauch and Moran 1994). This assignment will result in a turn occurring from T35 to N38. After biotinylation of residue H23C, streptavidin binding occurred only on the side of VDAC addition to the membrane (*cis*) and created a channel with "type 2 conductance", where the channel exists in the closed state and shows no voltage dependence (Song *et al.* 1998). These results support the previously described experimental data indicating that the N-terminus is mobile and furthermore indicate that it is in close proximity to the barrel opening.

The data obtained for residue N38, however, are less clear; membranes containing multiple N38C mutant channels are able to bind streptavidin from both sides. This produces "type 1 conductance", where channel conductance is decreased compared with the wild-type, but voltage-induced gating is still observed, indicating that N38 is not required for the latter process (Song *et al.* 1998). In contrast, a single N38C VDAC is able to interact with streptavidin only from one side of the membrane, suggesting that the *cis/trans* nature of binding to multiple channels results from insertion of this variant in both orientations across the membrane. Based on the presence of the IMS-localized N-terminus, and β-strand breaking P36 in the centre of a predicted β-rich region (residues 27–46), we placed the first β-strand (β1) from G27 to N34, N38 in a cytosolic loop, followed by a β-strand (β2) continuing to K46 (Bay and Court 2002). This placement agrees well with Neural Network assignments of a cytosolic loop including N38 (Fig. 1.3B).

1.6.4 Residues 47–104

Residues S47 to V52 are not predicted to form a β-strand. The presence of T53 in an IMS turn fits well with biotinylation data placing both residues T53 and H23 facing the IMS (Song *et al.* 1998). Replacement of the ScVDAC residue corresponding to D50 in NcVDAC with a lysine created a pore with normal anion selectivity, as expected if this residue does not contribute to the charged nature the of barrel (Blachly-Dyson *et al.* 1990).

The next β-strand (β3) could extend from S54 to K64 based on hydropathy alignment profiling (Rauch and Moran 1994). This assignment is supported by labelling of bovine VDAC E72 (corresponding to NcVDAC E58) with the hydrophobic compound dicyclohexylcarbodiimide, indicating that this residue is embedded in the membrane (De Pinto *et al.* 1993). Also, replacement of the ScVDAC equivalent of K60 and K64 in

NcVDAC produced less anion-selective pores, as expected for β-barrel residues (Blachly-Dyson *et al.* 1990). P65 is predicted to be associated with the short turn needed prior to the next putative β-strand (β4), extending from T69 to N75 (Bay and Court 2002, Runke *et al.* 2006). The placement of T69 and T53 on opposite sides of the OM is supported by *trans* streptavidin binding to the double mutant T53C/T69C (Song *et al.* 1998). Using the same approach, it was shown that A79 and T53 reside on the same side of the membrane (Song *et al.* 1998), supporting the proposal of a turn between residues N75 and A79.

Residues L80 to F100 are estimated to form two β-strands (β5-β6); however, the location of a short turn between the two strands is not uniformly predicted by the hydropathy plot (Benz 1994), the Neural Network (Fig. 1.3B), and the prediction algorithm of Rauch and Moran (1994) (see Fig. 1.3A). It is proposed that G93 is part of a turn, as glycine tends to end β-strands (Rauch and Moran 1994, Kyte and Doolittle 1982). Placement of the second β-strand in this region is supported by replacement of K95 with E in the *Neurospora* and yeast proteins (Runke *et al.* 2000, Blachly-Dyson *et al.* 1990). In both cases, a cation-selective channel was produced, as expected if a charged residue within the lumen is altered. Finally, P104 would act as an effective barrier to β-strand extension past L103. At this point a total of six β-strands are predicted and there is some general agreement between experimental data and predicted models (Figs. 1.4C, and 1.4D) (see Bay and Court 2002, Runke *et al.* 2006).

1.6.5 Residues 105–135

A single β-strand (β7) from residues A105 to K112 follows the loop starting at S101 (Bay and Court 2002, Runke *et al.* 2006). This β-strand placement is contradicted by the observation that ScVDAC variant K108E does not display altered ion selectivity, as might be expected if K108E (K109 in NcVDAC) is in the barrel lumen (Blachly-Dyson *et al.* 1990). It

is important to note, however, that not every charged residue in the barrel lumen is expected to have a large-scale impact on ion selectivity.

One or two β-strands may form between residues K112 and L130 (see Fig. 1.3A) (Rauch and Moran 1994, Benz 1994, Mannella *et al.* 1996). Dual biotinylation experiments show that T53C and T135C bind streptavidin in a *cis* fashion, placing them on the same side of the membrane (Stanley *et al.* 1995, Song *et al.* 1998) and arguing against the presence of two β-strands between K112 and L130. Furthermore, K112 is *trans* to T53 (Song *et al.* 1998).

A single β-strand (β8) between H123 to L130 is also supported based on the characteristics of the deletion variants 126porin and 120porin, which lack residues 126-136 and 120-143, respectively (Runke *et al.* 2006). β8 partially overlaps a β-strand predicted by Benz (Benz 1994) and Rauch and Moran (Rauch and Moran 1994).These deletion variants were engineered with two glycyl residues at the junction of the deletions to compensate for the lack of flexibility that may arise if all of the residues separating two β-strands are deleted. 126porin and 120porin have very limited pore forming ability, suggesting that β-strands were disrupted in those variants (Runke *et al.* 2006). β8 was positioned in these models to include R125 and R129 that influence ion selectivity and exclude K132 that does not (Blachly-Dyson *et al.* 1990). Its presence also maintains the relative orientations of K112 and T135 (Song *et al.* 1998). In contrast, a large extramembrane loop (S120 to H144) was predicted in this region by Casadio *et al.* (Casadio *et al.* 2002).

Together, these data support the placement of two β-strands, β7 between A105 to K112 and β8 between H123 to L130, followed by a turn including P134 (Fig. 1.4D).

1.6.6 Residues 136–212

Limited data were available for the segment A136 to S211, making it very difficult to identify regions of β-strand propensity (Bay and Court 2002). Dual biotinylation mutant T53C/D156C was *cis* for streptavidin binding, assigning D156 to either a turn or the end of a β-strand on the same side of the membrane as H23, T53, A79, and T135. Hydropathy data (Benz 1994) support the latter placement. Furthermore, ScVDAC residue D156 (also D156 in NcVDAC) is speculated to sit outside the barrel, since the yeast D156K variant did not display altered channel selectivity (Blachly-Dyson *et al.* 1990, Peng *et al.* 1992). This leaves us with two possibilities (Fig. 1.4C). Either the same large IMS loop includes T135 and D156 or two very short β-strands separate these two residues.

Deletion variants were analyzed to help resolve this dilemma (Runke *et al.* 2006). 147porin, lacking residues 147-151, does not form pores, suggesting that the ninth β-strand includes some or all of residues 147–151. β9 (E145 to S152) places D156 outside the membrane where it would not contribute to ion selectivity, and D152 in the membrane (Blachly-Dyson *et al.* 1990). However, D156 is likely on the same side of the membrane as T135, suggesting its placement in the IMS (Song *et al.* 1998). To reconcile these data, a long loop in the IMS that spans T135 to D156 could be introduced and the assumption made that only D152 interacts with the channel in a way that regulates ion selectivity. However, this arrangement would leave only residues 157–164 to create a short β-strand and a loop to connect to β10 (see below). Therefore, D156 is placed in a loop in the Runke *et al.* model, where it could be accessible from the IMS (Runke *et al.* 2006).

T183 and S190 are *trans* to T53 and exposed to the cytosol. T183 is eight residues to the C-terminal side of P174; accordingly, we predicted a β-strand from approximately I162 to

H172 (Bay and Court 2002), which combines segments of two β-strands predicted by hydropathy analysis (Benz 1994). Another region of uncertainty includes S190 to S211, both of which rest on the cytosolic side of the membrane, as demonstrated by biotinylation assays (Song *et al.* 1998). Again, this arrangement can be achieved through prediction of one large loop or two short β-strands. Antibodies directed against residues 195–210 of NcVDAC do not bind intact mitochondria (Stanley *et al.* 1995), arguing against a large cytosolic loop. The possibility remains, however, that such a large loop interacts strongly with the barrel lumen, leaving it inaccessible to the antibody. Replacements in ScVDAC that correspond to NcVDAC S192 (D192K; Blachly-Dyson *et al.* 1990), K206 (K205E), and K212 (K212E; Thomas *et al.* 1993) do not change ion selectivity, supporting the absence of a membrane-bound segment in this region.

A β-strand in the position of β10 (Y165 to H172) is predicted by all algorithms, except that of Rauch and Moran (Rauch and Moran 1994) (Fig. 1.4A) and is supported by the limited pore formation of deletion variant 166porin, lacking residues 166-170, (Runke *et al.* 2006). β10 has been placed to expose R164 to the cytosol, as this residue is not involved in ion selectivity, and to position P174 outside of the β-strand. Support for a second β-strand within this region (β11) (A178 to N185) is the limited pore formation by the nested deletion variants 173porin (173-184) and 177porin (177-182) (Runke *et al.* 2006). The deletion in 173porin is predicted to disrupt β11 and the turn between β10 and β11 (Fig. 1.4D), and this variant also has a significantly increased level of random sequence, which likely contributes to its inability to form pores. This region contains a predicted β-strand that is proposed by all models except that of Casadio *et al.* (Casadio *et al.* 2002). In the current transmembrane

arrangement, β11 is also needed to maintain S190 on the same side of the membrane as N38, T69, and K112 (Runke *et al.* 2006).

The placement of two β-strands (β12-13) within this region is supported by the lack of pore formation by 195porin, lacking residues 195-198, and the likelihood that W209 resides in a hydrophobic environment, as shown by Trp fluorescence of the detergent-solubilized His$_6$-porin (Runke *et al.* 2006). The two strands must arrange S190 and S211 on the same side of the membrane, and keep N198 (E198 in yeast) and K212 in positions where they do not participate in ion selectivity. Finally, at least some of residues 195–210 must be exposed to the IMS. Given the number of residues available in this region, β12 and β13 are proposed to be only six residues long, the minimum needed to span the membrane (Koebnik *et al.* 2000).

1.6.7 Residues 213–283

All or part of β14 is predicted by all algorithms (Rauch and Moran 1994, Benz 1994, Casadio *et al.* 2002, Bay and Court 2002), and spanning residues from T216 to I227 (Fig. 1.3A and 1.4). In the Runke *et al.* model, β14 is placed between residues E220 and I227, leaving P229 in the IMS. Replacement of E220 does not affect ion selectivity; if it resides in β14, it must be in a position that limits its contribution to ion selectivity. The presence of P229 places a turn in the IMS. Dual biotinylation results indicating that T53 and R240 are *cis* with respect to streptavidin binding suggest that this loop also includes R240 (Song *et al.* 1998). A loop spanning 228-242 is also predicted from the deletion variants 228porin and 238porin which formed large pores, suggesting that residues 228–232 and 238–242 are not involved in β-strand formation (Runke *et al.* 2006). Within this region, K234 contributes to

ion selectivity; this observation is compatible with a large loop that can enter the pore and contribute to the charge characteristics of the channel. 228porin forms a cation-selective pore, and the deleted segment includes D228, whose absence would decrease the net negative charge in the region, and therefore would be unlikely to directly shift the ion selectivity toward cations. Therefore, residues 228–232 are not direct determinants of ion selectivity, but perhaps interact with a region of the protein that is. P229 is also absent in 228porin, which may alter the topology of the loop that contains it, perhaps interrupting interactions responsible for gating. K234 and K236, which are required for the stable assembly of yeast VDAC1 into the mitochondrial OM (Smith *et al.* 1995), are also within this proposed loop.

A hydrophobic region separates R240 and D264, both of which are *cis* to T53 (Song *et al.* 1998). Part of this region is predicted to form a β-strand (Rauch and Moran 1994, Benz 1994); however, two strands must exist to place both R240 and D264 on the IMS side of the membrane. The N-terminal segment of this region is not amphipathic, but it could reside in the barrel, with lumen-facing hydrophobic residues interacting with another part of VDAC or another protein. By exchanging K248 with E in ScVDAC (N248 in NcVDAC), a channel with altered ion selectivity was created, supporting the placement of this residue in the barrel (Thomas *et al.* 1993). In contrast, the ion selectivity of ScVDAC R252E (Blachly-Dyson *et al.* 1990) is not changed, placing this residue outside the barrel.

The deletion variant 242porin (242-248) results in formation of unstable pores (Fig. 2, Runke *et al.* 2006) suggesting that β15 occurs from residues A243 to L250. This β-strand placement would be necessary to place at least some of segment 251–268 facing the cytosol, where it would be accessible to antibody binding (Stanley *et al.* 1995) (Fig. 1.3A). Placing a β-strand between residues A243 and L250 places N248 (K248 in yeast) inside and R252

outside of the membrane, as predicted in Blachly-Dyson *et al.* (Blachly-Dyson *et al.* 1990). β15 was not predicted by Benz (Benz 1994) or Song *et al.* (Song *et al.* 1998).

In the Runke model, β16 (V-255 to S-262) places V255 in the membrane (Blachly-Dyson *et al.* 1990) and D264 on the same side of the membrane as R240. A β-strand at the position of β16 is predicted in all models (Fig. 1.3 and residues E253 to D264 in Mannella *et al.* (Mannella *et al.* 1996)). Finally, all of the models presented in the literature (for examples, see Figs. 1.3A, 1.4A, and 1.4B) predict that the region spanning residues K273 to S283 forms a β-strand. ScVDAC variant D282K displays altered ion selectivity, further supporting a membranous location for the C terminus (Thomas *et al.* 1993). However, this putative β-strand cannot be accommodated in the TM arrangement because it would create an odd number of β-strands. The truncation variant ΔCporin lacking residues 269–283 forms pores in artificial bilayers (Court *et al.* 1996), supporting the absence of a β-strand in this region. Finally, an epitope between 272 and 283 is accessible in mitochondria with ruptured outer membranes, suggesting that this segment resides in the IMS (Stanley *et al.* 1995). This prediction is also compatible with the fact that K267 and K274 do not contribute to ion selectivity. A role for E282 (D282 in yeast) in the process is possible if the C-terminus interacts with the channel, as may be suggested by fluorescence analysis (Fig. 1.4D) (Runke *et al.* 2006). In these studies, deletion of the C-terminus increases Trp fluorescence, suggesting that the C-terminal segment interacts with one or both of the two Trp residues, thereby quenching fluorescence of the detergent-solubilized protein. It is noteworthy that the amino and carboxyl termini of two β-barrel proteins of the outer membrane protein import machinery TOM40 (Künkele *et al.* 1998), and Tob55/Omp85 (Gentle *et al.* 2004) are also likely exposed to the IMS.

1.7 VDAC Folding in vivo: relevance to bacterial porin folding?

Understanding the *in vivo* assembly of integral β-barrel proteins into the outer membrane of gram-negative bacteria or mitochondria will provide a basis for analyzing the folded state of isolated porins, which in turn is essential for accurately determining the structure of the protein. In the following sections, the basic steps of transport and assembly of mitochondrial and bacterial porins into their respective outer membranes will be compared and contrasted.

1.7.1 Sorting Signals

In bacteria, integral outer membrane protein import has been best characterized for *E. coli* PhoE porin, and to a lesser extent for OmpF and OmpA. The precursor bacterial porin protein is translated in the cytoplasm with a cleavable, hydrophobic N-terminal signal sequence (Freudl *et al.* 1988) and targeted to the inner membrane secretory (Sec) protein complex (Manting and Driessen 2000). Additional C-terminal sorting information has been identified in *E. coli* PhoE. A variant PhoE protein, ΔF330, lacking the C-terminal Phe residue demonstrated slower insertion kinetics than did wild-type PhoE, suggesting that this residue is required for directing porin insertion into the OM (Jansen *et al.* 2000).

In contrast, mitochondrial porin is synthesized outside of the organelle, in the cytosol (Mihara *et al.* 1982, Freitag *et al.* 1982). Poorly defined sorting information is located in the C-terminal portion (residues 269-283) of the *Neurospora* protein as deletion of this region prevents import into isolated mitochondria (Court *et al.* 1996). Further, Lys residues at positions 234 and 236 in *Saccharomyces* VDAC1 are required for assembly in the outer membrane (Smith *et al.* 1995, Angeles *et al.* 1999).

1.7.2 Transport into the intermembrane/ periplasmic space

In bacteria, the precursor porin traverses the cytoplasmic membrane via the Sec complex, to enter the periplasmic space. In contrast, VDAC import into the outer membrane first involves transport across the translocase of the outer mitochondrial membrane complex (TOM complex), into the inter-membrane space (reviewed by Rapaport 2005). The TOM complex is composed of the core Tom40 protein and subunit proteins Tom22, Tom7, Tom6, and Tom5. The signal required for targeting to the TOM complex is unknown, but apparently does not include the first 20 residues of the putative N-terminal α-helix (Court *et al.* 1996). Like many mitochondrial precursor proteins, mitochondrial porins targeted to the Tom20 complex (Schleiff *et al.* 1997, Schleiff *et al.* 1999) are sorted by the core TOM complex (Sherman *et al.* 2005, Sherman *et al.* 2006). Competitive import assays of porin into isolated yeast and *Neurospora* mitochondria, with intact or proteolytically-cleaved receptors, indicated that besides Tom20, mitochondrial porin insertion is dependent on the central receptor Tom22, and other components of the core complex, Tom5 and Tom7 (Krimmer *et al.* 2001). The *Neisserial* porin PorB can also utilize this import pathway, emphasizing that both bacterial porin and mitochondrial porin share signals recognized by the TOM complex (Muller *et al.* 2000, Muller *et al.* 2002).

Within the periplasmic space, bacterial porin begins to fold into an assembly-competent form that is possibly assisted by the chaperones Skp (Harms *et al.* 2001) and SurA (Lazar and Kolter 1996). Although chaperone-bacterial porin interactions have been demonstrated *in vitro*, the extent of chaperone assisted folding *in vivo* is uncertain since lipopolysaccharide (LPS) involvement has also been shown to assist OmpF assembly (Traurig and Misra 1999). However, even the LPS interaction with OmpF is not essential,

suggesting a variety of folding and assembly chaperones may be involved preparing porin for insertion into the OM (Visudtiphole *et al.* 2005).

As with bacterial porins, mitochondrial porins reside between two membranes before final assembly into the OM. Chaperone-mediated folding of mitochondrial porin within the intermembrane space has been proposed to involve the hetero-oligomeric components TIM8/13 which transport precursors from the TOM complex to the TIM23 translocase complex in the mitochondrial inner membrane (IM) (reviewed by Rapaport 2005). Yeast mutants lacking either TIM8 or TIM13 demonstrated reduced levels of β-barrel protein import, including that of VDAC suggesting that these proteins to play a dual role in protein import to the OM and to the IM (Hoppins *et al.* 2004). Homology between TIM8/13 and bacterial chaperones Skp and SurA protein has not been demonstrated to date.

Other possible VDAC chaperones that have been identified from two-hybrid screens involving human VDAC1 are the interacting proteins: dyenin light chain Tctex-1 and mitochondrial heat-shock protein 70/peptide-binding protein 74 (PBP74). Both proteins modulate mitochondrial channel forming activity in BLB experiments and co-localized with enhanced green fluorescence protein –human VDAC1 fusion proteins in HeLa cells (Schwarzer *et al.* 2002). The relevance of these findings is not clear, as mt-hsp70 is localized to the matrix, and not the intermembrane space (Schmidt *et al.* 2001, Voos and Rottgers 2002) and Tctex-1 is a cytoplasmic homodimeric protein that plays a role in actin remodelling within the cytosol (Chuang *et al.* 2005) and dyenin-cargo delivery from the post-Golgi to the plasma membrane (Yeh *et al.* 2006). Additionally, an as yet unannotated 54-kDa protein referred to as the VDAC modulator has been isolated from the IMS and alters porin channel formation and conductance in BLB experiments, suggesting that it may also

participate in VDAC assembly into the OM (Liu and Colombini 1991, Liu and Colombini 1992, Liu and Colombini 1992, Liu *et al.* 1994).

1.7.3 Integration into the outer membrane

VDAC is assembled into the outer membrane by the topogenesis of mitochondrial outer-membrane β-barrel proteins (TOB-SAM) complex (reviewed by Paschen *et al.* 2005). The TOB-SAM complex is a hetero-oligomer composed of the core component Tob55, as well as Tob37 and Tob38. *In organello* import of radiolabelled mitochondrial porin into mitochondria demonstrated that precursor porin is associated with His-tagged Tob55, supporting a role for Tob55 in porin topogenesis in the outer membrane (Paschen *et al.* 2003). Although the TOB-SAM complex appears similar to the chloroplast Toc complex based on β-barrel structure and function, only Tob55 has primary sequence homology to bacterial Omp85 (reviewed by Paschen *et al.* 2005), an essential protein involved in outer membrane protein assembly (Voulhoux *et al.* 2003, Gentle *et al.* 2004). Inactivation of the *omp85* gene in *Neisseria meningitidis* resulted in the accumulation of precursor PorA and PorB within the periplasm, indicating that Omp85 plays an important role in porin assembly into the bacterial OM (Voulhoux *et al.* 2003).

In summary, it appears that both bacterial and mitochondrial porins have similar mechanisms for importing and assembling into the OM, an observation that supports the endosymbiont theory of mitochondrial evolution (Paschen *et al.* 2003). However, unlike mitochondrial porin, all bacterial porins must undergo translocation across the inner membrane before assembling into the OM, creating a requirement for cleavable N-terminal targeting sequences not needed for VDAC precursor protein. Perhaps the α-helical N-terminus has some distant relationship to these bacterial Sec targeting sequences, although

the observation that deletion of the N-terminal 20 residues does not prevent import into isolated organelles suggests that a targeting function has been lost (Court *et al.* 1996). However, the amino-terminal sequences of fungal mitochondrial porins are one of the most highly conserved parts of the protein, suggesting that this segment has gained other functional relevance (Young *et al.* submitted). Similarities in porin folding, particularly at the OM assembly stage, likely will direct future studies into the *in vivo* assembly and folding of bacterial and mitochondrial porins.

1.8 OmpA folding in vitro: a β-barrel folding archetype

Given the complexities of the *in vivo* systems, many of the folding studies of bacterial porin have been carried out *in vitro*. Bacterial porin folding in detergents and artificial bilayers has been well characterized using a variety of biophysical techniques. *Escherichia coli* OmpA has been used extensively as a model for β-barrel folding. This protein has two functional domains; the first 171 residues of the N-terminus comprise the eight transmembrane β-stand barrel domain and the last 154 residues of the C-terminus form a globular periplasmic domain that interacts with peptidoglycan (Koebnik 1995). Structural characterization of this protein has primarily focused on the truncated N-terminal β-barrel region and a three-dimensional structure has been determined by X-ray diffraction (Pautsch and Schulz 1998) as well as by NMR spectroscopy (Arora *et al.* 2001) techniques. The β-barrel fragment of OmpA has served as a useful archetype for folding studies, both in detergents and lipid bilayers, due to its relatively small size, its monomeric state, and the availability of a well characterized three-dimensional structure. To simplify the discussion below, this N-terminally truncated variant will be referred to as OmpA.

Most detergent-based folding experiments involving OmpA utilize urea-denatured protein, which like many porins, has the ability to refold in a variety of detergents following isolation from inclusion bodies (Kleinschmidt and Tamm 1996, Kleinschmidt *et al.* 1999). Denatured OmpA refolds only in the presence of zwitterionic or non-ionic detergents with minimum acyl chain lengths ranging from C_8-C_{12}, which meet the requirement for detergent packing around the hydrophobic perimeter of the β-barrel. OmpA refolding is also influenced by the micellar state of the detergent; refolding could only be demonstrated at or above the critical micelle concentration of each detergent (Kleinschmidt *et al.* 1999). The precise mechanism for this detergent-induced OmpA refolding is not clear. OmpA refolding in the Kleinschmidt *et al.* experiments appears to occur as a two-state folding transition proceeding from random structure in monomeric detergent to β-strand in micellar detergent concentrations (Kleinschmidt *et al.* 1999).

The first instance of using mixed systems to refold OmpA denatured by urea or SDS was performed by the Henning lab, where denatured OmpA was diluted into Triton X-100 in the presence of LPS, resulting in refolded protein (Schweizer *et al.* 1978). The refolding process was described as spontaneous since the refolded protein was observable immediately after detergent-LPS addition. Similar experiments performed by Dornmair *et al.* (Dornmair *et al.* 1990) also demonstrated spontaneous re-folding of OmpA. The protein was extracted from the outer membrane, boiled in SDS and then diluted into the non-ionic detergent octyl-β-glucoside (OG). In this case, refolding occurred without LPS addition (Dornmair *et al.* 1990). To determine the requirements for re-folding in detergent, SDS-denatured OmpA was refolded in the presence of different concentrations of OG. Protein refolding occurred above an OG weight fraction (X_{OG}) of 0.7 (Ohnishi *et al.* 1998, Ohnishi and Kameyama 2001).

Based on this system, a pathway of refolding involving four kinetic phases was deduced from the three experimentally obtained rate constants (Ohnishi and Kameyama 2001). These rate constants were derived from OmpA CD and fluorescence spectra monitored over a period of days as the weight fraction of OG was increased. The spectral profiles were used to calculate the re-folding kinetics of OmpA in the detergent mixtures. The experimentally derived equations indicated the logarithmic dependence of OmpA re-folding on the weight fraction of OG and suggested that two potential folding intermediates may occur along the detergent refolding pathway during the spontaneous refolding of OmpA in detergent (Ohnishi and Kameyama 2001).

The characterization of the folded and unfolded states of OmpA along with other Omps in both detergents and membranes has been facilitated by monitoring the shift in apparent molecular mass when the proteins are electrophoresed through SDS-polyacrylamide gels. Schweizer *et al.*(1978) first demonstrated that unboiled OmpA migrated through, or electrophoresed through the gels with an apparent molecular mass of 30 kiloDaltons (kDa) whereas the boiled species had an apparent mass of 35 kDa. The folded state of the 30 kDa species of OmpA on SDS-PAGE could be correlated to channel activity in BLB experiments, since only protein isolated from these bands had demonstrated gating activity (Arora *et al.* 2000). Refolded OmpA can also be identified by its protease resistance since the 35 kDa species demonstrate higher susceptibility to degradation by proteases on SDS-PAGE gels (for examples see Dornmair *et al.* 1990, Kleinschmidt and Tamm 1996, Kleinschmidt *et al.* 1999).

Although, OmpA appears to spontaneously refold in detergents, OmpA membrane insertion and folding mechanisms have been examined in much greater detail. As in detergent-OmpA refolding experiments, urea-denatured protein also inserts and refolds

within artificial phosphatidyl choline-based lipid bilayers (Surrey and Jahnig 1992, Surrey and Jahnig 1995). In experiments similar to those first described for bacteriorhodopsin and UDP-glucuronosyltransferase (Scotto and Zakim 1988), OmpA spontaneously inserts into liposomes from a urea-denatured state but only at temperatures of 30°C and above (Surrey and Jahnig 1992). Three transitions along the OmpA *in vitro* folding pathway were identified and two intermediates, a misfolded water-soluble form and a partially folded, membrane-inserted form, were detected (Surrey and Jahnig 1995, Kleinschmidt and Tamm 1996). This three step mechanism of OmpA refolding within liposomes has been refined further through the application of time-resolved Trp fluorescence quenching (Kleinschmidt *et al.* 1999, Kleinschmidt and Tamm 1999, reviewed in Kleinschmidt 2003, Tamm *et al.* 2004, Kleinschmidt 2006 see Fig. 1.5). In the first, most rapid step (occurring within seconds to minutes), the urea solubilized protein is unfolded (U) and upon dilution in water rapidly becomes a hydrophobically collapsed water-soluble intermediate (I_W). The slower second stage (taking minutes to hours) involves three membrane bound intermediates, where the first intermediate is formed when I_W associates with the membrane (I_{M1}) in an aggregated arrangement characterized by disordered Trp residues. This aggregated form proceeds to the second intermediate (I_{M2}) where β-structure begins to develop but lacks tertiary contacts; this form has been termed a "molten disk". The β-hairpin loops within I_{M2} translocate to the centre of the lipid bilayer and lack correct tertiary arrangements forming the final "molten globule" intermediate (I_{M3}) that proceeds to the native state (N).

This kinetic folding mechanism of OmpA is influenced by a variety of factors such as membrane thickness, elasticity, and curvature, which affect both the kinetics and refolding of OmpA (Kleinschmidt and Tamm 1996, Kleinschmidt *et al.* 1999, Kleinschmidt and Tamm

2002, Marsh *et al.* 2006, Pocanschi *et al.* 2006). From these studies OmpA insertion occured

spontaneously only into highly curved SUVs, where as spontaneous insertion into unstrained

LUVs required phospholipid chain lengths of 12 C atoms only (Kleinschmidt and Tamm

1996, Kleinschmidt *et al.* 1999, Kleinschmidt and Tamm 2002).

A folding pathway parallel to that described for OmpA has recently been determined

for a larger β-barrel porin, FomA of *Fusobacterium nucleatum*. FomA is composed 14 TM

antiparallel β-strands and is assembled in artificial bilayers via a mechanism similar to the

three step kinetic folding mechanism shown for OmpA (Pocanschi *et al.* 2006). Overall, the

insertion and folding kinetics of FomA were much slower than those of OmpA. The insertion

kinetics of both proteins were dependent on membrane thickness and lipid bilayer content.

Since the elasticity and curvature of liposomes containing varying phosphatidylcholine lipid

head groups influenced the insertion and folding kinetics of both OmpA and FomA, the

folding of larger porins may have a greater dependence on particular lipid composition than

does OmpA (Pocanschi *et al.* 2006).

To complicate the application of the OmpA folding studies described above to *in vivo*

folding studies, two conformations of full length OmpA, have been suggested to form *in vitro*

and possibly *in vivo*. The first is a two-domain 171-residue, 8-TM β-barrel with 154 residue

periplasmic C-terminus and the second is a minor form that exists as a single β-barrel domain

consisting of all 325 residues (Sugawara and Nikaido 1992, Sugawara and Nikaido 1994,

Arora *et al.* 2000). A single domain β-barrel conformation of OmpA is proposed to occur *in*

vivo based on the detection of OmpA C-terminal epitopes on the cell surface by antibodies

against peptides within the OmpA C-terminus domain which is proposed to reside within the

Figure 1.5 Cartoon diagram summarizing OmpA intermediates during insertion and folding into artificial membranes as determined by time-resolved Trp fluorescence quenching (TDFQ) experiments (reviewed by Tamm *et al.* 2004, Kleinschmidt 2006). The unfolded state (U), water-collasped intermediate (I_W), three membrane bound intermediates (I_{M1}, I_{M2}, and I_{M3}), and the native conformation (N) of OmpA are shown in the diagram. Trp residues W7, W57, W102, and W143 examined by TDFQ experiments are indicated by blue circles. Red lines with or without connecting red filled rectangles (β-strand) represent OmpA protein in the various stages of folding. Black arrows indicate each stage during OmpA insertion and folding into the phospholipid bilayer.

U

I_W

Molten globule

I_{M1} I_{M2} I_{M3} N

Molten disk

Phospholipid Bilayer

W7

W15   W102   W143

W57

W1   W57   W57

W7   W143

W1   W57   W57

W7   W143

W7   W15   W57   W143

W102   W143

W7

W15   W7

W7

W15   W7

W57   W143
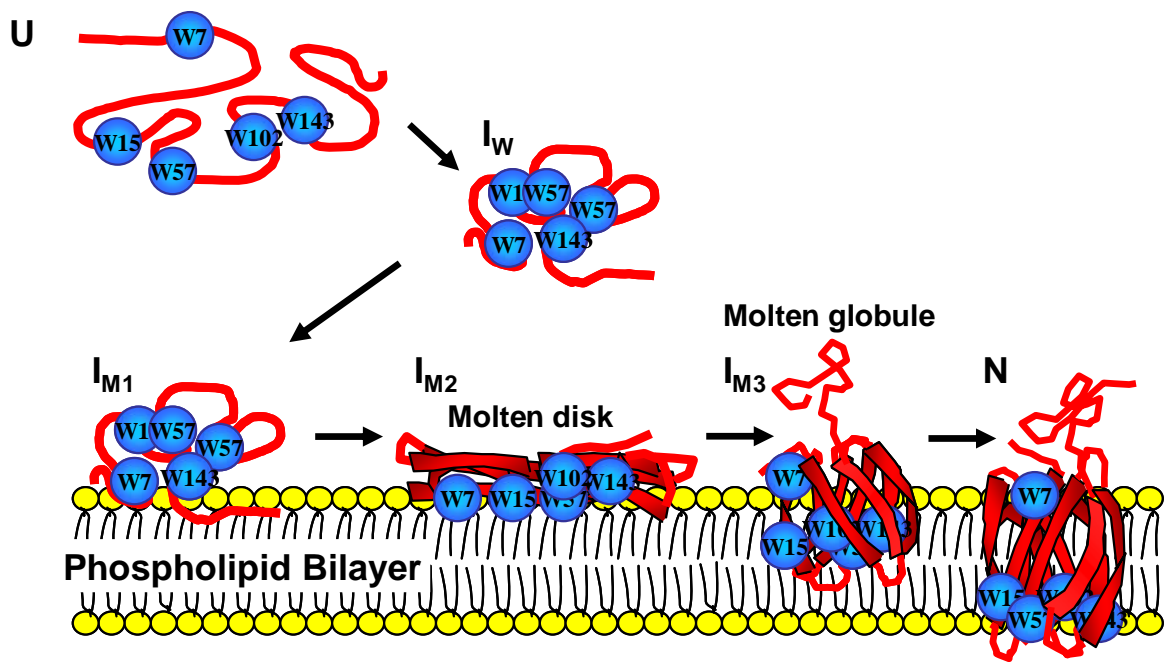
periplasm (Martin *et al.* 1993, Rawling *et al.* 1995, Singh *et al.* 2003). Planar lipid bilayer experiments involving full length OmpA protein at room temperature have also demonstrated two populations of channels: a major subset of small pores with conductances of 50-80 picoSiemens (pS) and a minor set of larger pores with 260-320 pS (Arora *et al.* 2000). Larger pores could not be demonstrated in BLB experiments using N-terminal domain OmpA protein suggesting that both domains are required to produce the both small and large channels (Arora *et al.* 2000). The channel size of full length OmpA appears to be influenced by temperature since small, low conductance channels are formed at temperatures of $\leq 21^{\circ}C$ (Zakharian and Reusch 2003, Zakharian and Reusch 2005) and upon incubation at higher temperatures channels could be converted to larger pores with increased conductance. Characterization of this transition revealed that it was irreversible, as incubation of full length OmpA large pores at physiological temperatures ($\geq 26^{\circ}C$) produced greater numbers of large pores with high channel conductance. At $37^{\circ}C$ only large pores were observed (Zakharian and Reusch 2005). These large pores could only be converted back to small pores in the presence of denaturants such as SDS or urea. Based on these findings a two-domain OmpA structure may represent a partially-folded intermediate that is kinetically stable only at or below room temperature and will be converted to a one-domain β-barrel at higher temperatures (Zakharian and Reusch 2005). However, this one-domain OmpA state has not been structurally characterized apart from these experiments.

Overall, examination of the folding of C-terminally truncated OmpA in liposomes reveals that similar steps in the folding mechanism may apply to its folding in mixed detergent systems and that can be used to reflect the folding of much larger porins. The three

step folding mechanism of OmpA contrasts the three-stage mechanism proposed for integral α-helical membrane proteins (Engelman *et al.* 2003).

1.9 VDAC folding in vitro: self catalyzed oriented insertion

Unlike OmpA, the folding mechanism of mitochondrial porins has not been characterized in detail. Folding studies involving mitochondrial porins have primarily focused on its channel forming ability and orientation in black lipid bilayer experiments. The first studies that focused on mitochondrial porin insertion and orientation within these artificial bilayers were examined by Zizi *et al.* (1995). These experiments utilized two yeast VDAC variants (E145K or E152K), both with asymmetric voltage-dependence, meaning that gating could be induced only when the electric field was oriented in one direction across the membrane. Since the asymmetry was observed in the presence of a single channel or hundreds of channels, it was assumed that all channels were inserted in the same orientation. However, the initial channel orientation was random and the orientation of subsequent channels depended on the orientation of the pre-inserted VDAC within each membrane. This phenomenon was referred to as "auto-directed insertion".

Studies by Xu *et al.* (Xu and Colombini 1996, Xu and Colombini 1997) expanded on this phenomenon by using native *Neurospora crassa* VDAC to characterize the folding ability of mitochondrial porin in various denaturing and non-denaturing solvents, prior to insertion into the artificial bilayers. In these BLB experiments, membranes were prepared from phospholipids and detergent-solubilized VDAC was added to one side of the compartment (the *cis* chamber). Perfusion of one chamber or the other with 2 M guanidinium chloride or 2 M urea in the presence of VDAC resulted in bursts of channel insertion; all channels were oriented in the same way. When urea solubilized porin was perfused in the

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trans chamber, channel insertion increased by 10-60 fold in comparison to perfusion of the

cis chamber. In contrast, when chambers were perfused with VDAC solubilized in sarcosine,

a natural N-methyl derivative of glycine, channel insertion did not occur, suggesting that the

denaturants enhance an insertion competent conformation of the protein (Xu and Colombini

1996). In bilayers devoid of inserted VDAC, perfusion with 5 M urea or 0.001 % Triton

X100 solubilized VDAC resulted in a longer delays (240 sec) in channel formation than when

added to the opposite side of initial channel addition/ insertion (14 sec) (Xu and Colombini

1997). This suggests that porin insertion is self-catalyzed by previously inserted VDAC and

that this insertion is enhanced by denaturing solvents added to the opposite side of initial

VDAC insertion.

Even though mitochondrial porins appear to possess self-catalyzed auto-directed

insertion ability, questions such as how the initial porin channels form within these artificial

membranes and how their orientation is determined still remain. Additionally, the

concentrations of denaturing solvents used in the experiments by Xu *et al.* (1996, 1997) were

exceedingly high in comparison to those in the OmpA folding studies, where maximum urea

concentrations were in the mM range. However, as for bacterial porins, these experiments

demonstrate that mitochondrial porins solubilized in detergents or denaturing solvents can

insert and form conductive channels in artificial bilayers.

1.10 Thesis Objectives

As discussed in the above sections, the precise topology of mitochondrial porins

within mitochondrial OM remains elusive. It is clear that structural analysis that goes beyond

determining the secondary structure of mitochondrial porin is crucial to the understanding of

the structure and function of this protein. Thus, the overall objective of this study is to

investigate the folded states of mitochondrial porins in membrane mimetic systems. Ultimately, these results obtained from this work may reveal a detergent-based system able to promote a stable, β-rich, folded arrangement of mitochondrial porins suitable for analysis by NMR spectroscopy. Recombinant His_6-tagged *Neurospora crassa* porin (His_6-porin) was used as the starting material, and detergent systems were chosen for porin folding, as they have been used successfully for studies of bacterial β-barrel proteins.

Three specific approaches were used:

i) To examine the folded state of His_6-porin in several single detergent systems.

ii) To identify mixed detergent systems capable of promoting a β-rich folded state of His_6-porin.

iii) To examine the role of sterols on porin arrangement in detergent and lipid-based systems.

The tools used in these investigations were circular dichroism spectropolarimetry in the near and far UV regions, fluorescence spectrophotometry, and ultraviolet (UV) absorbance spectroscopy. Additionally, liposome-embedded His_6-porin was probed by proteolytic digestion, and its channel forming ability measured through liposome swelling assays. These approaches also required the examination of model compounds in detergent, as such reference data were not available. This thesis presents the first extensive characterization of mitochondrial porins in detergent and liposomes, and provides the first system for generating a detergent-folded state of the protein that has strong similarities to the membrane-embedded form.

CHAPTER 2 Spectroscopic analysis of the conformations of mitochondrial porin solubilized in detergent.

The material presented in chapter 2 was written with the intent to submit to the Biophysical Journal in parallel with the material in Chapter 3. I completed all of the experiments outlined in this chapter. The data were analyzed and the manuscript was written with guidance and feedback from my advisor and Dr. J. O'Neil, who are co-authors on the manuscript.

2.1 Abstract

Far-UV circular dichroism (CD) spectropolarimetry has been the predominant tool for direct structural analysis of mitochondrial porins. In this study, the conformation of detergent-solubilized hexahistidinyl-tagged *Neurospora crassa* porin was probed by fluorescence, near-UV CD and UV absorption spectroscopy. N-acetyl and O-alkyl-ester derivatives of tryptophan and tyrosine were examined to model the interactions between the detergents and the amino acid side chains in the protein. Lauryl dimethylamine oxide (LDAO) promotes a β-sheet rich conformation, with most of the tyrosine residues in the hydrophobic interior of the protein-detergent complex. Sodium dodecyl sulfate (SDS) and dodecyl phosphocholine (DPC) promote different amounts of α-helix and β-strand, and tyrosine exposure is higher in SDS. In all three detergents, the two tryptophan residues in porin reside in hydrophobic environments that are weakly asymmetric, suggesting transient tertiary interactions. Porin solubilized in these detergents can be incorporated into liposomes by freeze-thaw methods, and form functional channels. Membrane insertion is accompanied by increased levels of β-strand and loss of protease sensitivity. Analysis of these partially-

folded states that are competent for membrane insertion provided the starting point for mixed

detergent folding systems described in Chapter 3.

2.2 Introduction

Porins, the most abundant proteins in the mitochondrial outer membrane (for example see

Mayer *et al.* 1993), are predicted to span the lipid bilayer as β-barrels (Fig. 2.1), as do their

bacterial namesakes (Mihara and Sato 1985, Kleene *et al.* 1987, reviewed in Bay and Court

2002). The resulting channels permit the mitochondrial entry and exit of molecules,

including substrates and products of the electron transport chain and oxidative

phosphorylation, cofactors for mitochondrial enzymes, nucleotides for RNA and DNA

synthesis, and cytoplasmically synthesized proteins (reviewed in Blachly-Dyson and Forte

2001). Interactions with many of these solutes are known to regulate the pore behaviour in

black lipid bilayers, and presumably they have *in vivo* regulatory activity (Blachly-Dyson and

Forte 2001). Furthermore, mitochondrial porin interacts with cytosolic hexokinase (Arora *et

al.* 1993), and intermembrane space enzymes including creatine kinase (Brdiczka *et al.* 1994).

Together with the inner membrane ADP/ATP carrier and cyclophilin-D, porin is believed to

be a component of the permeability transition pore that may be the conduit for cytochrome *c*

release upon initiation of apoptosis (reviewed by Crompton *et al.* 2002). Interaction partners

in the outer membrane such as the pro- and anti-apoptotic regulators of the Bcl-2 family

(Shimizu *et al.* 1999, Shimizu *et al.* 2000, Vander Heiden *et al.* 2001, Rostovtseva *et al.*

2004) further implicate porin in the regulation of apoptosis, which in turn is critical to key

processes such as development, aging and the destruction of abnormal or virally-infected

cells (Jacotot *et al.* 2000, Jacotot *et al.* 2001, Tsujimoto and Shimizu 2002). To

Figure 2.1. Model for the transmembrane arrangement of *Neurospora* mitochondrial porin (Bay and Court 2002, Runke *et al.* 2006). The N-terminus is at the left and proposed to reside in the intermembrane space. Potential β-strands are indicated by filled rectangles, and loops and turns by thin lines between them. The putative limits of the β-strands are indicated; it should be noted that neither the number of strands nor the precise position of any one strand has been confirmed by high resolution methods. Proteases typsin (cleave protein at K and R residue sites) and chymotrypsin (cleaves protein at W, Y, and F residues sites) have 28 and 29 predicted cut sites respectively located throughout this protein sequence.

CYTOSOL

| N 34 | A 40 | K 64 | T 69 | A 87 | L 94 | K 112 | H 123 | S 152 | Y 165 | N 185 | N 198 | K 212 | E 220 | L 250 | V 255 |
| G 27 | S 47 | L 57 | W 74 | L 80 | N 101 | A 105 | L 130 | E 145 | Y 171 | A 178 | A 203 | W 209 | Y 225 | Y 247 | S 262 |

Y155

Y194
Y193

Y176

Y22

N

C

INTERMEMBRANE SPACE

55
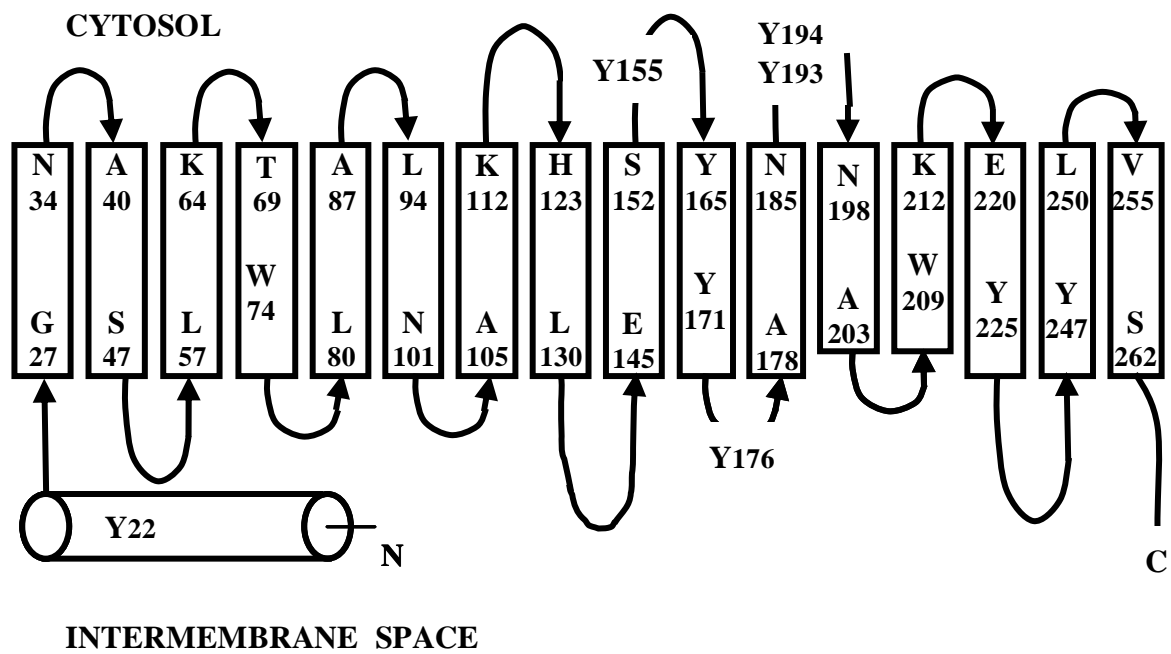
understand the abilities of this protein to form a gated, ion-selective pore and its roles in many important cellular processes, high-resolution structural information is required.

Numerous approaches have been taken to investigate the activity and structure of mitochondrial porin (see Chapter 1, reviewed by Bay and Court 2002). These studies have relied on detergent-solubilized porin, initially isolated from mitochondria in Triton X-100 (Colombini 1980, Roos *et al.* 1982), Freitag *et al.* 1982), lauryl dimethylamine oxide (LDAO; Aljamal *et al.* 1993), or Genapol X-080 (Troll *et al.* 1992). Detergent-solubilized native porin inserts into black lipid bilayers upon the application of voltage and forms conductive channels (Colombini 1980; Freitag *et al.* 1982). The pores are anion-selective in their high conductance open state, but are converted to a slightly cation-selective partially-closed state with increased voltage, leading to their alternative designation as voltage-dependent anion channels (VDAC, Colombini 1980). Detergent resuspended mitochondrial porins reconstituted into liposomes have also demonstrated channel forming activity (Colombini 1980, Colombini *et al.* 1987). These liposome-based systems have been useful for the characterization of pore forming ability by porin and its isoforms (Xu *et al.* 1999, Shao *et al.* 1996, Komarov *et al.* 2004), and to determine influences on channel formation and substrate diffusion of other interacting molecules (Bathori *et al.* 2006, Colombini *et al.* 1987, Madesh and Hajnoczky 2001) or proteins (Shimizu *et al.* 1999, Shimizu *et al.* 2000, Shimizu and Tsujimoto 2000, Gonzalez-Gronow *et al.* 2003).

More recent work has utilized recombinant mitochondrial porins that are isolated from inclusion bodies in *E. coli* and renatured in detergents; these proteins, following pre-incubation with ergosterol or cholesterol, can also be inserted into black lipid bilayers, where they form channels functionally indistinguishable from those of porin isolated from

mitochondria (Popp *et al.* 1996; Popp *et al.* 1997; Koppel *et al.* 1998). Far-UV CD spectropolarimetry has confirmed that the native and recombinant porins from fungal mitochondria and pea root plastid, dissolved in LDAO or Genapol X-080, all have high β-strand content (40-70%; Koppel *et al.* 1998, Popp *et al.* 1997, Runke *et al.* 2000, Runke *et al.* 2006). *N. crassa* mitochondrial porin embedded in liposomes is also β-strand rich, suggesting that the protein in detergent forms β-strands similar to those found in the membrane (Shao *et al.* 1996). However, the tertiary conformations of the protein in these detergents or in liposomes have not been examined and it is not known whether the protein exists as a molten globule under detergent conditions or forms tertiary arrangements expected for membrane reconstituted porins.

X-ray crystallography has been used to investigate the structures of numerous bacterial porins (summarized in Popot and Engelman 2000), and the structures of bacterial outer membrane protein X (OmpX, Fernández *et al.* 2001, Fernández *et al.* 2004) and the transmembrane domain of OmpA (Arora *et al.* 2001) have been determined recently by nuclear magnetic resonance (NMR) spectroscopy. The inherent stability of folded bacterial porins (for example see (Dornmair *et al.* 1990)) aids the acquisition of high (mM) concentrations of folded protein needed for these studies. These approaches have not been applied to mitochondrial porins, likely due in part to difficulties resulting from the conformational instability of the protein in the detergent systems used (Shao *et al.* 1996, Bay and Court, unpublished results). In addition, mitochondrial porins are significantly larger (~30 kDa) than the bacterial porins studied by NMR (~20 kDa).

For mitochondrial porins, spectroscopic analysis of detergent and membrane associated proteins is the first essential step toward determination of the folded state.

Secondary structure predictions and in vitro analyses of wild-type and variant porins have led to models for the transmembrane arrangement of porin (see Fig. 2.1 for one example), but cannot reveal the precise positions of the membrane-spanning segments, loops and termini in the folded protein (reviewed in Bay and Court 2002). Therefore, in this study, mitochondrial porin of *N. crassa* was solubilized in a collection of detergents to examine the conformation of the protein, with the goals of characterizing the folded states of porin solubilized in commonly used detergents and following reconstitution in artificial membranes (liposomes). The ultimate goal is to identify a system suitable for solubilization of high concentrations of folded protein for structural analysis by NMR spectroscopy. The detergents chosen (Genapol X-080 (Troll *et al.* 1992), LDAO (Koppel *et al.* 1998), DPC (Arora *et al.* 2001), OG (Shao *et al.* 1996), DDM (Ahting *et al.* 1999), and SDS (Popp *et al.* 1997) have been used successfully in electrophysiological or structural studies of bacterial or mitochondrial β-barrel proteins.

Several approaches to investigating mitochondrial porin in detergents and artificial membranes were used in this study: secondary structure content and tertiary interactions involving tryptophan and tyrosine were investigated by CD in the far and near UV regions, respectively. CD and fluorescence spectroscopy of membrane-bound porin was used, although there were some limitations due to light scattering by liposomes. Exposure of tyrosine residues to the aqueous environment was estimated by second derivative analysis of UV spectra and the hydrophobicity of the environment surrounding tryptophan residues was determined by fluorescence. Model tyrosine and tryptophan compounds were also investigated in the same detergents, to form a reference dataset useful for the present and future studies of membrane proteins in detergent. Taken together, these studies represent the

first characterization of detergent-solubilized amino acid model compounds and mitochondrial porin by fluorescence and UV absorption, and the first investigation of tertiary interactions in mitochondrial porin reconstituted in detergents and liposomes.

2.3 Materials and Methods

2.3.1 Lipids, detergents, and amino acid derivatives

Egg yolk L-α-phosphatidyl choline, egg lecithin L-α-phosphatidic acid, NAc-W-NH$_2$, NAc-Y-NH$_2$, SDS, ethylene glycol, urea and NAc-W-OEth were obtained from Sigma (St. Louis, MO), DPC, DDM, OG and LDAO from Anatrace (Maumee, OH). BOC-Y-OMe (FLUKA, Oakville, ON) and Genapol X-080 from Caledon Laboratories (Georgetown, ON). CMCs of single detergents in 50 mM sodium phosphate pH 7 (Table 1) were measured for this study using the method of dye solubilization (Kleinschmidt $et\ al.$ 1999).

2.3.2 Expression and purification of His$_6$-porin

The cloning of the cDNA for $N.\ crassa$ mitochondrial porin into the pQE-9 vector (Qiagen, Toronto, ON), which encodes an N-terminal His$_6$-tag has been described previously (Popp $et\ al.$ 1996). Protein expression was carried out using the QIA$express\ E.\ coli$ M15 [pREP4] strain from Qiagen.

The recombinant His$_6$-porin used in this study was expressed in $E.\ coli$ and purified in 8 M urea, using the procedure described in (Runke $et\ al.$ 2000), with the exception that protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The yield of His$_6$-porin was 2-5 mg/L. Porin was eluted from immobilized nickel nitrolotriacetic acid (Ni-NTA) in 8 M urea, 0.1 M EDTA , 0.1 M Na$_2$HPO$_4$, 10 mM Tris•Cl pH 6.8, followed by overnight dialysis in the same buffer lacking EDTA. The protein concentration was

determined using the calculated extinction coefficient at 280 nm of 24,050 M^{-1} cm^{-1}. Protein

purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was

estimated to be at least 95%.

2.3.3 Solvent and detergent solubilization of His$_6$-porin

Dialyzed His$_6$-porin was acetone-precipitated and the dried pellets were resuspended

either in 8 M urea or in aqueous detergent solutions buffered in 50 mM sodium phosphate pH

7. Washing the pellet in acetone to remove urea following the initial precipitation greatly

reduced the solubility of the protein; a requirement for low levels of denaturant for re-folding

the bacterial porin OmpGm has also been reported (Conlan and Bayley 2003). The

resuspended samples were mixed by repeated inversion overnight at room temperature (22°-

25^oC), centrifuged at 10,000 g for 15 minutes, and the optically-cleared supernatants were

collected. Where indicated, protein samples were concentrated by ultrafiltration through

Centricon™ columns (MW cutoff 10,000; Amicon, Inc., Beverly, MA).

2.3.4 Liposome stock preparation

His$_6$-porin was reconstituted into small unilamellar vesicles (SUV) using

modifications of the procedures described by (Shao *et al.* 1996). Briefly, a 10:1 (w/w)

mixture of egg yolk L-α-phosphatidyl choline and egg lecithin L-α-phosphatidic acid

phospholipid was dissolved in chloroform and evaporated in a fumehood at room temperature

(22-25^oC) overnight. Evaporated lipids were resuspended in 50 mM sodium phosphate buffer

at pH 7 or stored at -20^oC in a dessicator until needed. The resulting milky suspension was

diluted 1:1 with phosphate buffer pH 7 and sonicated on ice until the microtip (Fisher

Scientific Sonifier Model 300) could clearly be observed through the haze to yield the stock

SUV suspension. As described in Traikia *et al.* 1997; Traikia *et al.* 2000, the unsonicated

stock solution of dried lipids resuspended in buffer constitutes multilamellar vesicles (MLV)

with diameters usually varying from 1-10 μm. This solution was sonicated to form the stock

SUV solution, which should contain SUVs 30- 60 nm in diameter. Freeze-thaw treatment of

the stock SUV solution would then generate fragile large unilamellar vesicles (LUV) that

tend to break apart and reform SUVs (Traikia *et al.* 1997; Traikia *et al.* 2000). Thus, the

proteoliposomes described in this study are mainly SUVs.

2.3.5 Proteoliposome preparation and swelling assays

Proteoliposomes were prepared based on a modified procedure for Type B (CD grade)

liposomes as described by (Shao *et al.* 1996). In short, the sonicated 20 mg/ml stock

phospholipid was diluted 1:1 with a 1 in 2 dilution of detergent-solubilized His_6-porin in 50

mM sodium phosphate buffer pH 7. Initial detergent concentrations were 3.5 mM SDS, 2

mM DPC, or 7.5 mM LDAO and initial His_6-porin concentrations ranged from 0.015 – 0.030

mg/ml (0.5 to 1 μM). The resulting suspension was subjected to three cycles of freezing in

liquid nitrogen for 1 min and thawing for 20 min at room temperature. Upon the last thawing

in the freeze-thaw cycle, samples were diluted with 1.5 volumes of 50 mM sodium phosphate

pH 7 resulting in a cloudy solution and contained 6.6 mg/ml of total phospholipid. Final

concentrations of detergent in the swelling assay samples were 0.58 mM SDS, 0.33 mM

DPC, and 1.25 mM LDAO, which are below their CMC values (Table 2.1). Final

concentrations of His_6-porin ranged from 2.5 – 5.0 μg/ml (80- 166 nM). Negative controls for

liposome swelling experiments were prepared with detergents only and lacked His_6-porin.

Liposome swelling (Shao *et al.* 1996) was measured on an Ultrospec 3100 pro

spectrophotometer at an absorbance of 400 nm. Liposome samples prepared as described

above were diluted 1/100 into 50 mM sodium phosphate pH 7 buffer to a final volume of 0.5

ml and measured in a 1 cm quartz cuvette. After 300-400 seconds, liposomes were initially

diluted with 40 μl of the iso-osmotic phosphate buffer pH 7, followed by an addition of 40 μl

of the hyperosmotic 1 M sucrose after 600-700 seconds. Liposome re-swelling, is indicated

by a gradual decrease in A_{400nm} according to Bangham's Law described by the equation

$A = k/V$ where UV absorbance (A) is inversely proportional to liposome volume (V) based

on the refractive index of lipid particles (k) (Bangham *et al.* 1978). Liposome re-swelling was

followed for 1200 or 1800 seconds after sucrose addition.

Swelling assays were also performed to assess the ability of urea and detergent-

solubilized His_6-porin to insert into liposomes as described for *E. coli* OmpA (Surrey and

Jahnig 1995). Liposomes for porin insertion experiments were prepared as described above

for liposome swelling except that the stock 20 mg/ml SUV suspension was diluted 1: 1 with

50 mM sodium phosphate buffer pH 7. After the three freeze-thaw cycles, 0.25 volumes of

detergent-resuspended porin and 1.25 volumes of 50 mM sodium phosphate pH 7 were

added, to create a final 1:1.5 dilution. To encourage insertion, samples were incubated at

room temperature, 30°C, and 42°C overnight.

2.3.6 Reconstitution of detergent-solubilized His_6-porin in liposome for spectroscopic

analysis

His_6-porin was resuspended in detergent at concentrations (3.5 mM SDS, 2 mM DPC,

and 7.5 mM LDAO) that upon 1:3 or 1:4 dilution in phosphate buffer resulted in final

detergent concentrations near the CMC (0.9-1.0 mM SDS, 0.6-1.2 mM DPC, and 3.1-3.8 mM

LDAO). His_6-porin concentrations varied from 5-10 μM in the different detergents. The

protein was diluted into liposomes (2 mg/ml) buffered in 50 mM sodium phosphate pH 7.

Liposome-detergent-His$_6$-porin mixtures were then subjected to one of three methods for reconstituting His$_6$-porin: freeze-thaw, sonication, and insertion. The freeze-thaw procedure was essentially as described for liposome swelling. Porin incorporation by sonication of the liposomes/protein/detergent mixture was achieved by sonication on ice in four 1 minute bursts and 30 second rests between bursts until the sample was optically clear. Insertion-type liposomes were created as described above. Following treatment, the resulting proteoliposomes were placed on a rotating mixer overnight at room temperature (22-25°C), followed by centrifugation at 10,000 g for 10 min to remove any precipitated material. Following centrifugation, the pelleted material was resuspended in 8 M urea and measured by UV absorption spectroscopy at 280 nm to determine the amount of insoluble protein, which was then used to estimate the final concentration of His$_6$-porin in the supernatant. In some cases, following spectroscopic analysis, liposome swelling assays of these samples were performed with samples diluted to 0.66 mg/ml phospholipid.

2.3.7 Isolation of *N. crassa* mitochondria

Mitochondria were isolated from *N. crassa 97-20 mus his*$^-$ (Ninomiya *et al.* 2004) strain according to the method described by Harkness *et al.* 1994 (Harkness *et al.* 1994). Briefly, *N. crassa* conidia were used to inoculate Vogel's medium supplemented with (0.2 g/L) histidine and grown at 30°C for 14-16 hrs. Mycelia were filtered out from the media and ground with a mortar and pestle, with 2 ml of SEM (250 mM sucrose, 1 mM EDTA, 9 mM MOPS pH 7.5) and 1.5 g quartz sand added per g of filtered mycelia for 1 min on ice.

Ground *Neurospora* mycelia were centrifuged in a Sorvall SS34 rotor (Mandel, Guelph, ON) at 4,000 rpm, 4°C for 10 min to pellet cell debris and the supernatant was centrifuged at 12,000 rpm for 12 min, at 4°C to pellet mitochondria. Mitochondrial pellets

were resuspended in 1 ml of chilled SEM and brought to 20 ml SEM and centrifugation steps described above were repeated. The final mitochondrial pellet was resuspended in SEM to final concentration of 1 mg/ ml mitochondrial protein as determined by a Bradford assay (Sigma).

2.3.8 Protease digestions of isolated mitochondria and proteoliposomes

Isolated mitochondria (1 mg/ml) in SEM were used directly or microcentrifuged for 3 min at 4°C at 13,000 rpm to pellet mitochondria, which were then resuspended into an identical volume of detergent (3.5 mM SDS, 20 mM DPC, or 50 mM LDAO) buffered at pH 7 with sodium phosphate. Mitochondrial digestions contained 0.5 mg/ml of mitochondria and detergent concentrations of 1.75 mM SDS, 25 mM LDAO, and 10 mM DPC. All samples were digested with 0.15 mg/ml trypsin or chymotrypsin for 10 or 30 min intervals at room temperature.

His$_6$-porin reconstituted in liposomes was digested after spectroscopic examination. Each liposome sample was digested as described for mitochondria and contained 0.008-0.015 mg/ml (0.25- 0.5 μM) His$_6$-porin and 1 mg/ml phospholipid.

Detergent-solubilized His$_6$-porin samples were digested with trypsin and chymotrypsin under the same conditions as for mitochondria except that times were 30 or 60 minutes. The final concentration of His$_6$-porin was 0.075 mg/ml (2.5 μM) and the detergent concentrations were 1.75 mM SDS, 25 mM LDAO, and 10 mM DPC. All protease digestions were arrested by adding phenylmethylsulfonlyfluoride to a final concentration of 20 mM and 4:1 dilution into Laemmli buffer (Laemmli 1970) containing 8 M urea. All reactions were stored at -20°C until loaded onto SDS-PAGE gels containing 2 M urea, which

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was necessary to aid migration of the protein into the gel in the presence of high concentrations of lipid.

2.3.9 Western blots of proteoliposomes and *N. crassa* mitochondria

All protease digested samples were loaded onto 0.1 % SDS- 3 M urea- 14% PAGE gels and electrophoresis was performed with the Mini-Protean® II system (Bio-Rad Laboratories, Hercules, CA). Gel loading was as follows: 5 μl of 0.5 mg/ml mitochondria in SEM or detergent, 10 μl of 7.5- 15 μg/ml His$_6$-porin in 1 mg/ml phospholipids, and 1 μl 75 μg/ml His$_6$-porin in detergent. Gels were blotted with a Trans-Blot® Cell system (Bio-Rad) onto nitrocellulose 0.45 μm membranes (Bio-Rad) overnight at 4°C and stained with Ponceau S (Sigma) to visualise the Benchmark protein ladder (Invitrogen, Burlington, ON).

Immunoblotting was carried out at room temperature using rabbit antibodies directed against residues 7- 20 of *N. crassa* mitochondrial porin (αNcPor-N, generated by R. Lill, at Universität München). Membranes were blocked for 30 minutes with a 5 % (w/v) skim milk/ 10 mM Tris-Cl, 0.15 mM NaCl, pH 7.5 (TBS) and incubated in 5 % (w/v) skim milk/ TBS with a 1: 1000 dilution of anti 7- 20 *N. crassa* mitochondrial porin antiserum for 1 hour. Membranes were washed twice with 0.1 % (v/v) Triton X-100/ TBS for 30 minutes each and twice with TBS for 10 minutes each before soaking in 5 % (w/v) skim milk / TBS with 1: 5000 dilution of anti-rabbit alkaline phosphatase (Sigma) antibody for 1 hour. Blots were washed twice with 0.1 % (v/v) Triton X-100/ TBS and TBS as described above. Membranes were soaked in detection buffer (0.1 M NaCl, 0.1 M Tris-Cl pH 9.5) for 2 minutes and soaked in 1: 100 dilution of CDP-star (Roche Diagnostics, Indianapolis, IN) in detection buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl) for 1 minute. Blots were placed in plastic and exposed

for 1- 2 hrs on X-Omat Blue XB-1 Scientific Imaging film (Kodak, Rochester, NY) before image development.

2.3.10 Fluorescence spectrophotometry

Fluorescence spectroscopic analyses of urea-, ethylene glycol-, and detergent-solubilized model compounds and His_6-porin were performed using a Shimadzu RF-1501 fluorometer or a JASCO-810 (FMO-427S) spectropolarimeter/fluorometer. All emission spectra were measured in a 1-cm path length quartz cuvette after excitation at 275 nm or 296 nm. Detergent- solubilized His_6-porin samples were measured at $A_{280\,nm}$ of 0.01, which corresponds to a protein concentration of 0.42 µM. All His_6-porin-liposome samples were diluted 1:10 in 50 mM sodium phosphate buffer pH 7 to a final liposome concentration of 0.2 mg/ml and His_6-porin concentrations ranging from 0.1-0.2 µM. His_6-porin-liposome samples were normalized to a concentration of 0.42 µM with reference to His_6-porin in urea. Two sets of model compounds were used: $NAc-W-NH_2$ and $NAc-Y-NH_2$, which have been characterized in solvents of varying polarity (Cowgill 1967, Garcia-Borron *et al.* 1982, Nayar *et al.* 2002) and BOC-Y-OMe and NAc-W-OEth, which are more hydrophobic and therefore are more likely to partition into the hydrophobic interior of the detergent micelles. His_6-porin contains two tryptophan and nine tyrosine residues. Samples of individual model compounds, and 2:9 Trp:Tyr mixtures, were prepared at molar concentrations close to those of the individual residues in porin and all of the protein and model compound fluorescence spectra were normalized with reference to His_6-porin in urea. The sums of the spectra obtained from the individual components equalled those from the 2:9 mixtures, indicating a lack of interaction between the fluorophores (for example see Fig. 2.3).

Fluorescence scans were repeated three times and the averaged spectra were used for analysis. Increased light scatter prevented accurate determination of higher wavelength regions 15-20 nm from the excitation wavelength. To determine tyrosine emission maxima in solutions containing both tyrosine and tryptophan, the second derivatives of the averaged spectra were obtained with Jasco Spectra Analysis Software, Version 1.53.04, using data at 1-nm intervals and a window of 9-13 nm, depending on the noise in the spectrum. This analysis was used to aid the determination of λ_{max}Tyr and λ_{max}Trp in the porin spectra (Table 2.3; Garcia-Borron *et al.* 1982)

2.3.11 Ultraviolet (UV) absorption spectroscopy

UV absorption spectra were obtained on an Ultrospec 4000 spectrophotometer, using a 1-cm path length cell and a porin concentration of 5 µM. Six spectra were obtained for each sample and averaged for further analysis. The second derivatives of the averaged spectra were obtained as described above, using data at 0.5 nm intervals and a window of 6.5 nm. Tyrosine exposure (α) was calculated using the method described by (Ragone *et al.* 1984), where r is the ratio of short (283 nm) to long (~291 nm) wavelength trough depths. For a given detergent, $\alpha = (r_n\text{-}r_a)/(r_u\text{-}r_a)$ where, $r_n = r$ for the protein in that detergent, $r_a = r$ for the 2:9 mixture of NAc-W-NH$_2$/NAc-Y-NH$_2$ in EG, and $r_u = r$ for the unfolded protein (in urea). Light scattering in the UV spectrum from 200-270 nm prohibited accurate measurements of the absorption spectra of liposome-His$_6$-porin samples.

2.3.12 Circular dichroism (CD) spectropolarimetry

CD spectra were acquired on a JASCO J-810 spectropolarimeter-fluorometer calibrated with (+)-10-camphorsulfonic acid and purged with N$_2$ at 20 L/min (Manley and

O'Neil 2003). CD spectra of 1.5 μM detergent-solubilized His_6-porin samples and porin-liposome samples containing 0.5-2.0 μM His_6-porin were measured in the far UV region (190-250 nm) using 0.05-0.10-cm path length quartz cuvettes at 22^oC, a scan rate of 10 nm/min and a response time of 8 s. Examination of all porin liposome samples by CD determined that high initial concentrations of His_6-porin in SDS were necessary to maintain 1-2 μM of protein in the SUV samples after centrifugation. CD spectra were corrected by baseline subtraction and were converted to mean residue ellipticity (MRE) according to the formula: $[\theta]_M = M\theta / \{(10)(l)(c)(n)\}$ where $[\theta]_M$ is 10^{-3} deg cm^2 $dmol^{-1}$, M is the molecular weight of His_6-porin (31 402 g/mol), θ is the measured ellipticity in millidegrees, l is the path length of the cuvette in cm (0.1 cm), c is the protein concentration in g /L, and n is the number of amino acid residues in the protein (295). Detergent-solubilized His_6-porin spectra were deconvoluted with the CONTINLL algorithm (Provencher and Glockner 1981, van Stokkum *et al.* 1990) in the DichroWeb package (Whitmore and Wallace 2004). Light scattering by the liposome samples prevented reliable acquisition of spectra below 200 nm and thus prohibited deconvolution with secondary structure prediction algorithms.

Temperature was controlled during thermal denaturation experiments of detergent-solubilized His_6-porin using the Peltier device in the spectropolarimeter. These experiments were carried out with a 1^oC / min ramp speed; θ (in mdeg) was monitored at 208 or 216 nm at 1^oC intervals, and full spectra were collected at 5^oC intervals. Liposome sample experiments were monitored at 217 nm at 1^oC intervals, and full spectra were collected at 10^oC intervals. Using the program *Mathematica*[TM], the resulting denaturation curves were fitted to the equation $Y_{obs} = \dfrac{Y_f + m_f T + (Y_u + m_u T) \bullet e^{(-\Delta H + T\Delta S)/(RT)}}{1 + e^{(-\Delta H + T\Delta S)/(RT)}}$ (Privalov and Potekhin 1986,

Lopez-Llano *et al.* 2004) where Y_{obs} is the observed ellipticity, Y_f and Y_u are the ellipticities of the low and high temperature forms of the protein, respectively, m_f and m_u are the slopes of the curves at low and high temperatures, respectively, R is the gas constant, and ΔH and ΔS are the enthalpy and entropy of unfolding. The equation fits the thermal dependence of the ellipticity to a two-state unfolding transition. $T_m = \Delta H / \Delta S$, where T_m is the midpoint of the transition and the concentrations of folded and unfolded protein are equal. T_m serves as a quantitative indicator of the thermal stability of the protein, as the unfolding reactions are, in most cases, irreversible.

Near-UV (245-330 nm) CD spectra of 33 µM His_6-porin in detergent were measured with a JASCO J-810 spectropolarimeter-fluorometer in a 1-cm path length jacketed quartz cuvette at room temperature, with a scan rate of 2 nm/min, and a response time of 8 s. Spectra of His_6-porin reconstituted in liposomes were measured in a 5-cm path length jacketed quartz cuvette at room temperature with a scan rate of 10 nm/ min, and a response time of 8 seconds. Scans were measured in triplicate for averaging to reduce noise. Molar ellipticity was calculated from the baseline corrected spectra according to the formula: $[\theta] = M\theta / \{(10)(l)(c)\}$ where $[\theta]$ is the molar ellipticity in degrees cm^2 $dmol^{-1}$, θ is the measured ellipticity in millidegrees, l is 1 cm or 5 cm, and c is the protein concentration in g/ L.

2.4 Results

2.4.1 Solubility of His_6-porin in detergent systems

To obtain high concentrations of folded recombinant porin, a practical system for purification and detergent solubilization is needed. Highly purified His_6-porin can be recovered from inclusion bodies at high concentrations (> 1 mg/mL) by extraction from *E.*

coli in urea or guanidinium chloride followed by Ni-NTA affinity chromatography (Popp *et al.* 1996, Koppel *et al.* 1998). Urea was used in this study, owing to the difficulty of spectroscopic analysis of proteins dissolved in guanidinium salts. Given the cost of many detergents, it is impractical to exchange the urea for detergent by dialysis, so the protein was precipitated from urea with acetone, and the resulting pellet dissolved in sodium-phosphate buffered detergent solution. Six detergents, namely SDS, an ionic detergent, LDAO and DPC, zwitterionic detergents, and Genapol X-080, OG, and DDM, non-ionic detergents, were examined for their ability to solubilize acetone-precipitated porin. The results are presented in Table 2.1, along with the measured CMCs of each of the detergents. The highest concentrations of His_6-porin (750 µM) were obtained with SDS. At high concentrations, DPC and LDAO solubilize His_6-porin to concentrations of about 66 µM and 25 µM, respectively; further concentration of the protein by ultrafiltration was not possible due to precipitation of detergent/protein complexes and concentration of the detergent. His_6-porin is least soluble in the non-ionic detergents Genapol X-080, OG, and DDM (~2 µM; Table 2.1); increased concentrations of these detergents do not solubilize higher concentrations of protein. Instability has been reported for bacterial porins dissolved in Genapol X-080 (Conlan and Bayley 2003) and recombinant mitochondrial porins in OG (Koppel *et al.* 1998). DDM- and OG-solubilized His_6-porin rapidly form a visible precipitate during ultrafiltration, suggesting that the protein exists as soluble aggregates in these detergents. Therefore, the non-ionic detergents were not used further in this study.

2.4.2 Reconstitution of His_6-porin into liposomes

Modified type B liposomes (Shao *et al.* 1996) were used for osmotic swelling assays to determine if any of the detergent-solubilized porins studied herein were capable of forming

Table 2.1. Solubility of acetone-precipitated His_6-porin after resuspension in single detergents or solvents in 50 mM sodium phosphate pH 7. The protein concentrations indicated were determined from optically clear samples obtained after centrifugation.

Detergent / Solvent	CMC (mM) in 50 mM sodium phosphate	Concentration (μM) of solubilized His_6-porin
8 M urea		> 1 000
3.5 mM SDS	0.9 - 1.0	66
75 mM SDS	0.9 - 1.0	750
15 mM DM	0.9 - 1.9	2
50 mM OG	12.5 - 15.0	2
18 mM Genapol X-080	0.7 - 1.4	2
50 mM LDAO	3.1 - 3.8	5
300 mM LDAO	3.1 - 3.8	25
10 mM DPC	0.6 - 1.2	5
100 mM DPC	0.6 - 1.2	66

channels in membranes. Following freeze-thaw treatments, all detergent-solubilized His_6-porin preparations were capable of forming pores in liposomes (Fig. 2.2), despite differences in secondary structural content when the protein was solubilized in detergents alone (see below). Liposomes that incorporated His_6-porin in SDS (SDS-His_6-porin-liposomes) demonstrated the most rapid osmotic response to sucrose, with a change of -0.01 $A_{400\ nm}$ over 20 min. Different batches of SDS-His_6-porin-liposomes had the most reproducible swelling patterns of all detergent-solubilized His_6-porin samples examined (data not shown), which may indicate that SDS maintains more protein in a conformation amenable for incorporation into artificial membranes. Heating proteoliposomes to $42^{o}C$ (data not shown) or $65^{o}C$ (Fig. 2.2A) did not abolish osmotic shrinkage but did reduce the swelling phase (to -0.006 $A_{400\ nm}$ over 20 min), which agrees with previous experiments that used liposomes containing mammalian and fungal porins (Bathori *et al.* 1993, Shao *et al.* 1996). Heating SDS-His_6-porin-liposomes to $100^{o}C$ produced non-swelling response patterns (Fig. 2.5A) indistinguishable from SDS-liposomes lacking His_6-porin (data not shown). DPC-His_6-porin-liposomes were also osmotically responsive and heating of these proteoliposomes to $65^{o}C$ resulted in partial loss of the swelling response (Figs. 2.2B). Swelling rates of LDAO-His_6-porin-liposomes were not consistent between batches. In cases where osmotically-responsive liposomes were generated, heating to $65^{o}C$, enhanced the re-swelling phase (Fig. 2.2C), suggesting that increased temperature either drives pore formation by membrane-associated protein, or increases the activity of existing pores. Heating of both DPC- and LDAO-His_6-porin-liposomes to $100^{o}C$ eliminated swelling.

Urea-solubilized *E. coli* OmpA assembles into the membrane upon dilution into a liposome suspension (Surrey and Jahnig 1995). Therefore, swelling assays were also

Figure 2.2. Liposome swelling assays. SDS-His$_6$-porin-liposomes were prepared by the freeze-thaw method **(A-C)** or the insertion method **(D)**. Swelling was measured either directly (solid line) or following heating of the proteoliposomes to 65°C (dashed lines) or 100°C (dotted lines). Liposomes were diluted within 300-400 s by addition of 40 μl of the iso-osmotic phosphate buffer, causing a decrease in A$_{400\ nm}$. Addition of 40 μl of hyperosmotic 1 M sucrose at 600-700 s resulted in liposome shrinkage, and a sharp increase in A$_{400\ nm}$. Reswelling of osmotically responsive proteoliposomes is detected as a gradual decrease in optical density. Relative A$_{400\ nm}$ values were plotted, on the same scale, to allow the individual plots to be clearly discernable from each other.
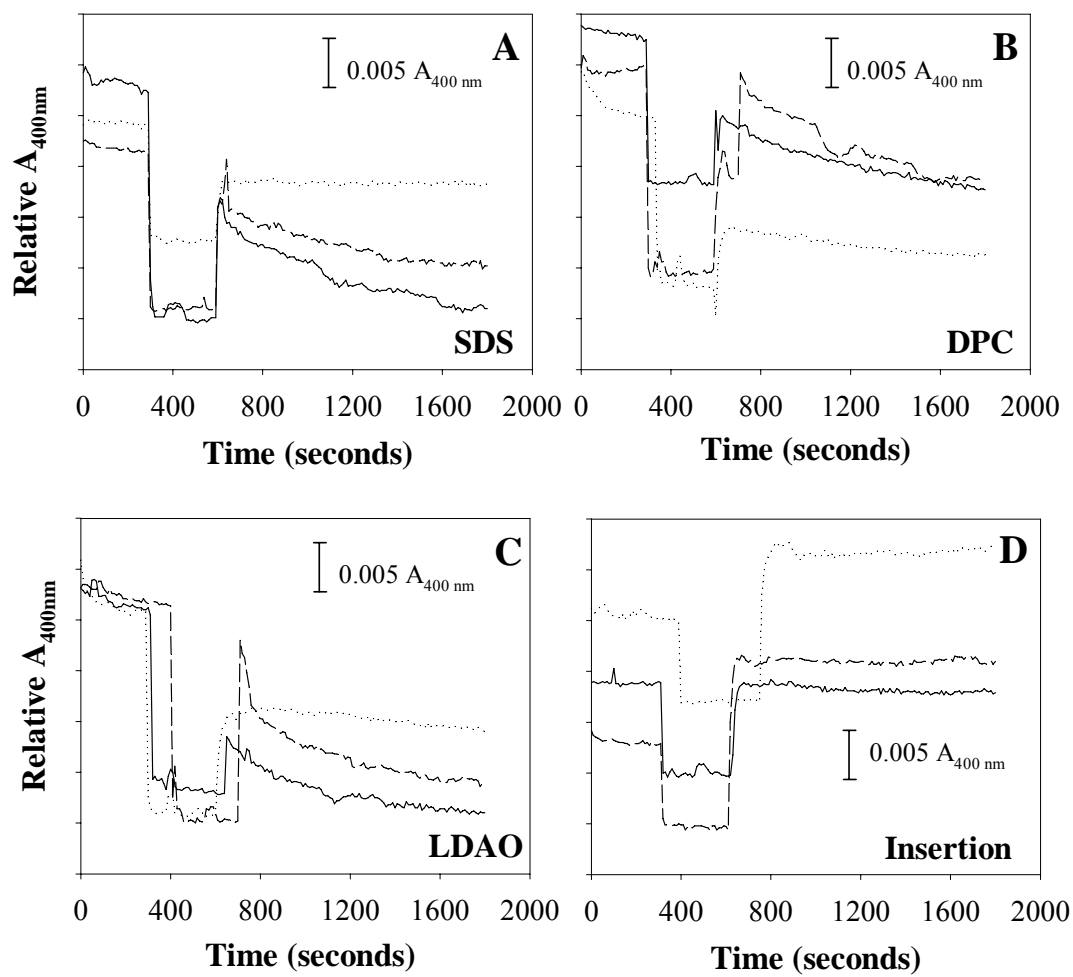
performed to determine if urea- or detergent- solubilized His_6-porin possesses the capacity to insert into liposomes in the absence of membrane disruption by freeze-thaw or sonication. However, urea-solubilized His_6-porin did not produce detectable channels in liposomes and porin was recovered with insoluble material following centrifugation of the sample (data not shown). Swelling was not observed at increased concentrations of His_6-porin (9 μg/ml or 0.3 μM) or increased temperature (30 or 42°C). Incubation of detergent-solubilized porin with preformed liposomes also did not generate osmotically-responsive proteoliposomes.

2.4.3 Protease sensitivity of mitochondrial porin and detergent-solubilized His_6-porin reconstituted into liposomes

Protease sensitivity is frequently used as a criterion for refolding of bacterial porins, such as OmpG in detergent (Conlan and Bayley 2003) or OmpA following reconstitution into artificial membranes (Dornmair *et al.* 1990, Kleinschmidt and Tamm 1996). *Neurospora* mitochondrial porin assembled in the outer membrane also displays resistance to high levels of protease (Freitag *et al.* 1982). Therefore, protease sensitivity of native mitochondrial porin, and detergent-solubilized His_6-porin prior to and following reconstituting into liposomes was examined. Immunoblotting was performed to allow detection of the low amounts of His_6-porin (< 2 μM) maintained in liposomes and of porin in the mixture of mitochondrial proteins. Trypsin (T) and chymotrypsin (C) were also detected as 24 or 25-kDa bands on the blots due to sequence similarity between T and C and the porin peptide (residues 7-20) used to generate antibodies for this study.

Western blots of undigested *N. crassa* mitochondria in SEM show an intense band of intact porin at 30 kDa (Fig. 2.3A); the weak band at 28 kDa likely arises from slight

proteolysis of porin during isolation or incubation at room temperature in the absence of added protease. The full-length protein is highly resistant to digestion with T or C (Fig. 2.3A). Following a 30 min digestion with 0.15 mg/ml T or C the intensity of the 30-kDa band is unchanged (Fig. 2.3A). Undigested 3.5 mM SDS solubilized mitochondria gave rise to four bands; the major one of 30 kDa corresponds to intact monomeric porin and the minor 28 kDa, 21 kDa and 20 kDa bands are proteolytic fragments resulting from the activity of mitochondrially-associated proteases during the 30-min incubation at room temperature (Fig. 2.3A). The presence of these fragments only in the SDS-solubilized mitochondrial preparations suggests that porin is arranged in a more protease sensitive conformation in this detergent. This is confirmed by the complete absence of the 30 kDa band after 30 min of digestion by T and C (Fig. 2.3A). Western blots of undigested or T or C digested mitochondria resuspended in either 50 mM LDAO (data not shown) or 20 mM DPC (Fig. 2.3A) also demonstrated a single band at the molecular mass of 30 kDa when immunoblotted with αNcPor-N. Longer digestions with T or C did not reduce the intensity of the 30 kDa band (data not shown).

Protease-sensitivity of His_6-porin in liposomes was also investigated. His_6-porin migrates more slowly than mitochondrial porin (30 kDa) and has an apparent molecular mass of 32 kDa. A small amount of a 28-kDa proteolytic fragment was also seen. Remarkably, undigested FT SDS-His_6-porin-liposomes also contained two additional species of molecular masses of approximately 90 and 65 kDa (Fig. 2.3B), suggestive of trimers and dimers of the protein. Following digestion with trypsin, the majority of the full-length protein remained, but the putative oligomers were no longer detectable. Similar results were obtained for FT

Figure 2.3. Protease sensitivity of mitochondrial porin. **A)** *N. crassa* mitochondria (2.5

µg/lane) were resuspended in buffer alone (SEM), 3.5 mM SDS or 20 mM DPC and

incubated at room temperature for 30 min in the absence (-) or presence of 0.75 µg of trypsin

(T) or chymotrypsin (C). The last lane contains trypsin only, which is indicated by an

asterisk. Following electrophoresis through urea-SDS-PAGE gels, samples were

immunoblotted and probed with αNcPor-N antibodies. **B)** His_6-porin in liposomes. His_6-

porin was solubilized in either SDS or DPC and incorporated into liposomes by freeze-thaw

(FT), insertion (IN) or sonication (SO), as described in Materials and Methods. Samples were

incubated in the absence (–) or presence (+) of 7.5 µg trypsin for 30 min prior to analysis as

described in A). Arrow, position of wells in the gel; filled circles, multimers of His_6-porin,

open circle, minor degradation product of His_6-porin; asterisks, cross-reacting trypsin. **C)**

Detergent-solubilized His_6-porin. His_6-porin was solubilized in 3.5 mM SDS, 20 mM DPC or

50 mM LDAO and subjected to trypsin digestion as described for panels A and B. An image

of the Coomassie Blue stained urea-SDS-PAGE gel is shown. In all panels, the positions of

the marker proteins in the Benchmark^{TM} ladder (Invitrogen) are indicated to the left of each

panel.

A Mitochondria
   SEM   SDS   DPC
   - T C - T C - T C   T

50
40
30
25
20
10
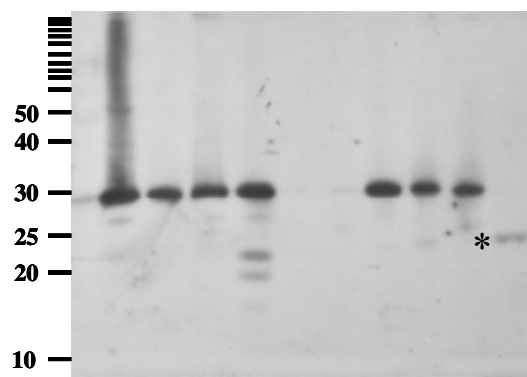
B Liposomes
   SDS   DPC
   FT IN SO   FT IN SO
   - T - T - T   - T - T - T

220
100
80
60
50
40
30
25
20
15
10
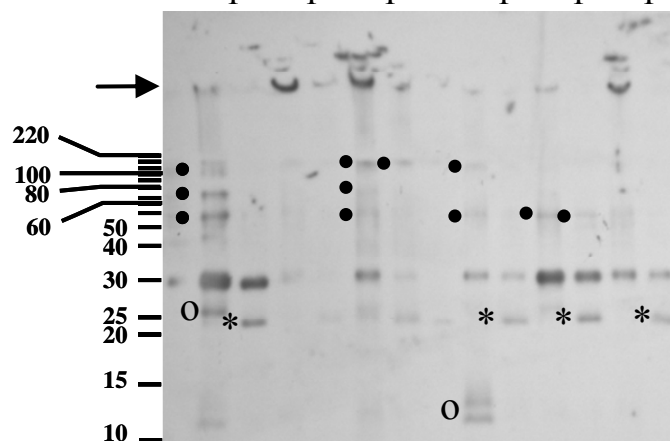
C Detergent
   SDS   DPC   LDAO
   - T C - T C T C

45
36
29
24
20
14
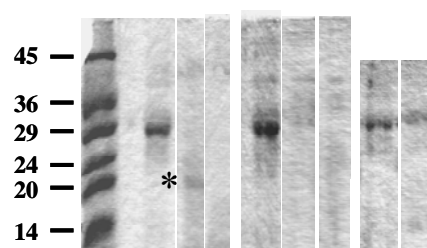
DPC-His$_6$-porin-liposomes, except that the band intensity was about half of that of the SDS-containing samples, likely due to accumulation of protein in the wells during electrophoresis (Fig. 2.3B). In addition, the 90-kDa band species was present in lower amounts in the DPC-dissolved protein.

Analysis of His$_6$-porin protease resistance by immunoblotting of INS and SON SDS-His$_6$-porin-liposomes proved difficult due to the high amount of His$_6$-porin retained in the wells of the gel (Fig. 2.3B). In both samples, some of the 32 kDa His$_6$-porin band remained following digestion with either trypsin or chymotrypsin (Fig. 2.3B). Immunoblots of undigested INS and SON DPC-His$_6$-porin-liposomes showed bands at molecular masses of 65 and 32 kDa, corresponding to dimeric and monomeric forms of His$_6$-porin (Fig. 2.3B). Interestingly, the 90 kDa band was not observed in undigested DPC-His$_6$-porin-liposome samples but this may be due to a reduced amount of His$_6$-porin that entered the gel. T and C digestion of INS and SON DPC-His$_6$-porin-liposomes resulted in two signals on the blot, an intense 32-kDa His$_6$-porin band and a minor 28-kDa species (Fig. 2.3B). Similar results were obtained for INS and SON LDAO-His$_6$-porin liposomes (data not shown). Thus, at least some of the protein in both the INS and SON LDAO- and DPC-His$_6$-porin-liposomes acquires protease-resistance upon introduction following incubation with liposomes in the presence or absence of sonication.

The ability to maintain high concentrations of His$_6$-porin in detergent alone enabled protease digests to be examined on Coomassie blue stained SDS-PAGE gels. Undigested His$_6$-porin solubilized in 3.5 mM SDS, 20 mM DPC and 50 mM LDAO resulted in a single band at 32 kDa (Fig. 2.3C). High molecular weight bands at 90 or 65 kDa were not observed on these gels and subsequent western blots also lacked these bands (data not shown). The 32-

kDa band was retained in LDAO-solubilized His$_6$-porin samples following digestion with T or C after 0.5, 1, and 2 hrs (data not shown). 20 mM DPC- and 3.5 mM SDS-solubilized His$_6$-porin was highly sensitive to proteolysis by both T and C (Fig. 2.3C).

2.4.4 Characterization of His$_6$-porin reconstituted into liposomes by far-UV CD

Circular dichroism spectropolarimetry in the far UV region (200-250 nm; Fig. 2.4) was used to determine the secondary structural content of His$_6$-porin reconstituted by several different methods into liposomes. CD spectropolarimetry measures differences in absorption of left handed versus right handed circularly polarized light by asymmetric molecules. In the far UV region, the chromophore is the peptide bond and characteristic CD spectra arise for protein folded in secondary structures (Johnson 1990). SDS-His$_6$-porin liposomes were the only ones to maintain a high enough concentration of protein for examination by CD spectropolarimetry. Although light scattering by the liposomes prohibited collection at wavelengths below 200 nm, SUVs similar to those used here have been shown to minimize distortions from differential light scattering and absorption flattening observed in CD spectra of membrane suspensions (Wallace and Mao 1984, Mao and Wallace 1984).

The far-UV CD spectrum of FT SDS-His$_6$-porin-liposomes indicated that the protein was rich in β-strands, based on the sharp λ_{min} at 217 nm (Fig. 2.4B). This spectrum was similar to that obtained from native *N. crassa* mitochondrial porin reconstituted into liposomes (Shao *et al.* 1996). CD spectra of SDS-His$_6$-porin-liposomes prepared by INS and SON had broader λ_{min} values also centred around 217-218 nm (Fig. 2.4C and 2.4D), which may reflect differences in β-strand arrangements since the latter preparations were incapable of producing pores by osmotic swelling assays.

Figure 2.4. Far-UV CD analysis and thermal denaturation of porin reconstituted from SDS into liposomes. Proteoliposomes were prepared as described in Materials and Methods. His$_6$-porin concentrations ranged from 1-2 μM in final concentrations of 0.9 mM SDS. **A)** Thermal denaturation of FT (upper solid line), INS (dotted line) and SON (lower solid line) type SDS-His$_6$-porin-liposomes was followed at MRE of 217 nm. Filled symbols indicate the MRE following cooling of the sample to 20°C: SON, square; INS, circle; FT, triangle. **B-D)** Full CD spectra obtained during the experiments shown in A). Spectra were taken at 20°C (solid line), 60°C (short dashed line), and 90°C (long dashed line) and following cooling back to 20°C (dotted line). **B)** FT, **C)** SON and **D)** INS-type proteoliposomes were used for these experiments.
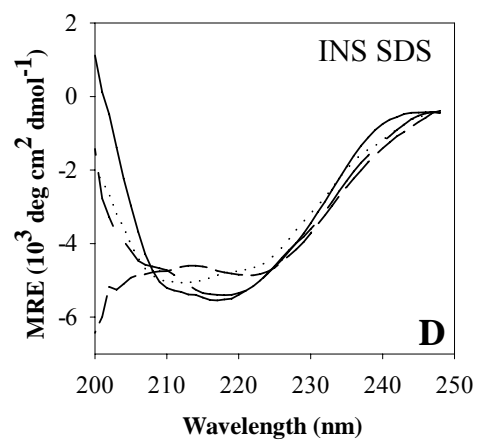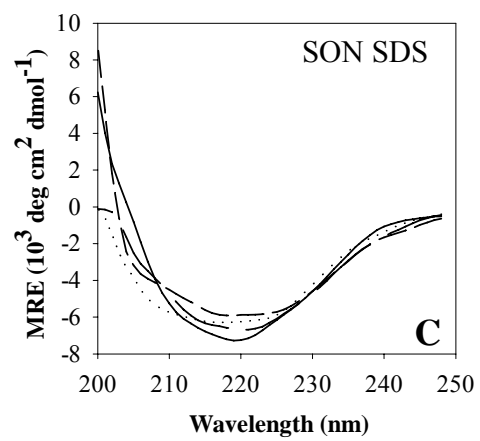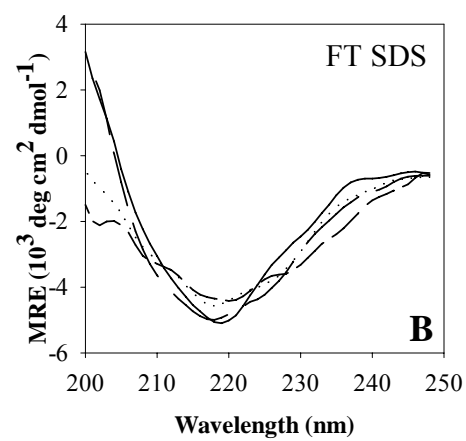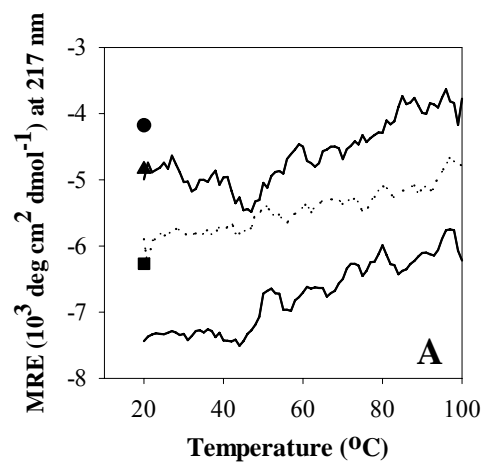
The significant conformational alterations from the α-helical (λ_{min} at 208 nm with a shoulder at 220 nm) spectra in SDS (Fig. 2.5A) to high β-strand content (λ_{min} at 217 nm) in liposomes (Fig. 2.6) indicates that His_6-porin underwent a massive conformational change upon interaction with the artificial membrane. The role of the lipid in this change is further supported by the observation that dilution of His_6-porin in 3.5 mM SDS with phosphate buffer to an SDS concentration of 0.9 mM resulted in precipitation of porin, not β-strand formation. Also, CD spectra of His_6-porin FT liposomes prepared in the presence of 3.5 mM

SDS produced spectra characteristic of those from porin in SDS alone (with λ_{min} at 208 nm, data not shown). Hence, the presence of phospholipid and detergent at or below the CMC, are required for development of β-strands. Thermal unfolding of SDS-His_6-porin-liposomes was performed to assess the stability of the protein secondary structure within the membrane. Thermal denaturation of FT-SDS-His_6-porin-liposomes produced a gradual increase in ellipticity at temperatures above 50^oC and the ellipticity did not change upon cooling from 100^oC to room temperature (Fig. 2.4A and 2.4B). CD spectra (200- 250 nm) of FT-SDS-His_6-porin-liposomes did not change upon heating from 20°-50^oC (Fig. 2.4B). The CD spectra measured at 60^oC and above demonstrated gradual broadening at the λ_{min} with slight reductions in negative ellipticity at the λ_{min} and larger decreases at wavelengths below 210 nm. Despite λ_{min} broadening and spectrum flattening, it appears that β-strand content was maintained at high temperatures (90^oC, Fig. 2.4B). As observed for thermal denaturation curves monitored at 217 nm, the full length CD spectrum of 20^oC cooled FT SDS-His_6-porin-liposomes was very similar to the spectrum obtained at 100^oC except at wavelengths < 205 nm and indicates that heating the protein results in some irreversible alterations to secondary structure content.

Thermal denaturation profiles monitored at 217 nm of SON-SDS-His$_6$-porin-liposomes (Fig. 2.4C) resemble those of the FT-SDS-His$_6$-porin-liposomes where ellipticity values remain unchanged upon heating to 45°C and heating the protein above 50°C results in a gradual reduction in negative ellipticity (Fig. 2.4D). A constant gradual decrease in negative ellipticity was observed for INS-SDS-His$_6$-porin-liposomes upon heating from 20°C to 100°C (Fig. 2.4D). Irreversible changes in conformation occurred at high temperatures since ellipticity values did not change upon cooling of both SON- and INS-SDS-His$_6$-porin-liposomes to 20°C (Fig. 2.4A). Above 40°C, CD spectra of SON-SDS-His$_6$-porin-liposomes were broader around λ_{min} and a small shoulder appeared at 205 nm (Fig. 2.4C). In contrast, CD spectra of INS-SDS-His$_6$-porin-liposomes demonstrated this 205 nm shoulder at temperatures above 30°C and a shift in the λ_{min} from 217 to 209 nm occurred above 70°C (Fig. 2.4D), indicating an alteration to a more α-helical type conformation. Cooling both SON- and INS- SDS-His$_6$-porin-liposome preparations to 20°C resulted in CD spectra intermediate to the 60°C and the 90°C spectra of each preparation (Fig. 2.4C and 2.4D).

Overall, CD spectra of His$_6$-porin reconstituted into liposomes by freeze-thaw have sharp λ_{min} and curves characteristic for high β-strand content. CD spectra of SON and FT SDS-His$_6$-porin-liposomes maintain β-strand rich curves at high temperatures in comparison to spectra of INS SDS-His$_6$-porin-liposomes. Combined with swelling data, these observations suggest that FT His$_6$-porin-liposomes contain temperature-tolerant β-barrels.

2.4.5 Characterization of detergent-solubilized His$_6$-porin by far-UV CD

Circular dichroism spectropolarimetry in the far UV region (190-250 nm; Fig. 2.5) was used to compare the secondary structures of His$_6$-porin dissolved in urea and in each of the detergents with those in liposomes. The results presented in Table 2 were obtained by

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deconvolution of the CD spectra with CONTINLL and similar values were obtained using SELCON3, CDSSTR (Whitmore and Wallace 2004) and the convex constraint algorithm (CCA, Perczel *et al.* 1992) (data not shown). As expected in a denaturing system, the CD spectrum of His_6-porin in 8 M urea (Fig. 2.5A) shows little or no ellipticity above 210 nm, indicating a lack of secondary structure in the protein; this spectrum was not amenable to deconvolution. In contrast, significant secondary structure was observed in the detergent-solubilized porins. His_6-porin dissolved in 3.5 mM SDS (Fig. 2.5A) and in 50 mM SDS (data not shown) exhibit the characteristic α-helical spectrum with a minimum near 208 nm and a shoulder near 222 nm as reported by others (Shao *et al.* 1996, Popp *et al.* 1997, Koppel *et al.* 1998). Deconvolution of the spectrum yields 23% α-helix and 20% β-strand, with the remainder existing as β-turns and unordered structure (Table 2.2). The stability of the SDS-solubilized protein was assessed by thermal unfolding (Fig. 2.5B and 2.5C). Upon heating to $95^{o}C$, a linear increase in ellipticity at 208 nm was observed, indicating that a cooperative unfolding process did not occur (Fig. 2.5B). Slight shifts in the entire far UV CD spectrum were observed as temperature increased (Fig. 2.5C), and deconvolution revealed a small loss of α-helix (23% to 17%) with a concomitant increase in random structure, and little change in β-sheet content at $60^{o}C$, but not at $95^{o}C$ (Table 2.2). Upon cooling back to $20^{o}C$, the MRE at 208 nm returned to its original value (Fig. 2.5B), and the CD spectrum was indistinguishable from that obtained prior to heating (Fig. 2.5C), indicating that the protein regained its original structure (Table 2.2). Overall, the protein had greater temperature stability in liposomes than in detergent, as λ_{min} of ~ 217 nm was maintained at high temperatures (Fig. 2.4D) and only a slight linear increase in ellipticity at the minimum was observed (Fig. 2.4A).

Figure 2.5. Far-UV CD spectra of His$_6$-porin in detergent. **A)** Spectra were obtained, at 20°C, from 1.5 μM samples of porin solubilized in 8 M urea (dotted), 50 mM LDAO (short dashed), 20 mM DPC (long dashed), and 3.5 mM SDS (solid line). Melting curves for 1.5 μM His$_6$-porin in **B)** 3.5 mM SDS or **D)** 50 mM LDAO (open triangles) or 20 mM DPC (open circles) were generated by following the MRE at 208 nm in **B)** or 216 nm in **D)**. The solid lines in **D)** are corresponding thermal denaturation curves obtained as described in Materials and Methods. The circle in **B)** and the filled triangle and circle in **D)** indicate the MRE at the indicated wavelength upon cooling of the protein from 95°C to 20°C. **C)** Spectra of the SDS-solubilized protein at 20°C (solid line), 60°C (short dashed), 95°C (long dashed), following cooling to 20°C (dotted) are presented. **E)** and **F)** Spectra of the DPC- and LDAO-solubilized protein**.** Lines indicate the same temperatures as in **C)**, except that the short dashed line in D) represents 80°C.
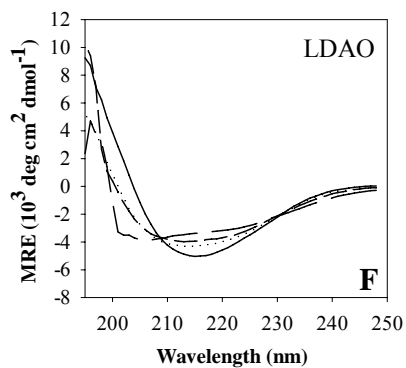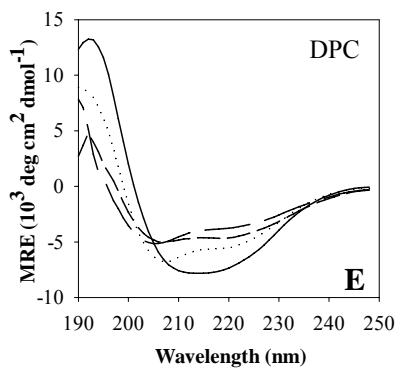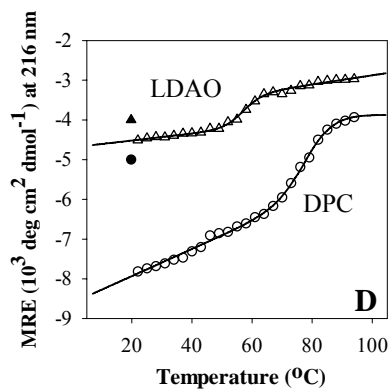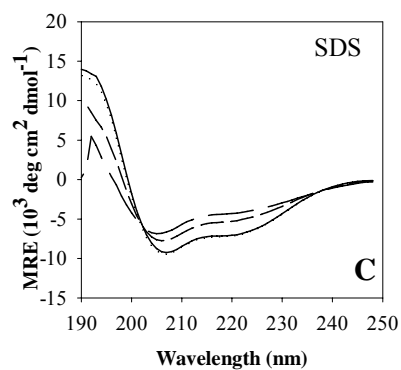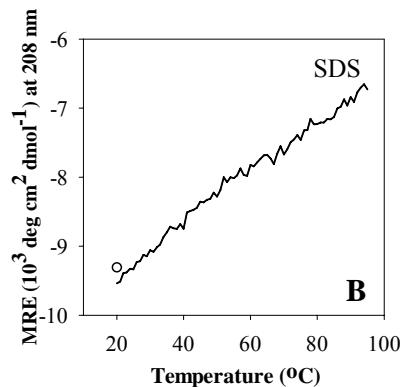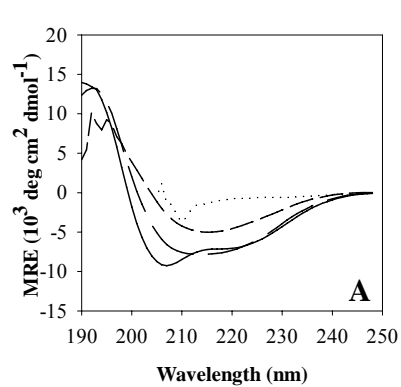
The spectra of the protein solubilized in 10-20 mM DPC exhibit a broad minimum extending from 214 to 218 nm (Fig. 2.5A), whereas in 100 mM DPC, the spectrum has the characteristic minima of an α-helical rich protein (data not shown). However, deconvolution of the spectra suggests that both preparations contain the same α-helix content (16-17%) but that at low detergent concentrations there is a small increase in β-sheet content (30%) compared to higher detergent concentrations (26%).

To determine the stability of the protein in 20 mM DPC thermal denaturation was followed at 216 nm. The sigmoidal melting curve suggests a two-state cooperative transition and curve-fitting as described in Materials and Methods yields a Tm of 79.0^{o}C (Fig. 2.5D and 2.5E). Deconvolution of the spectra showed a loss of α-helix and a concomitant increase in random structure; β-sheet content was unchanged. Protein precipitation occurred during heating; a visible precipitate was observed at the end of the experiment and an increase in absorption, indicating light scattering was noted near 90°C. The heat denaturation was not completely reversible; the protein refolded to a conformation similar to that at the midpoint of the denaturation curve (Fig. 2.5; Table 2.2). At the conclusion of the experiment, precipitated protein was removed by centrifugation and $A_{280\ nm}$ measurements indicated that approximately 2/3 of the protein remained in solution.

The CD spectra of porin dissolved in LDAO exhibit a single broad minimum at 216 nm, characteristic of proteins with predominantly β-strand secondary structure (Fig. 2.5F). Deconvolution of the spectrum confirmed that in 75 mM LDAO the β-strand is highest (39%). Higher LDAO concentrations appear to promote more β-structure than do lower concentrations (Table 2.2). During thermal unfolding, the CD spectra of the LDAO-

Table 2.2. Analysis of His$_6$-porin solubilized in urea or detergents. Far-UV CD spectropolarimetry was performed on 1.5 μM porin samples; the resulting spectra were deconvoluted with CONTINLL (Whitmore et al 2004). The CD spectra collected at 20°C were repeated at least three times and the standard deviations were less than 1%. The average value is presented. The thermal denaturation CD experiments were carried out once.

Detergent / Solvent	Temp (^{o}C)	α-helix (%)	β-strand (%)	turn & unordered (%)
8 M urea	20°C	n/d	n/d	n/d
50 mM LDAO	20°C	16	32	53
75 mM LDAO	20°C	19	39	42
3.5 mM SDS	20°C	23	20	56
	60°C	17	23	59
	95°C	24	19	64
	95→20°C	24	20	57
50 mM SDS	20°C	31	21	48
10 mM DPC	20°C	16	30	54
100 mM DPC	20°C	17	26	57
20 mM DPC	20°C	20	29	52
	80°C	10	34	57
	95°C	11	29	60
	95→20°C	17	25	58

n/d, not determined

solubilized protein undergo a small change in MRE at 216 nm, and the spectrum broadens in this region, indicating a decrease in ordered structure. As in DPC, the melting curve was sigmoidal, indicating a two-state cooperative transition, but in LDAO the T_m was lower (57^oC). High temperature spectra were not deconvoluted because data could not be collected to 190 nm, likely due to increased light scattering. Unlike the case with DPC, a visible precipitate was not observed after heating but nonetheless, the folding is irreversible, and the spectrum after cooling resembles that observed near the midpoint in the denaturation curve (60^oC).

2.4.6 Fluorescence of tryptophan model compounds in detergent

Fluorescence spectrophotometry probes the environments surrounding both tyrosine and tryptophan residues in protein. Initially, to determine the influence of detergent headgroups and acyl chains on fluorescence of His$_6$-porin, Trp and Tyr model compounds were examined. The fluorescence properties of the model compounds reflect their partitioning among the microenvironments in an aqueous detergent solution, namely water, the micelle surface, and the hydrophobic interior (Spyroacopoulos and O'Neil 1994). Compounds of differing hydrophobicities were used, individually, and in the 2:9 molar ratio that is present in mitochondrial porin. Pairs of model compounds were chosen, with differing hydrophobicities, to probe different microenvironments in the detergent solutions. The amide compounds NAc-Y-NH$_2$ and NAc-W-NH$_2$ are commonly used as models for aromatic amino acids in proteins (for example Cowgill 1968, Garcia-Borron *et al.* 1982, Lee and Ross 1998, Nayar *et al.* 2002, Noronha *et al.* 2004, Guzow *et al.* 2005). BOC-Y-OMe (N-t-butyloxycarbonyl-D-tyrosine-methyl ester) and NAc-W-OEth (N-Acetyl L-tryptophan ethyl ester) are more hydrophobic than the standard N-acetyl derivatives and therefore were chosen

as better probes of the interiors of detergent micelles. As seen in Table 2.3 and discussed below, each pair of model compounds had similar fluorescence properties in phosphate buffer and the commonly used solvents ethylene glycol and methanol hence comparisons between the two model compounds can be made.

Tryptophan fluorescence following excitation at 296 nm will be described first, with respect to the classification system devised by Burstein and colleagues to describe the microenvironments of Trp in proteins (Burstein *et al.* 1977, Burstein *et al.* 1973, Reshetnyak *et al.* 2001). Tryptophan in a highly hydrophobic environment without any possibility for hydrogen bonding exhibits highly quenched fluorescence at about 307-308 nm (Class A). Tryptophan in a slightly more polar environment exhibiting more flexibility and hydrogen bonded in a 1:1 exiplex fluoresces at 316 nm (Class S), whereas Trp hydrogen-bonded in a 2:1 exiplex fluoresce at about 331 nm (Class I), with higher efficiency. Tryptophans that are partially exposed to solvent in flexible regions of a protein fluoresce near 342 nm (Class II) and tryptophan in model compounds and unfolded proteins that are highly exposed to bulk water fluoresce at 347-352 nm (Class III).

Phosphate buffered aqueous solutions were used by Cowgill for the fundamental series of experiments characterizing Trp and Tyr model compounds and protein fluorescence (for example Cowgill 1966, Cowgill 1967, Cowgill 1968, Cowgill 1968, Cowgill 1970). Eight molar urea is commonly used to unfold proteins and expose their constituent amino acids to the surrounding aqueous environment (Cowgill 1966), although recent work has demonstrated that transient structure may exist under these conditions (Whittington *et al.* 2005). Both Trp model compounds display class III fluorescence in phosphate buffer with λ_{max}Trp of 350-351 nm, similar to that in urea (Table 2.3). The relative fluorescence of the

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model compounds in urea is about twice as high as that in phosphate buffer; increases have been reported by others (14-20% increase, Eftink 1994, McGuire and Feldman 1973). In both solvents, N-Ac-W-OEth fluorescence is about half of that of N-Ac-W-NH$_2$. Cowgill (Cowgill 1970) reported reduced fluorescence efficiency of N-acetyltryptophan methyl ester (0.5) compared to N-acetyltryptophan (1.4), although no data for N-Ac-W-NH$_2$ were provided.

Ethylene glycol is frequently used to mimic the hydrophobic interior of a protein (DEC ~ 38.7; Havel 1996 and methanol is commonly used to measure the effects of a nonpolar solvent on model compounds (DEC ~33; Lee and Ross 1998. λ_{max}Trp of the model compounds is blue-shifted in both solvents (338-344 nm, Class II) compared to that in urea and phosphate buffer. The blue-shift of λ_{max}Trp is slightly greater in methanol, compared to EG, as expected for a less polar solvent, but relative emission in EG is higher, perhaps due to effects of solvent viscosity on fluorescence quenching, as has been observed for tyrosine (Noronha *et al.* 2004. In methanol and EG, the fluorescence intensities of both model compounds are very similar to each other whereas in urea, there is a two-fold difference in intensity.

Comparisons of the fluorescence spectra of the model compounds in the solvents described above to those of detergent-solubilized compounds provide information regarding both the hydrophobicity of the microenvironment surrounding the molecule, and the quenching of fluorescence by detergent moieties. In both 50 mM LDAO and 20 mM DPC, λ_{max}Trp (337-343 nm) was in the range observed for both compounds in methanol and EG (338-344 nm), indicating that N-Ac-W-OEth and N-Ac-W-NH$_2$ partitioned significantly into the hydrophobic interiors of the detergent micelles. In these detergents the λ_{max}Trp of N-Ac-

Table 2.3. Summary of fluorescence data collected for His_6-porin and amino acid model compounds solubilized in various detergents or solvents. Intesity data were normalized to that of His_6-porin in 8 M urea.

		Fluorescence			
		Tyr (Ex 275 nm)		**Trp (Ex 296 nm)**	
Detergent or Solvent	**His$_6$-porin or Model Compound**	λ_{max}	**Int$_{max}$**	λ_{max}	**Int$_{max}$**
8 M Urea	His$_6$-porin	299	nd*	348	0.06
	NAc-Y-NH$_2$	300	0.18		
	BOC-Y-OMe	300	0.21		
	NAc-W-NH$_2$			350	0.17
	NAc-W-OEth			351	0.10
	W-NH$_2$ + Y-NH$_2$†	301	nd	350	0.02
	W-Oeth + BOC-Y‡	301	nd	352	0.13
Phosphate buffer	NAc-Y-NH$_2$	301	0.07		
	BOC-Y-OMe	300	0.08		
	NAc-W-NH$_2$			351	0.11
	NAc-W-OEth			351	0.04
	W-NH$_2$ + Y-NH$_2$	302	nd	350	0.10
	W-Oeth + BOC-Y	300	nd	350	0.05
Ethylene gylcol	NAc-Y-NH$_2$	302	0.68		
	BOC-Y-OMe	302	0.54		
	NAc-W-NH$_2$			344	0.28
	NAc-W-OEth			344	0.20
	W-NH$_2$ + Y-NH$_2$	301	nd	345	0.30
	W-Oeth + BOC-Y	300	nd	343	0.24
Methanol	NAc-Y-NH$_2$	301	0.25		
	BOC-Y-OMe	300	0.21		
	NAc-W-NH$_2$			341	0.07
	NAc-W-OEth			338	0.07
	W-NH$_2$ + Y-NH$_2$	301	nd	339	0.13
	W-Oeth + BOC-Y	300	nd	338	0.09

Table 2.3, continued

3.5 mM SDS	His$_6$-porin	303	nd	336	0.03
	NAc-Y-NH$_2$	301	0.13		
	BOC-Y-OMe	299	0.12		
	NAc-W-NH$_2$		0.26	351	0.11
	NAc-W-OEth		0.10	347	0.04
	W-NH$_2$ + Y-NH$_2$	301	0.29	350	0.12
	W-Oeth + BOC-Y	300	0.15	346	0.06
50 mM SDS	His$_6$-porin	302	0.09	336	0.03
	NAc-Y-NH$_2$	299	0.10		
	BOC-Y-OMe	301	0.10		
	NAc-W-NH$_2$		0.15	345	0.07
	NAc-W-OEth		0.07	341	0.03
	W-NH$_2$ + Y-NH$_2$	300	0.16	345	0.07
	W-Oeth + BOC-Y	301	0.10	342	0.04
50 mM LDAO	His$_6$-porin	bd§	bd	333	0.08
	NAc-Y-NH$_2$	302	0.12		
	BOC-Y-OMe	299	0.02		
	NAc-W-NH$_2$		0.36	349	0.23
	NAc-W-OEth		0.26	345	0.17
	W-NH$_2$ + Y-NH$_2$	301	0.40	351	0.25
	W-Oeth + BOC-Y	300	0.27	345	0.17
20 mM DPC	His$_6$-porin	299	0.26	343	0.06
	NAc-Y-NH$_2$	301	0.19		
	BOC-Y-OMe	301	0.16		
	NAc-W-NH$_2$		0.43	348	0.25
	NAc-W-OEth		0.35	343	0.22
	W-NH$_2$ + Y-NH$_2$	301	0.43	350	0.24
	W-Oeth + BOC-Y	301	0.37	343	0.22

Liposomes	**Method**				
SDS	Deterget Alone	303	nd	336	0.03
SDS	FT	hs ¶	hs	329	0.04
SDS	SON	hs	hs	334	0.07
SDS	INS	hs	hs	335	0.09

* nd, not determined due to overlapping Tyr and Trp emission

† W-NH$_2$ + Y-NH$_2$, 2:9 molar mixtures of NAc-W-NH$_2$ and NAc-Y-NH$_2$

‡ W-Oeth + BOC-Y, 2:9 molar mixtures of NAc-W-OEth and BOC-Y-OMe.

§ bd, Tyr fluorescence below detection levels

¶ hs, high scatter at low wavelengths prevented analysis of Tyr fluorescence

W-OEth (337-339 nm) was shifted further to the blue than that of the amide compound (342-343 nm), indicating that this model compound resides on average in a more hydrophobic environment and interacts with both the hydrophobic interior of the detergent micelle and the solvent (intermediate between Classes I and II fluorescence). Based on these observations, N-Ac-W-OEth appears to be a better model for Trp in protein folded in zwitterionic-detergent. The relative fluorescence intensity of the model compounds in both detergents is similar to that in methanol, indicating a lack of quenching by the LDAO and DPC micelles, and further suggesting that the viscosity of the microenvironments in the micelles of both of these detergents is more similar to that of methanol than EG.

In contrast, the lack of a blue-shift in λ_{max}Trp for both model compounds (347-351 nm; Class III) in 3.5 mM SDS indicates that neither N-Ac-W-NH$_2$ nor N-Ac-W-OEth spends much time in the hydrophobic interior of the detergent micelle. However, in 50 mM SDS, these compounds display Class II fluorescence (λ_{max}Trp 341-345 nm; Table 2.3), indicating increased exposure to a hydrophobic environment. The blue-shift is the greatest for N-Ac-W-OEth, indicating that, as in DPC and LDAO, it partitions into a more hydrophobic environment than N-AcW-NH$_2$. At both SDS concentrations, the fluorescence of both model compounds is similar to that observed in phosphate buffer, indicating a lack of quenching.

Mixtures of either the amide or the alkylester model compounds were prepared in the 2:9 ratio present in His$_6$-porin. λ_{max}Trp was unchanged by the addition of a Tyr model compound to the solution (Table 2.3); generally the fluorescence intensities at λ_{max}Trp were slightly higher in the mixtures, likely due to overlapping Tyr fluorescence.

2.4.7 Tryptophan fluorescence of His$_6$-porin in detergent

In most models for porin structure, both tryptophan residues reside in membrane-spanning β-strands, one Trp residue is located near the centre of each half of the protein (reviewed by Bay and Court 2002; see Fig. 2.1 for example). Given the hydrophobic character of Trp and secondary structure predications that place them in β-strands, it would be expected that these residues would be involved in hydrophobic interactions with either adjacent β-strands or the lipids surrounding the β-barrel; without structural data these interactions cannot be identified. Likewise, in detergent-solubilized porin Trp residues could interact with other hydrophobic regions of the protein or with the interior of the detergent micelle.

Trp fluorescence dominates the spectra of urea-solubilized His$_6$-porin following excitation at 275 nm (Fig. 2.6A). λ_{max}Trp is near 348 nm, as determined following excitation at 296 nm (see Table 2.3), and is characteristic of tryptophan residues exposed to an aqueous environment (Class III). Data from His$_6$-porin in phosphate buffer, EG and methanol is not available, as the protein is insoluble in these solvents. Therefore, porin fluorescence is compared to that of the urea-solubilized model compounds. In urea, λ_{max}Trp of His$_6$-porin is similar to that of both tryptophan model compounds, but the fluorescence intensity is at least 40% lower that that of both NAc-W-NH$_2$ and NAc-W-OEth in urea (Table 2.3), likely due to quenching by peptide carbonyl groups in the unfolded protein (Cowgill 1967).

The fluorescence spectra of His$_6$-porin in 20 mM DPC and in 50 mM LDAO (see Fig. 2.6D and 2.6E) are also dominated by the tryptophan signal. λ_{max}Trp is blue-shifted to 337 and 333 nm, respectively (Table 2.3), which suggests Class I fluorescence in DPC and

Figure 2.6. Fluorescence analysis. Fluorescence spectra were obtained following excitation at 275 nm of 0.42 μM His$_6$-porin (solid line), BOC-Y-OMe (dotted line) , and NAc-W-OEth (dash-dot-dot line) solubilized in **A)** 8 M urea **B)** methanol (MeOH) **C)** EG **D)** 20 mM DPC **E)** 50 mM LDAO or **F)** 3.5 mM SDS. **G)** Fluorescence of His$_6$-porin in proteoliposomes prepared by the freeze thaw (solid line), insertion (dotted line) or sonication (dashed line) methods. λ_{ex} was 275 nm. All emission values were corrected for protein concentration. Note that the y-axis scale for panels A-C is larger than that for panels D-G.
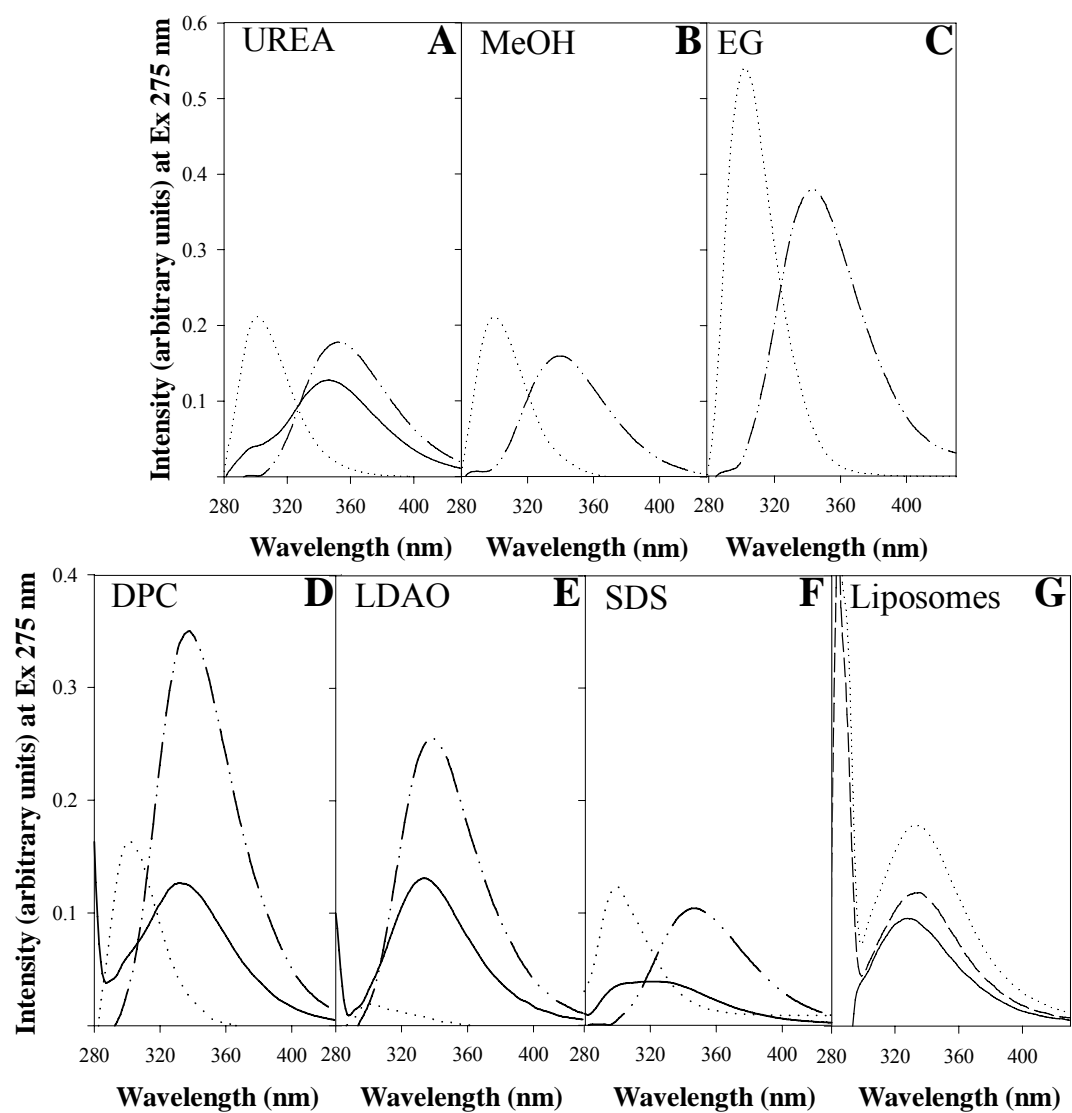
fluorescence intermediate between Classes I and II in LDAO. Thus, LDAO and DPC promote folded states in which the Trp residues are in environments of similar polarity, although the environment in LDAO appears slightly more hydrophobic. Relative Trp fluorescence intensity is similar in both detergents, indicating that the folded states of the protein do not place the Trp residues in close proximity to quenching groups.

The fluorescence spectrum obtained following excitation of SDS-solubilized His$_6$-porin at 275 nm is striking in that overall fluorescence emission is very low and intensity at λ_{max}Trp and λ_{max}Tyr are very similar. Excitation at 296 nm reveals that His$_6$-porin Trp fluorescence in SDS is strongly blue-shifted, (λ_{max}Trp ~ 336 nm; Class I/ Class II) and indicates that both tryptophan residues have very limited solvent exposure (Table 2.3). The intensity at λ_{max}Trp is reduced at least 50% compared to that in urea, DPC, and LDAO (Table 2.3). This strong quenching of porin fluorescence in SDS is likely due to a combination of the low dielectric of the micelles, and quenching by the sulfate head groups of the detergent and/or by the side chains of nearby moieties in the SDS-folded protein, such as protonated aspartate (14 residues in His$_6$-porin), glutamate (10 residues), histidine (14 residues), phenylalanine (17 residues) (Ladokhin 2000). His$_6$-porin fluorescence is unchanged at higher SDS concentrations (Table 2.3), supporting the conclusion that, unlike the model compounds, His$_6$-porin resides within the micelle at both high and low SDS concentrations.

2.4.8 Tryptophan fluorescence of His$_6$-porin reconstituted into liposomes

Fluorescence spectroscopy of Trp residues in bacterial porins (OmpF and OmpA) reconstituted into lipid vesicles has been employed to monitor protein refolding and membrane insertion events (Surrey and Jahnig 1995, Surrey *et al.* 1996). To ascertain the environment of Trp residues of His$_6$-porin in artificial membranes, fluorescence emission

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spectra were collected for each of the three preparations (FT, SON, and INS) of SDS-solubilized His$_6$-porin-liposomes following excitation at 275 nm (Fig. 2.6G), 280 nm, and 296 nm (Table 2.3). Precise examination of Tyr fluorescence was not possible due to the high degree of scatter of lower wavelength light by liposomes.

The emission spectrum of FT SDS-His$_6$-porin-liposomes following excitation at 275 nm had a sharp λ_{max}Trp of 330 nm and relative intensity of 0.1 (Table 2.3, Fig. 2.6G). This spectrum was striking in comparison to SDS-solubilized His$_6$-porin where the λ_{max}Trp is broad spanning the region of 315-335 nm and the relative intensity is 0.03 (Table 2.3, Fig. 2.3F). Following excitation at 296 nm, λ_{max}Trp of FT SDS-His$_6$-porin-liposomes was 329 nm, significantly blue-shifted from that of porin in detergent alone (336 nm); the relative fluorescence intensities were similar (Table 2.3; Fig. 2.6G). The emission spectra of SON and INS SDS-His$_6$-porin-liposomes following excitation at 275 nm have increased relative intensities compared to that of FT SDS-His$_6$-porin-liposomes (Table 2.3). λ_{max}Trp of SON SDS-His$_6$-porin-liposomes (λ_{ex} 296 nm) was slightly red-shifted compared to that of both INS and FT SDS-His$_6$-porin-liposomes, but was very similar to that of SDS-solubilized His$_6$-porin (Table 2.3, Fig. 2.6F).

Overall, the increased relative intensities at λ_{max}Trp in each emission spectrum of SDS-His$_6$-porin-liposomes in comparison to the spectrum of SDS solubilized His$_6$-porin support Trp location in an environment of increased hydrophobicity and/or reduced quenching by SDS headgroups. In general, the emission spectra for each preparation of SDS-His$_6$-porin-liposomes lacked the low intensity and broad λ_{max}Trp observed for SDS-solubilized His$_6$-porin, indicating that quenching by the detergent has been relieved. The higher relative intensity of the INS SDS-His$_6$-porin-liposomes emission spectrum, relative to that of the FT

liposomes, may also indicate that the Trp residues reside in a more hydrophobic environment. This observation is consistent with a β-barrel conformation in the osmotically-responsive FT liposomes, where Trp residues could be solvent-exposed in the channel (reduced intensity) and a collapsed β-structure in the INS liposomes (increased intensity).

2.4.9 Fluorescence of tyrosine model compounds in detergent

The detergent environments surrounding the Tyr model compounds were also investigated with fluorescence spectrophotometry following excitation at 275 nm. λ_{max}Tyr falls within the 299-302 nm range in all of the solvents and detergents tested (Fig. 2.6, Table 2.3). Given the +/- 3 nm precision of the fluorometer, these values can be considered to be the same. However, there are striking differences in the relative intensities of fluorescence that reflect the environments in which the model compounds reside.

The intensities of NAc-Y-NH$_2$ and BOC-Y-OMe fluorescence are similar in 8 M urea, and both are reduced by about 65% in phosphate buffer. The enhanced fluorescence intensity of both model compounds in urea is unexpected, as the quantum yield of NAc-Y-NH$_2$ reported by others is similar in water and in urea (Lee and Ross 1998). A minor (~10%) contribution to this effect may result from the use of an excitation wavelength of 275 nm, which is the λ_{max} excitation for tyrosine in urea, while that in water is 273.5 nm (Lee and Ross 1998). Solvent viscosity may also contribute to the increased Tyr emission in urea (Noronha *et al.* 2004); the viscosity of 8 M urea is 1.66 times that of water (Kawahara and Tanford 1966). Relative to that in phosphate buffer, increased fluorescence intensity was observed for both model compounds in the hydrophobic solvents methanol and EG; the increase was two-to-three fold greater in EG. As discussed above, the large differences in

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intensity in these two solvents may reflect their relative viscosities (0.6 cP for methanol, 19.9 cP for ethylene glycol).

In DPC, the high fluorescence intensity of both model compounds (0.16-0.19) indicates that they are exposed to an environment more similar to that in methanol (0.21-0.25) than to that in phosphate buffer (0.07-0.08). In striking contrast, Tyr fluorescence of BOC-Y-OMe is very strongly quenched in LDAO (0.02), suggesting close proximity of this model compound to quenching groups, most likely the headgroups of the detergent. In contrast, there is much less quenching of N-Ac-Y-NH$_2$ fluorescence (0.12), indicating that the moieties on the carboxyl and/or amino groups of the Tyr compounds promote different interactions of the model compounds with the detergent headgroups. For both model compounds, the fluorescence intensity in SDS, either at 3.5 or 50 mM, is similar to that in phosphate buffer, indicating little interaction between the model compounds and the hydrophobic regions of the SDS micelles. ^{1}H-NMR (data not shown) confirms that neither compound resides in the micelle in 3.5 mM SDS and that there is a small increase in the relative time that the hydrophobic BOC-Y-OMe spends in the interior of the micelle in 50 mM SDS, but this effect is not detectable by fluorescence.

2.4.10 Tyrosine Fluorescence of His$_6$-porin in detergent

Following excitation at 275 nm, fluorescence spectra of proteins are the result of both tyrosine and tryptophan emissions, and in most cases, are dominated by the tryptophan-derived signal (see Fig. 2.6). Second derivative analysis was used to estimate λ_{max}Tyr of His$_6$-porin in 8 M urea, DPC, LDAO and SDS; in all cases λ_{max}Tyr falls within the 299-303 nm range noted for the model compounds (Table 2.3). Both Trp and Tyr fluorescence contribute to intensity at λ_{max}Tyr (Fig. 2.6); therefore, only, general trends can be noted

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regarding the relative Tyr fluorescence intensities. Data from His_6-porin in phosphate buffer, EG and methanol are not available, as the protein is insoluble in these solvents.

The intensity of Tyr fluorescence in His_6-porin is very different from that of the model compounds. In urea, the intensity of the tyrosine fluorescence produced by His_6-porin is only about 10% of that of NAc-Y-NH_2 or BOC-Y-OMe in the same solvent (Fig. 2.6A, Table 2.3). Quenching of Tyr fluorescence in a protein can result from interactions with tryptophan and ionized carboxylates on glutamate and aspartate residues and interactions with other quenched tyrosine residues (Cowgill 1968). These interactions could reflect local backbone structure in urea (Whittington *et al.* 2005), random collisions between the tyrosine residues, and the quenching moieties, and/or resonance energy transfer (with Trp residues). Since the arrangement of His_6-porin in urea is highly unordered based on its far UV CD spectrum (Fig. 2.5A), resonance energy transfer is unlikely as it would require a stable conformation in which the residues are in close proximity.

Far-UV CD analysis demonstrated unique conformations of porin in DPC and LDAO; this conclusion is borne out by the differences in the Tyr microenvironments promoted by these detergents. Fluorescence of His_6-porin at λ_{max}Tyr is similar in 20 mM DPC (Fig. 2.6D) and 8 M urea (Fig. 2.6A), and lower than that of the model compounds in methanol (Fig. 2.6E), suggesting that, in DPC, the Tyr residues are either not exposed to a hydrophobic environment, or that Tyr fluorescence is quenched. In contrast, Tyr fluorescence in 50 mM LDAO (Fig. 2.6E) is negligible, likely due to quenching by detergent headgroups, as was seen for BOC-Y-OMe in LDAO. Weak fluorescence was obtained from His_6-porin in 3.5 mM SDS (Fig. 2.6F), as discussed with respect to Trp fluorescence above. The highly-quenched, blue-shifted Trp fluorescence contributes to the signal at λ_{max}Tyr; nonetheless the

fluorescence intensity at λ_{max}Tyr is low, suggesting that, in the SDS-promoted conformation, the tyrosine residues are likely solvent-exposed and may be in close proximity to quenching moieties.

2.4.11 Tyrosine exposure in detergent-solubilized His$_6$-porin

Conclusions regarding Tyr environments cannot be made from fluorescence data alone, as reduced Tyr fluorescence can reflect increased solvent exposure or increased fluorescence quenching. To discern between these two possibilities, UV absorption data were used to estimate tyrosine exposure to the aqueous environment (Table 2.4; Ragone *et al.* 1984). The Tyr and Trp components of the absorption spectra can be separated by second derivative analysis (SDUV; Ragone *et al.* 1984), in which the tyrosine and the tryptophan absorbance both contribute to a peak in the range of 280-285 nm, while a second peak derived from Trp absorbance is near 291 nm (Fig. 2.7A). The absorption maximum of Tyr varies with the polarity of the surrounding environment and is red-shifted and of a lower magnitude in a non-polar environment, such as EG, while the positions of both tryptophan-derived components remain unchanged as they are derived from the $^1L_b \leftarrow ^1A$ transition, which is insensitive to polarity (Ragone *et al.* 1985). A red-shift in Tyr absorption decreases the signal at 283 nm relative to that at 291 nm; the ratio of these values, *r*, therefore reflects the hydrophobicity of the Tyr environment. For example, in Table 2.4, *r* for both NAc-Y-NH$_2$ and BOC-Y-OMe in EG is 0.56-0.58, while in the polar solvent urea the values are higher: 2.55 and 1.77, respectively (see Fig. 2.7A and B). *r* is high (2.24-2.91) for the amide model compound mixtures in LDAO, DPC and 3.5 mM SDS (Fig. 2.7B), in agreement with the fluorescence data that indicated that NAc-Y-NH$_2$ is not partitioned into the hydrophobic

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Table 2.4. SDUV analysis of His$_6$-porin and model compounds. r and Y$_{exp}$ values were calculated as described in Materials and Methods. His$_6$-porin was insoluble in EG; hence there are no r data for this solvent. For the amide model compounds and His$_6$-porin, each experiment was repeated at least twice; averages are reported; standard deviations were less than 22%. For the hydrophobic compounds, the data reported are from a single experiment.

Detergent / Solvent	*r*		His$_6$-porin	Y$_{exp}$ (α)
	NAc-W-OEth + BOC-Y-NH$_2$	NAc-W-NH$_2$ + NAcY-NH$_2$		
EG	0.56	0.58		
8 M Urea	1.77	2.55	1.82	1.00
50 mM LDAO	0.10	2.64	0.82	0.23
10 mM DPC	0.50	2.91	0.85	0.25
3.5 mM SDS	1.51	2.24	1.39	0.77
50 mM SDS	1.08	1.71	1.45	0.70

Figure 2.7. SDUV absorption analysis. The 2:9 mixture of NAc-Trp-NH$_2$/NAc-Tyr-NH$_2$ was solubilized in **A**) 8 M urea (thick line), or EG (closed circles) or **B**) 3.5 mM SDS (open circles), 50 mM LDAO (filled squares), or 10 mM DPC (open squares). The second derivative plots of the UV absorbance spectra are presented. **C**) SDUV plots of the absorbance spectra of His$_6$-porin; the symbols are the same as those of panel B. For comparison, the tyrosine-derived minima in urea (filled circle) and ethylene glycol (filled triangle) are shown in panels B and C; they are highlighted by arrows.
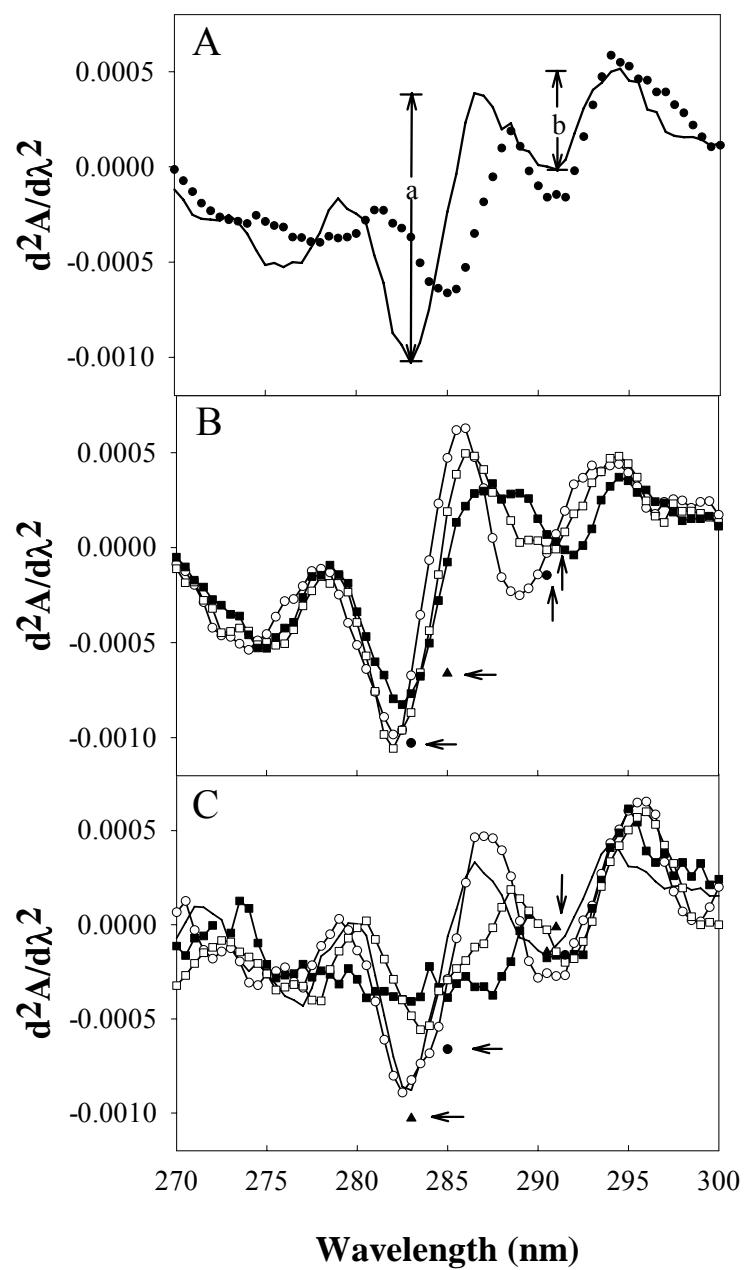
interiors of these detergent micelles. Similarly, r is high (1.51) for BOC-Y-OMe in 3.5 mM SDS, supporting the fluorescence data that suggest this compound is not in the hydrophobic interior of the micelle. In contrast, r is low for the hydrophobic model compound in DPC (0.5). Together with the similarity between the fluorescence spectra of BOC-Y-OMe solubilized in DPC and methanol, this suggests partitioning of this model compound into the DPC micelles. The lowest r value results from solubilization of the hydrophobic model compounds in LDAO, suggesting that the extremely low intensity of BOC-Y-OMe fluorescence in LDAO results from quenching of molecules of the detergent micelle headgroups.

The SDUV plot of His$_6$-porin in urea (Fig. 2.7C) is similar to that of the amide model compounds, and r is 1.82, similar to that in urea. Thus, the aromatic residues are solvent-exposed in the denatured protein. In contrast, r values near 0.8 were calculated for His$_6$-porin solubilized in DPC and LDAO (Fig. 2.7C), indicative of conformations in which the tyrosine residues are exposed to less polar environments, presumably within detergent micelles. As described in Materials and Methods, the r values for the amide model compounds, the protein unfolded in urea, and for the "folded" protein can be used to estimate α, the Tyr exposure (Ragone *et al.* 1984). In DPC and LDAO, Tyr exposure is calculated to be about 0.25, indicating that on average two or three of the nine tyrosine residues in the protein are solvent exposed (Table 2.4). Given these results, the very low intensity Tyr fluorescence by DPC- or LDAO-solubilized His$_6$-porin is best explained by a high degree of quenching of fluorescence. In contrast, Tyr exposure was high in SDS (Fig. 2.7C), with an r value of 1.39, and α of 0.77, indicating that 7 of the 9 Tyr residues are exposed to solvent, where they would exhibit lower intensity fluorescence, and be accessible to quenching by detergent

headgroups, or other exposed residues in the protein. These observations are compatible with the fluorescence data. Light scattering by liposomes prevented similar analyses of membrane-embedded His$_6$-porin.

2.4.12 Near-UV CD of His$_6$-porin in detergent and in liposomes

CD spectra obtained in the near-UV (260-330 nm) provide additional insight into the tertiary interactions in proteins. The 265-280 nm region is the most informative because both tryptophan (270-280 nm) and tyrosine residues (265-270 nm) absorb in this portion of the spectrum. His$_6$-porin could be solubilized at a concentration (≥ 25 µM) sufficient for this analysis in 8 M urea, 21 mM SDS, 300 mM LDAO and 100 mM DPC (Fig. 2.8A). In the region between 265 and 290 nm, the spectrum obtained in urea shows little indication of tertiary interactions, as noted for other proteins unfolded in urea (Reader *et al.* 2001, Masui *et al.* 1997). The negative ellipticity in the spectrum obtained in LDAO is of the same magnitude as in urea, suggesting an absence of tertiary structure in this detergent. The very weak ellipticity may reflect protein-protein interactions that occur in high concentration solutions. However, the ellipticity is at least an order of magnitude less than that observed during specific interactions between unfolded proteins, such as the self-association of partially-denatured bovine growth hormone (Havel *et al.* 1986).

In contrast to those in urea and LDAO, the near UV CD spectra of the protein dissolved in SDS and DPC show negative ellipticity suggesting that the protein is in a condensed state in which some of the aromatic residues exist in transiently stabilized tertiary interactions. The overall shape of the DPC and SDS spectra are similar, with a broad band

Figure 2.8. CD spectropolarimetry of His$_6$-porin in the near UV region. **A)** Spectra of 25-33 μM His$_6$-porin solubilized in 100 mM DPC (circles), 8 M urea (solid line), 300 mM LDAO (dashed line) and 21 mM SDS (triangles) were obtained as described in Materials and Methods. **B)** Spectra of His$_6$-porin in SDS-liposomes generated by the freeze-thaw (solid line), sonication (triangles) and insertion (circles) methods. All spectra were corrected for protein concentration.
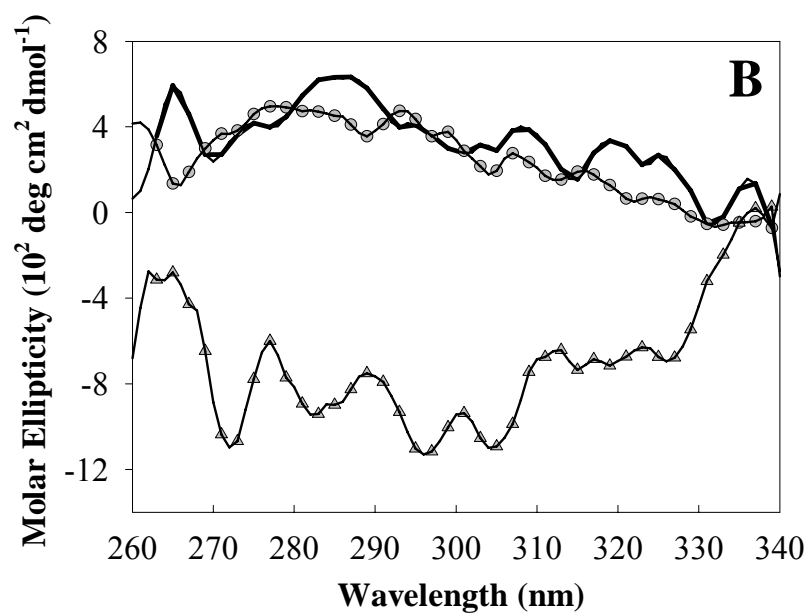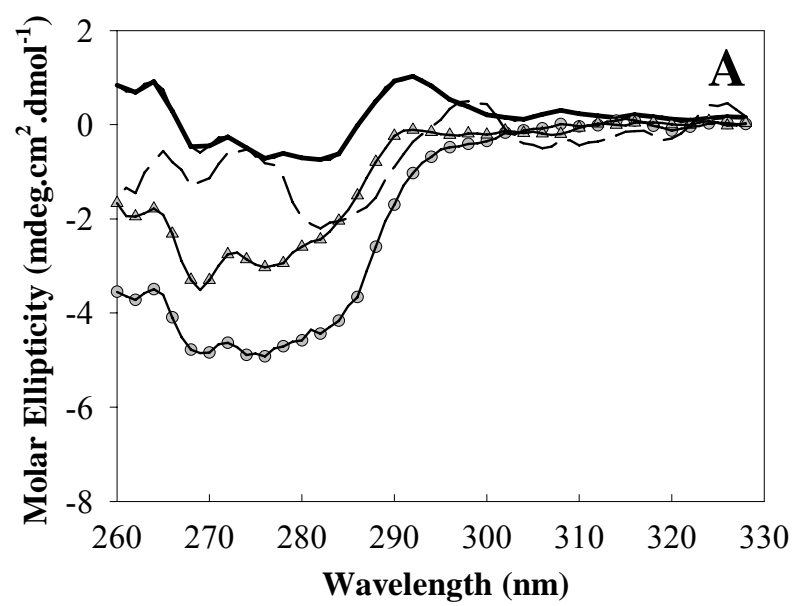
of negative ellipticity that spans both the tyrosine and tryptophan regions. The ellipticity is greater in DPC, suggesting stronger or more extensive interactions.

Near UV CD spectra of FT, SON and INS SDS-His$_6$-porin-liposomes were also examined (Fig. 2.8B). The spectrum of FT SDS-His$_6$-porin-liposomes had positive ellipticity in the 280-290 nm region. The near UV CD spectrum of INS SDS-His$_6$porin-liposomes also demonstrated positive ellipticity near 276 nm. In contrast, the near UV CD spectrum of SON SDS-His$_6$-porin-liposomes had strong negative ellipticity in the 310-270 nm region (Fig. 2.8B), similar to that observed for DPC and SDS solubilized His$_6$-porin, suggesting a condensed state with stronger tertiary contacts than those promoted by single detergents only.

2.5 Discussion

2.5.1 Porin solubility

The denaturant urea and the detergent SDS are able to solubilize acetone-precipitated His$_6$-porin at the highest concentrations. This protein is also moderately soluble in the ionic detergents LDAO and DPC, but poorly soluble in neutral OG, Genapol X-080 and DDM. Taken with data obtained by other groups, these results emphasize the importance of the method of protein purification in determining the most suitable detergent for solubilization of the protein. A thorough study by De Pinto *et al.* (1989) revealed that the Tritons, dimethylamine oxides, and zwittergents were best for isolation of porin from bovine heart mitochondria, with LDAO optimal for maintaining the protein in a membrane-insertion competent state. OG was successfully used by Shao *et al.* (1996) to solubilize porin from *Neurospora* mitochondria. The protocols used by these groups differed from the method presented herein in several critical ways: they did not involve refolding of the porin, only

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mild denaturation was needed to disrupt the membranes, and purification in LDAO recovers sterols as well (De Pinto *et al.* 1989).

Exchange of the urea, or guanidinium chloride, for detergent, to allow folding of the protein has been achieved previously by dialysis, and leads to high concentration of protein in LDAO (170 μM) or OG (34 μM; Koppel *et al.* 1998), but not in Genapol X-080 (DCB and DAC, unpublished results). However, routine preparation of highly concentrated porin in LDAO is prohibitively expensive using dialysis; instead, detergent exchange following acetone precipitation (Shao *et al.* 1996) of urea-solubilized His_6-porin was utilized in this study.

Several conclusions are apparent with respect to solubility of the acetone-precipitated His_6-porin in the various detergents tested. With the exception of OG, all of the detergents used have acyl chains of 12-13 residues. These lengths exceed the minimum chain lengths of 9-10 found by Kleinschmidt *et al.* (Kleinschmidt *et al.* 1999) to promote folding of the bacterial porin OmpA. Thus, the inability of octyl-glucoside to stably solubilize acetone-precipitated His_6-porin is likely due to the short length of the tail, rather than any characteristic of the headgroup. In this study, detergent concentrations greater than the CMC were required for optimal solubilization of the protein (data not shown), as also noted for OmpA (Kleinschmidt *et al.* 1999).

The highest levels of solubility were achieved with detergents with charged headgroups, the best being negatively-charged SDS, followed by the two zwittergents, LDAO and DPC. Since OG and DDM were unable to solubilize porin at useful concentrations, it appears that ionic charge, as in SDS and DPC, enhances the solubilization, presumably by disrupting inter-and intramolecular interactions in the precipitated protein and/or by

maintaining detergent-protein interactions required for solubility. Although the net charge on the headgroups of DPC and LDAO are both zero, the positively and negatively charged moieties in the head group of LDAO are in very close proximity, possibly limiting interactions with the precipitated protein and thereby reducing solubilization. In addition, the monomers of the uncharged detergents may be unable to effectively interact with the surface of the porin to create a stable protein-detergent micelle (Garavito and Ferguson-Miller 2001). A further hindrance to solubility in LDAO may be the increased β-strand content, because these structures have a preference for interacting with other segments of the protein to satisfy their hydrogen bonding potential, whereas individual α-helices can interact with lipid (Popot and Engelman 1990), or in this case, the hydrophobic portions of the detergent.

2.5.2 His$_6$-porin reconstituted into liposomes

Liposome swelling experiments involving His$_6$-porin solubilized by LDAO, DPC and SDS have demonstrated that, after FT in the presence of a SUV stock, each sample was capable of forming osmotically responsive pores in artificial membranes, as shown for porins isolated from mitochondria (Bathori *et al.* 1993, Shao *et al.* 1996). The spectroscopic features of His$_6$-porin in liposomes were similar to those observed for other β-barrel proteins – namely λ_{max}Trp near 330 nm, and a conformation rich in β-strand (Linke *et al.* 2000, OEP16; Kleinschmidt and Tamm 2002, Pocanschi *et al.* 2006). The fact that His$_6$-porin solubilized in a variety of detergents formed channels is interesting since each detergent promotes a different secondary arrangement with different levels of tertiary interactions (Table 2.2, Fig. 2.8). However, the effectiveness of pore formation varied from highly reproducible channel formation by SDS-His$_6$-porin to variable results with LDAO-porin. The effectiveness of SDS may relate to the α-helical structure it promotes – the protein may be

less prone to aggregation upon dilution into the SUV solution, allowing a greater fraction to interact with the membrane (Surrey and Jahnig 1992). Alternatively, SDS may be most effective at disrupting membrane packing, a requirement for insertion of membrane proteins into preformed membranes (see Scotto and Zakim 1988 for discussion). The osmotic response of some preparations of LDAO-His_6-porin liposomes increased following heating, suggesting that a kinetic barrier to assembly may exist under these conditions.

In general, the freeze-thaw procedure was the only one to produce osmotically-responsive proteoliposomes that were resistant to heating at 65°C. Sonication of liposomes in the presence of porin was not successful, suggesting that conditions created during the freezing and/or thawing steps, in addition to membrane rupture, were required for pore formation by His_6-porin. The shape of the resulting vesicles or defects in membrane packing may explain this observation (Surrey and Jahnig 1992). Some aggregation of the protein in the membrane may be necessary for pore formation, as shown for short β-sheet peptides $(xSxG)_6$ that form voltage-gated channels upon aggregation in lipids bilayers (Thundimadathil *et al.* 2005, Thundimadathil *et al.* 2005).

Dilution of His_6-porin into a suspension of preformed liposomes also did not lead to osmotically responsive proteoliposomes, in contrast to the folding of OmpA that can be achieved under similar conditions (Surrey and Jahnig 1992, Kleinschmidt and Tamm 1999). The reaction did not occur at 30°C, and 42°C, temperatures which are sufficient for breaking the kinetic barriers to in vitro folding and membrane insertion of OmpA (reviewed in Kleinschmidt and Tamm 1996). As the goal of the reconstitution experiments was to obtain folded, channel forming porin, in-depth examination of folding and membrane insertion was beyond the scope of this study.

In contrast to their variable osmotic responsiveness, proteoliposomes prepared by all three methods contain protease-resistant porins (Fig. 2.3). This suggests that in all of these cases, His_6-porin is interacting strongly with the lipid and/or with other His_6-porin molecules. The latter possibility is suggested by the oligomers observed on western blots of all three types of proteoliposomes (Fig. 2.3). Oligomers were not detected in detergent-solubilized His_6-porin preparations, in contrast to experiments utilizing purified rat liver mitochondrial porin (Zalk *et al.* 2005). A possible explanation for this discrepancy is that isolation of His_6-porin in urea prevents the formation of contacts required for multimeric interactions.

Oligomers of His_6-porin were also not detected in detergent- and SEM-solubilized mitochondria. Although the urea included in the SDS-PAGE gels presented in this study might disrupt such interactions, oligomers are also undetectable in immunoblots of gels lacking urea (data not shown). Furthermore, although mitochondrial porin may be able to oligomerize under certain conditions, interactions with other proteins (Roman *et al.* 2006) and porin-associated sterols (De Pinto *et al.* 1989) may prevent the formation of oligomers in the mitochondrial outer membrane. Electron microscopic analysis of two-dimensional arrays of mitochondria outer membranes from *N. crassa* did not detect any oligomeric arrangements of mitochondrial porin (Mannella 1989, Thomas *et al.* 1991, Guo and Mannella 1993). In addition, chemical cross-linking was required for detection of the oligomers of rat liver mitochondrial porin. Finally, although VDAC1 is abundant in rat cells, there are at least three isoforms of porin in this organism (Pavlica *et al.* 1990), and it was not ruled out that the hetero-oligomers were formed in the more recent experiments (Zalk *et al.* 2005).

2.5.3 The folded states of His$_6$-porin reconstituted into liposomes

The conformation of His$_6$-porin following reconstitution into liposomes was examined by CD spectropolarimetry and fluorescence spectroscopy. SDS-His$_6$-porin incorporated into liposomes by all three procedures is β-strand rich, but only in FT SDS-His$_6$-porin liposomes was the β-strand content stably maintained upon heating (Fig. 2.4), suggesting different interactions with the membranes in each preparation. Similarly, intrinsic Trp fluorescence is most intense in SON and INS SDS-His$_6$-porin liposomes, but in FT liposomes, fluorescence intensity (at λ_{ex} 296 nm) is similar to that of the protein in SDS, and λ_{max}Trp is the least red-shifted in comparison to SDS-His$_6$-porin, suggesting that the tryptophans reside in quite different environments in the osmotically-responsive and non-responsive forms of the protein.

Analysis of near-UV CD spectra also reveals differences among the proteoliposomes formed via the three methods. These spectra are dominated by positive ellipticity for the FT and SON-type proteoliposomes, and negative ellipticity for the INS-type (Fig. 2.7). In all cases, these data imply that tertiary contacts or intermolecular interactions involving the aromatic amino acids in porin are occurring in the membrane, but their nature is determined by the method used for porin reconstitution. As there are no near-UV data for natively folded mitochondrial porins, comparisons can only be made with bacterial proteins. The spectrum of OmpA in SDS is negative, but upon folding by the addition of OG, it is dominated by a positive signal (Ohnishi and Kameyama 2001), suggesting that the tertiary contacts in SDS are different from those in the β-barrel structure. Similarly, near-UV CD spectra with strong positive ellipticity have been reported for native bacterial proteins including the PorB class 2 (Minetti *et al.* 1997) and class 3 (Minetti *et al.* 1998) porins of *Neisseria*. Further, changes

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from positive to negative ellipticity in both the Tyr and Trp spectral regions have been observed during acid or thermal denaturation of several water-soluble β-rich proteins including the cardiotoxin analogue III, CTXIII, (Kumar *et al.* 1995, Sivaraman *et al.* 1997), CD40L (Matsuura *et al.* 2001) and TNF-α (Narhi *et al.* 1996). In all of these proteins, the aromatic amino acid residues reside in β-strands. However, this correlation is not absolute; for example, the near UV spectrum of the folded β-barrel of the src homology domain 3 of the growth factor receptor binding protein 2 displays strong negative ellipticity in the 280 nm region (Bousquet *et al.* 2000).

Although they do not form detectable pores in the membrane, it is possible that the forms of His_6-porin in the INS and SON liposomes resemble partially folded species of other β-barrel proteins. A detailed mechanism for folding and insertion of OmpA has been revealed by a series of elegant experiments using time resolved Trp fluorescence quenching (Kleinschmidt *et al.* 1999, Kleinschmidt, 1999 #462, see Fig. 1.5). The unfolded protein (U) becomes a hydrophobically collapsed water-soluble intermediate I_W upon dilution from urea into water. In the second stage this intermediate binds to the membrane (I_{M1}) and proceeds to intermediate (I_{M2}) where β-structure begins to develop at the membrane surface, but lacks tertiary contacts ("molten disk"). The β-hairpin loops translocate to the centre of the lipid bilayer lacking correct tertiary fold forming the final "molten globule" intermediate (I_{M3}) which then proceeds to the native state (N) (reviewed by Tamm *et al.* 2004). Trp fluorescence data suggest that porin in INS and SON-type proteoliposomes reside in a less hydrophobic environment than that in FT liposomes, which may indicate a partially inserted intermediate such as I_{M2}. However, it is not clear whether the weak tertiary contacts in His_6-porin detected by near-UV CD are compatible with the molten disk state as described for OmpA.

2.5.4 Detergent-promoted folded states

The secondary and tertiary structures of mitochondrial porin in urea, SDS, LDAO and DPC are unique, as demonstrated using a variety of spectroscopic techniques, most of which have not previously been applied to mitochondrial porins. As discussed by Nahri (Narhi *et al.* 1996), protein conformations are determined by both long and short range interactions established during folding of the protein, and each detergent was observed to promote a unique arrangement in terms of both secondary and tertiary structures.

Of the three detergents, DPC might be expected to promote correct folding of mitochondrial porins because 45% of the outer membrane is composed of the related lipid phosphatidylcholine (de Kroon *et al.* 1997). DPC does promote some β-strand structure (26-30%), but this amount is significantly lower than that obtained for porin isolated from mitochondria in OG (62-63%, Shao *et al.* 1996), and LDAO (45%, Koppel *et al.* 1998). Furthermore, the levels of unordered structure in DPC are high (>50%), suggesting that the DPC-solubilized porin is not in the same conformation as in mitochondria. In DPC, both Trp and most of the Tyr residues in His_6-porin were buried in the detergent/protein complex. Low tyrosine exposure has also been reported in *Neisserial* PorB porins folded in Zwittergent 3-14 (class 2 porin α = 0.09 (Minetti *et al.* 1997) and class 3 porin α ~ 0 (Minetti *et al.* 1998).

The DPC-promoted conformation is sensitive to heat denaturation, which is accompanied by a loss in α-helix, but not β-strand structure, suggesting that the α-helical structure plays an important role in maintenance of the protein in a soluble form. Reduction of this component at high temperature would then lead to protein precipitation, due to the constraints on maintaining β-strand rich protein in solution, as discussed above. In addition, fluorescence and near UV CD studies provide evidence for tertiary contacts in DPC-

solubilized porin; disruption of these contacts at high temperature may also contribute to precipitation of the protein.

Structural analysis of the protein in LDAO is particularly interesting, as this detergent is used for preparing porin for electrophysiological studies in artificial bilayers (Koppel *et al.* 1998). LDAO promoted high levels of β-strand content (32-39%), similar to the native protein in the same detergent (Koppel *et al.* 1998) but the absence of tertiary interactions suggests that the protein has not adopted its native conformation, but exists in a state that has been referred to as "molten globule" or "molten disk" (for example see Matsuura *et al.* 2001). This conformation requires tertiary mobility while maintaining interactions required for solubility of β-strands (Matsuura *et al.* 2001). Examples of β-strand rich molten globule proteins include CTXIII, Kumar *et al.* 1995, Sivaraman *et al.* 1997), which contains Tyr but no Trp residues, and Trp-containing proteins of the tumor necrosis factor family, CD40L (Matsuura *et al.* 2001) and TNF-α (Narhi *et al.* 1996). In the molten globule state, several arrangements are possible. One example is pockets of β-strand structure existing separately from large regions of unstructured protein. Alternatively, the protein may exist in a collapsed β-barrel, in which native β-strands form, but are maintained by interactions with the detergent rather than the adjacent β-strands as in a barrel structure.

Tyrosine exposure is very low under these conditions, suggesting that the C-terminal half of the protein, containing most of the Tyr residues (Fig. 2.1), is buried in the detergent/protein complex. The CD spectra obtained during thermal denaturation of LDAO-solubilized His$_6$-porin (Fig. 2.5D), resemble those of native porin in OG (Shao *et al.* 1996). However, unlike the OG-solubilized porin, His$_6$-porin in LDAO did not precipitate at high temperature (100°C), and partial refolding to a structure resembling that present at the 57°C

midpoint was observed upon cooling. The overall shape of the CD spectra at 65°C indicate similar changes in structure as reported by Shao *et al.* for porin in OG at 65°C (Shao *et al.* 1996). In contrast, thermal denaturation of β-strand in detergent-solubilized *Neisseria* PorB porins has a midpoint of 85-90°C (Minetti *et al.* 1998, Minetti, 1997 #260).

SDS-folded porin is predominantly α-helical, and contains the lowest levels of β-strand, and high levels of unordered structure (> 55%). α-helix formation is a common characteristic of many proteins in SDS (Visser and Blout 1971), including mitochondrial porin (Shao *et al.* 1996, Popp *et al.* 1997) and β-barrel bacterial porins (for example *E. coli* porin, Eisele and Rosenbusch 1990). Although the tryptophan residues are in a hydrophobic environment, most of the tyrosine residues are exposed, suggesting that the C-terminal half of the protein is folded very differently in SDS than in DPC and LDAO. This conformation is very stable during heating; only minor changes to secondary structure composition were observed, as reported for native porin in 35 mM SDS (Shao *et al.* 1996). These slight changes were completely reversible upon cooling (Fig. 2.2) and protein precipitation did not occur, in contrast to DPC-solubilized porin. Heating of the latter significantly reduced α-helical content from 20 to 11%, suggesting that maintenance of α-helix during heating is required to prevent precipitation. In spite of the non-native folded state, near UV CD spectra of SDS-solubilized porin indicates weak tertiary contacts in the protein, which in addition to detergent-protein interactions, may contribute to the overall stability of the protein in SDS.

Near UV CD spectra were obtained for DPC and SDS solubilized His$_6$-porin; in both cases, strong negative ellipticity in the 270-290 nm region was observed (Fig. 2.8). These spectra are unlike those of the protein in FT and INS SDS-His$_6$porin-liposomes. This

indicates that the weak tertiary contacts in SDS and DPC-solubilized His$_6$-porin are not characteristic of the membrane bound, osmotically-responsive conformation of His$_6$-porin.

2.5.5 Amino acid model compounds

Model compounds were critical for assessing the complex microenvironments provided by detergent systems. The N-acetyl-amide Trp and Tyr derivatives have been used extensively in fluorescence and UV absorption studies involving soluble proteins (for example, Nayar *et al.* 2002, Garcia-Borron *et al.* 1982, Cowgill 1967) or using organic solvents (for example, Nayar *et al.* 2002, Havel 1996, Guzow *et al.* 2005). Our work extends these studies to membrane proteins, by using the standard model compounds, and their hydrophobic alkylesters, and investigating their properties in detergent. Fluorescence and UV absorbance were used in combination to assess the interactions of the model compounds with detergent. In general, it should be noted that the useful concentrations for these studies are limited due to light scattering by detergent micelles, and that, in some cases, detergent solubility restricts the usable pH and temperature ranges.

There were two important observations from these model compound studies. First, detergent systems include multiple environments that prevent direct comparisons of detergent-solubilized protein with model compounds in simple solvents. Second, the commonly used hydrophilic model compounds NAc-Y-NH$_2$ and NAc-W-NH$_2$ are not suitable probes for the interior of detergent micelles, although they provide useful information about interactions between the detergent headgroups and amino acids exposed to the aqueous solution.

The multiple environments in detergent solutions are the hydrophobic core of the micelle, the micellar surface defined by the detergent headgroups, and the aqueous

environment surrounding the micelles. Like organic solvents, the hydrophobic environments provided by detergent micelles lead to blue-shifts in λ_{max} of Trp fluorescence (Table 2.3), and moieties in the detergent headgroups may quench this fluorescence. For example, LDAO almost completely quenches BOC-Y-OMe fluorescence, while SDS and DPC do not. This phenomenon is not observable with NAc-Y-NH$_2$ because it does not associate strongly enough with the micelle due to its relatively high polarity. Enhancements of Tyr fluorescence intensity were not observed in detergent, in spite of the predicted low dielectric in the micelle, suggesting that quenching is occurring. In contrast, in organic solvents, quantum yield increases with decreasing DEC (Lee and Ross 1998, Guzow *et al.* 2005). Therefore, the studies with model compounds revealed detergent-specific phenomena that provide context for future studies of detergent-solubilized membrane proteins.

There were striking differences between the interactions of the variants of the model compounds and each detergent, as seen in the fluorescence (Table 2.3) and UV absorbance data (Table 2.4, Fig. 2.7). NAc-W-OEth was the most useful gauge of hydrophobic environments in fluorescence studies, because, compared to NAc-W-NH$_2$, its fluorescence spectrum had a larger blue-shift upon interaction with micelles of all of the detergents studied (Table 2.3). Fluorescence analysis of the Trp model compounds also revealed that partitioning of the model compound into the micelle may be more sensitive to detergent concentration than is protein solubilization, as shown for SDS (Table 2.3). Finally, it is noteworthy that the correlation between blue-shifted λ_{max}Trp and increased fluorescence intensity in LDAO and DPC, but not in SDS, suggests relatively high viscosity in the interior of the LDAO and DPC micelles.

Tyrosine fluorescence was somewhat less informative, as clear differences in λ_{max}Tyr could not be discerned, and low level fluorescence intensity could result from either exposure to a watery environment, or from quenching and resonance energy transfer. However, UV absorption analysis of mixtures of Trp and Tyr model compounds (Table 2.4) confirmed that the hydrophobic BOC-Y-OMe partitioned more effectively into the interior of the detergent micelle than the more hydrophilic NAc-Y-NH$_2$ compound.

Taken together, the results presented herein establish a framework for future structural studies of mitochondrial porins in part by presenting a set of model compound data useful for interpreting fluorescence data of detergent-solubilized membrane proteins. Characterization of membrane-bound and detergent-solubilized His$_6$-porin by fluorescence and near UV CD is also an important step in developing strategies for solubilization of high concentrations of correctly-folded porin needed for NMR studies. This work also demonstrates that detergents (Genapol-X80, LDAO, OG) that are successful for black lipid bilayer studies are not necessarily useful for preparing high concentration porin solutions for structural analysis. The LDAO results in particular emphasize that competence for insertion into artificial membranes is not indicative of native-type folding in detergent. In fact, this observation supports the intriguing hypothesis that incompletely folded β-strand protein, rather than the natively-folded form, is required for insertion into artificial membranes under the conditions of "black-lipid bilayer" experiments. The next chapter will focus on mixed detergent systems, which have been used for refolding bacterial porins (for example, Ohnishi and Kameyama 2001), to obtain high concentrations of stably-folded protein.

CHAPTER 3 Two-step folding of recombinant mitochondrial porin in detergent.

The material presented in chapter 3 was written with the intent to submit to the Journal of Bioenergetics and Biomembranes. I completed all of the experiments outlined in this chapter. The data were analyzed and the manuscript was written with guidance and feedback from my advisor and Dr. J. O'Neil, who are co-authors on the manuscript.

3.1 Abstract

Precise information regarding the transmembrane topology of mitochondrial porins is essential for understanding the mechanisms by which this protein forms a channel in the outer membrane, and interacts with small solutes and proteins to regulate mitochondrial function. The lack of a system for maintaining high levels of properly folded protein has hindered the acquisition of high resolution structural data. In the current studies, several mixed detergent systems were analyzed for their ability to fold porin. A mixture of sodium dodecyl sulfate and dodecyl-β-D-maltopyranoside in a 1:6 molar ratio supports a β-strand rich conformation. In this state, the two tryptophan residues in the protein reside in hydrophobic environments and about half of the nine tyrosines are solvent exposed. Most importantly, heat-labile tertiary contacts were detected by near-UV circular dichroism spectropolarimetry. The SDS/DDM folded porin adopts a high molecular weight conformation that is resistant to proteolysis as is the native protein. The tertiary contacts and protease resistance of the SDS/DDM solubilized porin are very similar to those of the protein following functional reconstitution into liposomes. Thus, a system has been identified with the potential to solubilize high levels of mitochondrial porin in a state that will be useful for structural studies.

3.2 Introduction

One of the most abundant proteins in the mitochondrial outer membrane is mitochondrial porin, otherwise known as the voltage-dependant anion selective channel (VDAC). Like their bacterial namesakes, these channels are presumed to exist in β-barrels composed of antiparallel transmembrane β-strands. Reconstitution of these proteins into artificial membranes generates channels that passively transport solutes and display voltage–dependent gating; the channels are "open" and anion-selective at low applied voltages and exist in partially-closed, cation-selective states upon application of high voltages (reviewed by Benz 1994). In artificial membranes systems, recombinant mitochondrial porins that bear N-terminal hexahistidinyl-tags (His_6-porin) and were expressed in *Escherichia coli* are functionally indistinguishable from porins isolated from mitochondrial membranes (Popp *et al.* 1996, Koppel *et al.* 1998).

Mitochondrial porins have been shown to interact with small molecules and other proteins that participate in a variety of cellular processes. Examples of interaction partners include hexokinase (Arora *et al.* 1993) and creatine kinase (Brdiczka *et al.* 1994) involved in glucose metabolism. ATP (Rostovtseva and Colombini 1996,Rostovtseva and Colombini 1997), NADH (Zizi *et al.* 1994), and the inner membrane ATP/ADP carrier (reviewed in Crompton *et al.* 1999) also interact with porin and participate in processes involving oxidative phosphorylation. Furthermore, regulators of apoptosis, namely the Bcl-2 family of proteins (as reviewed by Shoshan-Barmatz *et al.* 2006) interact with VDAC in higher organisms, potentially controlling cytochrome *c* release and the induction of apoptosis. In many cases, these interactions modulate channel gating and reflect complex interactions of porin with its environment (reviewed by Blachly-Dyson and Forte 2001). The involvement of

mitochondrial porin in all of these cellular activities emphasizes the need for understanding the structural arrangement of this channel. However, in contrast to the wealth of information regarding bacterial porin structure, very little is known about the topology of mitochondrial porins.

Evidence supporting a β-barrel conformation has included structural predictions based on primary sequence on the properties of porin channels in artificial membranes and far-UV circular dichroism (CD) spectropolarimetry studies that revealed a β-strand rich secondary structure (40-70%) (Shao *et al.* 1996, Popp *et al.* 1997, Koppel *et al.* 1998, Runke *et al.* 2000). Electron microscopic analysis of two-dimensional and multi-lamellar arrays confirmed the barrel structure of porin, but the low resolution (nm) prevented the identification of membrane spanning segments (Mannella 1989, Thomas *et al.* 1991, Guo and Mannella 1993, Dolder *et al.* 1999). Other approaches to the determination mitochondrial porin structure have been hindered by the low yields of folded protein obtained from mitochondria, the low solubility of the recombinant protein in the detergents used for electrophysiological analysis, and the poor stability of mitochondrial porins in comparison to their bacterial counterparts (reviewed by Schulz 2000, Bay and Court 2002).

Isolation of native mitochondrial porin in quantities high enough for spectroscopic analysis (μg amounts) requires a large quantity of cells and numerous purification steps involving initial solubilization in detergent, such as Triton X-100 (Colombini 1980, Roos *et al.* 1982, Freitag *et al.* 1982), Genapol X-080 (Troll *et al.* 1992) or lauryl dimethylamine oxide (LDAO; Aljamal *et al.* 1993). The low yields of protein restrict the techniques available for the characterization of the folded state. Furthermore, many of these detergents are not compatible with spectroscopic analyses.

Recombinant His$_6$-porin expressed in *E. coli* has facilitated the purification of higher concentrations of protein (mg), but at the cost of the native folded state, since these proteins must be isolated from inclusion bodies (Koppel *et al.* 1998, Chapter 2). Isolation and folding have involved denaturation and subsequent solubilization in detergent, using dialysis to remove the denaturant while encouraging folding that presumably reflects that of the native porin (Koppel *et al.* 1998). Unfortunately, depending on the detergent used, this approach can be prohibitively expensive where high concentrations of protein are concerned.

Our previous studies utilizing His-tagged *Neurospora crassa* mitochondrial porin involved the use of single detergents for protein solubilization and reconstitution into liposomes and permitted spectroscopic characterization of the protein (Chapter 2). His$_6$-porin was purified in 8 M urea, acetone precipitated, and the dried pellet dissolved directly in the detergent of choice. High concentrations of His$_6$-porin were attainable in LDAO, dodecyl phosphocholine (DPC) and sodium dodecyl sulfate (SDS), but high β-strand content was only exhibited by the protein in LDAO. The near-UV CD spectra of the LDAO-solubilized protein display almost no ellipticity, suggesting that the protein exists in a molten globule conformation. Therefore, single detergent solubilization of His$_6$-porin is not suitable for further structural characterization of the recombinant protein, but it is still an attractive means for the solubilization of high concentrations of porin.

In addition, SDS-, LDAO-, and DPC-solubilized His$_6$-porin can be reconstituted into liposomes by freeze-thawing and thereby produce osmotically-responsive proteoliposomes (Chapter 2). Only SDS-His$_6$-porin could be reconstituted into liposomes at protein concentrations sufficient for analysis by both CD spectropolarimetry and fluorescence spectrophotometry. The CD spectrum of freeze-thawed SDS-His$_6$-porin-liposomes in the far-

UV region was characteristic of β-strand rich proteins and demonstrated positive ellipticity in near-UV region, as shown for other bacterial β-barrel porins (Ohnishi and Kameyama 2001, Minetti *et al.* 1997, Minetti *et al.* 1998). Although His_6-porin reconstituted into liposomes is a more relevant system for studying mitochondrial porin architecture, the high concentrations of protein required for further analysis by other high resolution spectroscopic techniques such as NMR spectroscopy cannot be obtained. Hence, a detergent based system for recombinant mitochondrial porin refolding is preferable.

Alternative detergent systems for solubilizing other membrane proteins involve mixtures of anionic and non-denaturing, non-ionic detergents. For example, the *E. coli* integral outer membrane protein A (OmpA) has been folded in a mixture of SDS and octyl-β-D-glucopyranoside (OG) (Dornmair *et al.* 1990, Ohnishi *et al.* 1998, Ohnishi and Kameyama 2001). SDS and dodecyl-β-D-maltopyranoside (DDM) systems have been used for solubilization of other membrane proteins including DsbB (Otzen 2003), and the P5 outer membrane protein of *Haemophilus influenzae* (Webb and Cripps 1999). The focus of many of these studies was to characterize unfolding kinetics upon the addition of SDS to protein folded in a non-ionic detergent (Ohnishi *et al.* 1998, Lau and Bowie 1997). However, in the case of OmpA, folding into a β-rich conformation could be achieved by adding OG to SDS-solubilized protein at a particular molar ratio of the two detergents (Ohnishi and Kameyama 2001). Alternatively, peptides have been used to probe mixed detergent systems.

In this study, mixed detergent systems were used to solubilize recombinant His_6-tagged *Neurospora crassa* mitochondrial porin. SDS and DPC were chosen for primary solubilization of the protein, based on the high solubility of His_6-porin in these detergents (Chapter 2). Several non-ionic and zwitterionic detergents were added as the secondary

surfactants and varying molar ratios of the two detergents were examined. The folded states

of the proteins in the resulting detergent mixtures were examined by CD spectropolarimetry

in the far-UV and near-UV regions, fluorescence spectrophotometry, and UV absorption

spectroscopy. To interpret the results obtained for His_6-porins in these binary systems,

aromatic amino acid derivatives of varying hydrophobicity were also examined to probe the

micellar environments experienced by the protein. Of the numerous mixed detergent systems

examined, only SDS/DDM solubilized His_6-porin was suitable for reconstitution into

liposomes and analysis by protease digestion.

3.3 Materials and Methods

3.3.1 Detergents and amino acid derivatives

DPC, DDM, and OG were purchased from Anatrace (Maumee, OH), and LDAO from

Calbiochem (San Diego, CA). Sigma (St. Louis, MO) was the supplier of N-acetyl-

tryptophan amide (NAc-W-NH$_2$), N-acetyl-tyrosine amide (NAc-Y-NH$_2$), SDS, urea and N-

acetyl-tryptophan ethyl ester (N-Ac-W-OEth). N-tert-butoxycarbonyl-tyrosine methyl ester

(BOC-Y-OMe), egg yolk L-α-phosphatidyl choline, and egg lecithin L-α-phosphatidic acid

were purchased from FLUKA (Oakville, ON).

3.3.2 Expression and purification of His$_6$-porin

The construct encoding the N-terminal His$_6$-tagged version of *Neurospora crassa* has

been described previously (Popp *et al.* 1996). Protein expression was carried in QIA*express*

Escherichia coli M15 [pREP4] (Qiagen, Toronto, ON). His$_6$-porin was purified in 8 M urea,

as described in (Chapter 2). Protein purity, as estimated by dodecyl sulfate-polyacrylamide

gel electrophoresis was at least 95%. Protein concentration was determined using the calculated extinction coefficient at 280 nm of 24,050 M^{-1} cm^{-1}.

3.3.3 Detergent solubilization of His$_6$-porin

Following dialysis against 8 M urea, His$_6$-porin was acetone-precipitated and the dried pellets were resuspended either in SDS (3.5 or 21 mM) or DPC (20 or 100 mM), buffered in 50 mM sodium phosphate pH 7. Following mixing by repeated inversion overnight at room temperature (22°-25^oC), samples were clarified by centrifugation at 10 000 g for 15 minutes. For mixed detergent systems, an appropriate amount of the second detergent, in powder form, was added and the samples mixed by inversion overnight. Insoluble aggregate was removed by centrifugation prior to analysis.

3.3.4 Liposome preparation and swelling assays

Modifications of the stock small unilamellar vesicles (SUV) preparation described by (Shao *et al.* 1996) were used for these studies. In brief, a 10:1 ($^w/_w$) mixture of egg yolk L-α-phosphatidyl choline and egg lecithin L-α-phosphatidic acid was dissolved in chloroform, evaporated at room temperature (22-25^oC) and resuspended in 50 mM sodium phosphate buffer at pH 7. The milky suspension was diluted 1:1 with 50 mM sodium phosphate buffer pH 7 and sonicated on ice until the solution appeared clear yielding the stock SUV suspension (20 mg/ml).

Liposomes used for swelling assays were prepared with the stock SUV according to a modified procedure described by (Chapter 2) for type B (CD spectropolarimetry grade) liposomes described by (Shao *et al.* 1996). In short, the SUV suspension was diluted 1:1 with 1 μM (0.030 mg/ml) His$_6$-porin solubilized in 1.75 mM SDS/ 15 mM DDM. The resulting

suspension was frozen in liquid nitrogen for 1 min and thawed for 20 min at room temperature, and this cycle was repeated three times. After the final freeze-thaw cycle, the samples were diluted with 1.5 volumes of 50 mM sodium phosphate pH 7 resulting in a cloudy solution that contained a final concentration of 6.6 mg/ml total phospholipid, 0.6 mM SDS, 5 mM DDM, and 166 nM (0.012 mg/ml) His_6-porin. Negative controls were prepared with detergents mixtures only and lacked His_6-porin.

Liposome swelling assays were performed to assess the ability of 1.75 mM SDS/15 mM DDM-His_6-porin to insert into liposomes as described for *E. coli* OmpA (Surrey and Jahnig 1995). Liposomes used for insertion experiments were prepared as described above except that the 20 mg/ml stock SUV was diluted 1:1 with 50 mM sodium phosphate pH 7 buffer and subjected to three freeze-thaw cycles. After the third cycle 0.25 volumes of 1 μM (0.03 mg/ml) His_6-porin solubilized in 1.75 mM SDS/15 mM DDM and 1.25 volumes of 50 mM sodium phosphate pH 7 buffer were added to the freeze-thawed SUV solution. To promote insertion, samples were incubated at room temperature, 30°C and 42°C overnight before measurement.

As described in (Chapter 2), liposome swelling was measured on an Ultrospec 3100 pro spectrophotometer as absorbance at 400 nm. Liposome swelling samples described above were diluted 1/100 into 50 mM sodium phosphate pH 7 buffer to a final volume of 0.5 ml and measured in a quartz cuvette. Liposomes were diluted with 40 μl of the iso-osmotic 50 mM sodium phosphate buffer pH 7 at 300-400 seconds followed by an addition of 40 μl of the hyperosmotic 1 M sucrose after 600-700 seconds. Liposome swelling in a sample is indicated by a gradual decrease in A_{400nm} over 1200 or 1800 seconds after sucrose addition.

3.3.5 Protease digestions of isolated mitochondria and His$_6$-porin reconstituted into liposomes or solubilized in mixed detergents

Mitochondria were isolated from *N. crassa 97-20 mus his*⁻ (Ninomiya *et al.* 2004) according to the method described in (Chapter 2). Mitochondrial pellets were resuspended in SEM (250 mM sucrose, 1 mM EDTA, 9 mM MOPS pH 7.5), or in 3.5 mM SDS/30 mM DDM buffered at pH 7 with 50 mM sodium phosphate to a final concentration of 1 mg/ml mitochondrial protein as determined by a Bradford assay (Sigma). Mitochondrial digestions contained 0.5 mg/ml mitochondrial protein in 1.75 mM SDS and 15 mM DDM. All samples were digested with a final concentration of 0.15 mg/ml trypsin for 10 min or 30 min intervals at room temperature.

His$_6$-porin reconstituted into liposomes was prepared for spectroscopic examination as described by (Chapter 2). Briefly, His$_6$-porin at a concentration of 2.5 – 5 μM in 3.5 mM SDS/ 30 mM DDM was diluted 1:4 into 50 mM sodium phosphate pH 7, which contained a final concentration of 2 mg/ml stock SUV. This liposome-detergent-His$_6$-porin mixture was subjected to one of three incorporation methods described by (Chapter 2): 1 cycle of freeze-thawing (FT), four 1 minute bursts of sonication (SON) on ice, or incubation with intact liposomes for direct porin insertion (INS). The resulting proteoliposomes were gently mixed overnight at room temperature (22-25°C) and centrifuged at 10 000 g for 10 min to remove precipitated material. Each proteoliposome sample was digested as described for mitochondria and contained 8-15 μg/ml (0.25-0.5 μM) His$_6$-porin, and 1 mg/ml phospholipid.

5 μM His$_6$-porin in 3.5 mM SDS/ 30 mM DDM was digested with trypsin and chymotrypsin under the same conditions as for mitochondria. Final concentrations of samples were as follows: 0.075 mg/ml (2.5 μM) His$_6$-porin and 1.75 mM SDS, and 15 mM DDM. All

protease digestions were stopped with phenylmethylsulfonlyfluoride to a final concentration of 20 mM and 4:1 dilution into Laemmli buffer (Laemmli 1970) containing 8 M urea. All reaction mixtures were stored at -20°C until needed. As a control for protease activity in the SDS/DDM system, BSA (Sigma) was also digested and demonstrated susceptibility to trypsin (data not shown).

3.3.6 Western blots of liposomes and *N. crassa* mitochondria

All protease digested samples were loaded onto 0.1 % SDS- 3 M urea- 14% PAGE gels; the urea was necessary to aid migration of the protein into the gel in the presence of high concentrations of lipid. Electrophoresis was performed with the Mini-Protean$^{®}$ II system (Bio-Rad Laboratories, Hercules, CA). Gel loading was as follows: 5 µl 0.5 mg/ml mitochondria in SEM or detergent, 10 µl of 7.5- 15 µg/ml His$_6$-porin in 1 mg/ml phospholipids, and 1 µl 75 µg/ml His$_6$-porin in detergent. Gels were blotted with a Trans-Blot$^{®}$ Cell system (Bio-Rad) onto nitrocellulose 0.45 µm membranes (Bio-Rad) overnight at 4°C and stained with Ponceau S (Sigma) to visualise the Benchmark protein ladder (Invitrogen, Burlington, ON).

Immunoblotting was carried out at room temperature using rabbit antibodies directed against residues 7- 20 of *N. crassa* mitochondrial porin (αNcPor-N, generated by R. Lill, at Universität München) following the same procedure described in Chapter 2. Blots were detected using CDP- star (Roche Diagnostics, Indianapolis, IN) and exposed for 1- 2 hrs on X-Omat Blue XB-1 Scientific Imaging film (Kodak, Rochester, NY).

3.3.7 Fluorescence spectrophotometry

Fluorescence spectroscopic analyses of detergent-solubilized model compounds and

His$_6$-porin were performed using a Shimadzu RF-1501 fluorometer or a JASCO-810 (FMO-427S) spectropolarimeter/fluorometer, as described in Chapter 2. The relatively hydrophilic (NAc-W-NH$_2$ and NAc-Y-NH$_2$), and hydrophobic (BOC-Y-OMe and N-Ac-W-OEth) model compounds were used for comparison with fluorescence by His$_6$-porin. Samples of individual model compounds, and Trp:Tyr mixtures in the same molar ratio as in His$_6$-porin (2:9), were prepared at molar concentrations close to those of the individual residues in porin and all of the protein and model compound fluorescence spectra were normalized with reference to His$_6$-porin in urea. The sums of the spectra obtained from the individual components equalled those from the 2:9 mixtures, indicating a lack of interaction between the fluorophores.

When necessary, the fluorescence spectra were smoothed using a moving average over a window of 5 nm. To determine the maximum fluorescence emission of tyrosine (λ_{max}Tyr) and tryptophan (λ_{max}Trp) in the porin or model compound spectra, the second derivatives of the averaged spectra were obtained with Jasco Spectra Analysis Software, Version 1.53.04, using data at 1-nm intervals and a window of 9-13 nm, depending on the noise in the spectrum (Table 3.2; Garcia-Borron *et al.* 1982).

3.3.8 UV absorption spectroscopy

UV absorption spectra were obtained on an Ultrospec 4000 spectrophotometer, as described in Chapter 2. Tyrosine exposure (α) was calculated using the method described in (Ragone *et al.* 1984, Chapter 2). Second derivative UV absorption spectra of proteoliposomes prepared for spectroscopic analysis could not be reliably measured due to high amounts of light scatter.

3.3.9 Circular dichroism spectropolarimetry

CD spectra were acquired on a JASCO J-810 spectropolarimeter-fluorometer calibrated with (+)-10-camphorsulfonic acid and purged with N_2 at 20 L/min (Manley and O'Neil 2003). CD spectra of 1.5 μM or 5 μM His$_6$-porin samples were measured in the far-UV region (195-250 nm) as described previously (Chapter 2). Following correction by baseline subtraction, CD spectra were converted to mean residue ellipticity (MRE) according to the formula: $[\theta]_M$ = $M\theta/[(10)(l)(c)(n)]$ where $[\theta]_M$ is 10^{-3} deg cm^2 dmol^{-1}, M is the molecular weight of His$_6$-porin (31,402 g/mol), θ is the measured ellipticity in millidegrees, l is the path length of the cuvette in cm (0.1 cm), c is the protein concentration in g /L, and n is the number of amino acid residues in the protein (295). Spectra were deconvoluted with the CDSSTR algorithm (Compton and Johnson 1986, Manavalan and Johnson 1987, Sreerama *et al.* 2000) in the DichroWeb package (Whitmore and Wallace 2004).

Near-UV (245-330 nm) CD spectra of 33 μM His$_6$-porin in mixed detergents were measured with a JASCO J-810 spectropolarimeter-fluorometer, as described in Chapter 2. Molar ellipticity was calculated from the baseline corrected spectra according to the formula: $[\theta] = M\theta / [(10)(l)(c)]$ where $[\theta]$ is the molar ellipticity in degrees cm^2 dmol^{-1}, θ is the measured ellipticity in millidegrees, l is 1 cm, and c is the protein concentration in g/ L.

Temperature was controlled during thermal denaturation experiments using the Peltier device in the spectropolarimeter. Experiments were carried out with a 1°C / min ramp speed; θ (in mdeg) was monitored at 208 or 216 nm (far-UV) and at 268 nm (near-UV) at 1°C intervals, and full spectra were collected at 5°C intervals. High tension (HT) voltage (absorbance) measurements were monitored during single wavelength and full spectra collection experiments.

Far-UV CD spectra of FT proteoliposomes in the SDS/DDM system could not be collected below 200 nm due to light scatter and were not deconvoluted for secondary structure estimates. Due to reduced signal to noise ratios in both the far UV- and near-UV CD spectra of FT SDS/DDM-His$_6$-porin-liposomes, collected data were not shown for all mixed detergent His$_6$-porin reconstituted into liposomes.

3.4 Results

Addition of a non-ionic detergent to SDS-solubilized OmpA β-barrel was shown to convert the α-helical rich structure to one predominantly β-strand (Ohnishi and Kameyama 2001). To determine if a similar approach would yield well-folded mitochondrial porin, several detergent systems were examined. SDS and DPC were chosen initially to solubilize His$_6$-porin, based on the high solubility of the protein in these detergents (Chapter 2). Under these conditions, β-strand content is low, and ranges from 17-25%. The non-ionic detergents, DDM and OG, and the zwitterionic LDAO were used as the second detergents in the system. With the exception of LDAO, several molar ratios of the two detergents were examined. Phase separation occurred at several LDAO/SDS ratios, limiting the mixtures that could be tested. For simplicity, mixtures will be described by each detergent, with its concentration in mM, as a subscript. For example, SDS$_{3.5}$DDM$_{7.5}$ is a mixture of 3.5 mM SDS and 7.5 mM DDM.

3.4.1 Characterization of mixed detergent-solubilized His$_6$-porin by far-UV circular dichroism spectropolarimetry

To determine the effects of secondary detergent addition on the folded states of the protein solubilized in DPC or SDS, far-UV CD was used (Fig. 3.1, Table 3.1). In 3.5 mM

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SDS, the far-UV spectrum of His_6-porin is typical of an α-helical rich protein, in that it has a minimum at 208 nm and a shoulder at 220 nm. Upon addition of 7.5 mM DDM, a small shift in the spectrum is observed (Fig. 3.1A), but the overall α-helical character is retained and structural content, as revealed by deconvolution of this spectrum is identical to that in SDS (Table 3.1). However, in $SDS_{3.5}DDM_{15}$, the spectrum of the protein has a broad minimum in the 210-220 nm range, more typical of a β-strand rich protein. This observation is confirmed by deconvolution, which reveals a loss of α-helix concomitant with an increase in β-strand to 28%. As the DDM concentration was increased to generate $SDS_{3.5}DDM_{30}$ or $SDS_{3.5}DDM_{45}$, this trend continued, and β-strand content reached 35 and 38%, respectively (Table 3.1). Throughout the SDS/DDM series, the level of turns and unordered structure remained unchanged.

Similar trends of increasing β-strand and decreasing α-helix occur upon addition of OG (12.5 to 100 mM) to His_6-porin in SDS and maximum levels of β-strand were reached in $SDS_{3.5}OG_{50}$ (Fig. 3.1B). Secondary structural content in $SDS_{3.5}LDAO_{40}$ (Fig. 3.1C) resembled that in the SDS/DDM and SDS/OG mixtures; the SDS/LDAO and SDS/OG mixtures are not useful for further structural studies due to the low porin solubility under these conditions (~1.5 μM, Table 3.1). Remarkably, the addition of either 10 or 100 mM DPC, which also promotes α-helix, increases the α-helical content of the protein to 52-58%, with a dramatic reduction in β-strand (7%). The very high α-helical content of SDS/DPC solubilized porin indicates that native folding has not been achieved.

SDS-solubilized His_6-porin is very stable during heating, and the limited unfolding that does occur is completely reversible (Chapter 2; Figs. 3.2A and 3.2B). Only the SDS/DDM system was capable of solubilizing porin at concentrations sufficient for this type

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of analysis (Figs. 3.2C and 3.2D, Table 3.1). During heating of $SDS_{3.5}DDM_{30}$-solubilized

porin to 45°C, there are only minor alterations to the secondary structural content. At 55°C,

an increase in the (HT) voltage or absorbance of the sample, as monitored by the CD

spectropolarimeter, indicated precipitation in the sample; absorbance measurements at the

end of the experiment demonstrated that about 2/3 of the protein remained in solution. At

55°C, the far-UV CD spectra broadened, and developed minima characteristic of higher

levels of α-helix. Deconvolution of these spectra confirms a steady loss in β-strand (35 to

28%) and constant α-helical content, with a significant increase in random structure (47 to

60%). Further large-scale changes did not occur at higher temperature. Unlike the situation in

SDS (Fig. 3.2A), the thermal denaturation is not reversible and the structural content upon

cooling to 20°C remains similar to that at 55°C and above (Table 3.1, Figs. 3.2C and 3.2D).

When the protein was solubilized initially in DPC, the same trend in secondary structure

content alterations upon addition of a second detergent were observed as for the SDS based

systems. Addition of 30 mM DDM, 100 mM OG or 50 mM LDAO (Figs. 3.1D-3.1F)

promoted structures with 33-38% β-strand, in contrast to the 25% present in the DPC-

solubilized protein. Of all the DPC-based systems, only DPC/LDAO is capable of

maintaining at least 5 μM porin in solution.

3.4.2 Fluorescence of tryptophan model compounds in mixed-detergent systems

Model compounds were used to determine how the addition of a second detergent to

SDS or DPC micelles affects the interactions of the aromatic amino acids, namely tyrosine

and tryptophan, with the micelles. Two variants of each compound were used, the relatively

hydrophobic O-alkyl-esters, BOC-Y-OMe and N-Ac-W-OEth, and the more hydrophilic

amide variants, N-Ac-Y-NH$_2$ and N-Ac-W-NH$_2$. In previous studies, it was shown that the

Table 3.1. Analysis of His$_6$-porin solubilized in mixed detergent systems. Far UV region CD spectropolarimetry was performed on either 1.5 or 5 μM porin samples as indicated; the resulting averaged spectra were deconvoluted with CDSSTR (Whitmore *et al.* 2004).

Solubilizing Detergent	Detergent added	Temp (^{o}C)	α-helix (%)	β-strand (%)	turns & unordered	Maximum Concentration of soluble porin (μM)
3.5 mM SDS		20	32	18	50	66
	7.5 mM DDM	20	33	17	50	5*
	15 mM DDM	20	23	28	49	5*
	30 mM DDM	20	18	35	47	5*
		35	14	36	50	5*
		45	14	33	52	5*
		55**	15	28	56	UD
		60	12	28	60	UD
		80	15	20	65	UD
		80→20	18	26	55	3.3*
	45 mM DM	20	13	38	49	5*
	12.5 mM OG	20	21	29	50	1.5
	25 mM OG	20	24	28	48	1.5
	50 mM OG	20	17	35	47	1.5
	100 mM OG	20	17	35	48	1.5
	40 mM LDAO	20	13	34	53	1.5
	10 mM DPC	20	58	7	36	5*
	100 mM DPC	20	52	7	41	5*
20 mM DPC		20	22	25	53	16
	7.5 mM DDM	20	14	33	54	1.5
	15 mM DDM	20	9	36	54	1.5
	30 mM DDM	20	9	38	53	1.5
	6.25 mM OG	20	13	29	58	1.5
	12.5 mM OG	20				1.5
	50 mM OG	20	7	35	57	1.5
	100 mM OG	20	6	38	56	1.5
	50 mM LDAO	20	16	33	51	5*

* Higher concentrations not tested.

** Precipitation of His$_6$-porin was detected by UV absorption at this temperature.

UD His$_6$-porin concentration in the sample was undetermined.

Figure 3.1. Far-UV CD analysis of 5 μM His$_6$-porin in mixed detergent systems. Spectra were obtained as indicated in Materials and Methods. **A)** SDS/DDM systems. Far-UV spectra obtained for His$_6$-porin in the following SDS/DDM mixtures: SDS$_{3.5}$ (solid line), SDS$_{3.5}$DDM$_{7.5}$ (dotted line), SDS$_{3.5}$DDM$_{15}$ (short dashes), SDS$_{3.5}$DDM$_{30}$ (dash-dot-dash), and SDS$_{3.5}$DDM$_{45}$ (long dashes). **B)** Mixed SDS/OG systems. Far-UV spectra obtained for His$_6$-porin in the following SDS/OG mixtures: SDS$_{3.5}$ (solid line), SDS$_{3.5}$OG$_{12.5}$ (dotted line), SDS$_{3.5}$OG$_{25}$ (short dashes), SDS$_{3.5}$OG$_{50}$ (dash-dot-dash), SDS$_{3.5}$OG$_{100}$ (long dashes). **C)** SDS/DPC and SDS/LDAO systems. Far-UV spectra obtained for His$_6$-porin in the following mixtures are indicated as follows: SDS$_{3.5}$ (solid line), SDS$_{3.5}$LDAO$_{40}$ (dotted line), SDS$_{3.5}$DPC$_{10}$ (short dashes), SDS$_{3.5}$DPC$_{100}$ (dash-dot-dash). **D)** DPC/DDM systems. Far-UV spectra obtained for His$_6$-porin in the following mixtures: 20 mM DPC (solid line), DPC$_{20}$DDM$_{7.5}$ (dotted line) DPC$_{20}$DDM$_{15}$ (short dashes), DPC$_{20}$DDM$_{30}$ (dash-dot-dash). **E)** DPC/OG systems. Far-UV obtained for His$_6$-porin in the following mixtures are indicated as follows: 20 mM DPC (solid line), DPC$_{20}$OG$_{12.5}$ (dotted line), DPC$_{20}$OG$_{25}$ (short dashes), DPC$_{20}$OG$_{50}$ (dash-dot-dash), and DPC$_{20}$OG$_{100}$ (long dashes). **F)** DPC and LDAO. Far-UV obtained for His$_6$-porin in the following mixtures are indicated as follows: 20 mM DPC (solid line), and DPC$_{20}$LDAO$_{40}$ (dotted line).
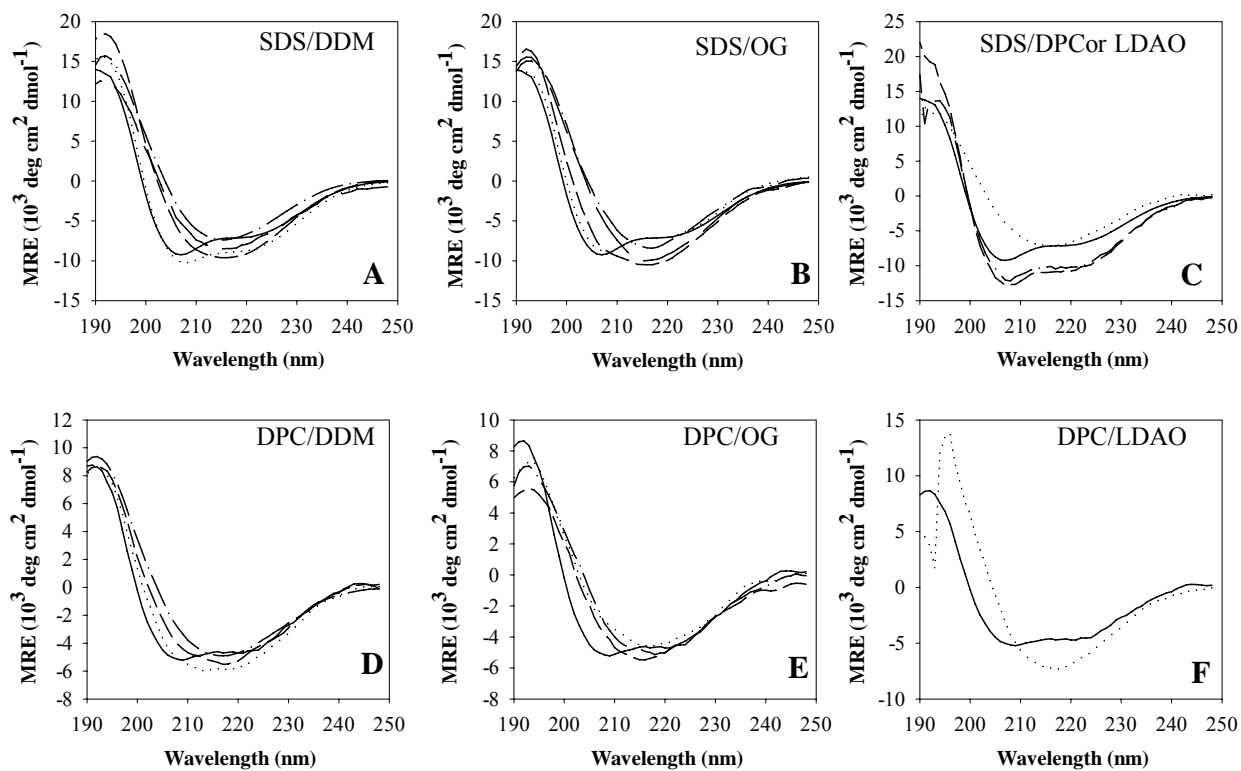
former compounds are better probes of the hydrophobic interiors of detergent micelles (Chapter 2). Trp fluorescence is described in relation to Burstein's classification system: Class S (λ_{max}Trp 316 nm), Class I (331 nm), Class II (342 nm) and Class III (347-351 nm). For these classes, increasing λ_{max}Trp is associated with increased solvent exposure of the Trp residues; Class III residues are completely exposed to the solvent (Burstein *et al.* 1973, Reshetnyak *et al.* 2001).

In 3.5 mM SDS, both model compounds display Class III fluorescence (Table 3.2), indicating that they are predominantly in the aqueous phase, although N-Ac-W-OEth interacts slightly more often with the micelle, as indicated by the blue shift in λ_{max}Trp (351 vs 347 nm), compared to that of N-Ac-W-NH$_2$. λ_{max}Trp of N-Ac-W-OEth blue-shifts to 341 (Class II) and 337 nm (Classes I/II) in SDS$_{3.5}$DDM$_{7.5}$ and SDS$_{3.5}$ DDM$_{15}$, respectively, indicating increased interactions between the micelles and the model compounds as the DDM concentration is increased. Relative to that in SDS$_{3.5}$DDM$_{15}$, a slight red-shift (343 nm (Class II)) occurs in SDS$_{3.5}$DDM$_{30}$, indicating that the interactions with micelles of higher DDM concentration are reduced. The fluorescence of N-Ac-W-NH$_2$ also blue-shifts upon addition of DDM to the SDS micelles; in this case, fluorescence shifts from Class III in SDS$_{3.5}$ and SDS$_{3.5}$DDM$_{7.5}$, to Class II in higher relative DDM concentrations (340 nm in SDS$_{3.5}$DDM$_{30}$).

In SDS, quenching of model compound Trp fluorescence by the detergent headgroups occurs (Chapter 2). In parallel with the blue shift in λ_{max}Trp for N-Ac-W-OEth, fluorescence intensity increases in SDS$_{3.5}$DDM$_{7.5}$ and SDS$_{3.5}$DDM$_{15}$ (Table 3.2), indicating that the quenching is reduced in the mixed micelles. However, the intensity in SDS$_{3.5}$DDM$_{30}$ is similar to that in SDS$_{3.5}$DDM$_{7.5}$. Thus, the changes in micellar environments do not parallel the relative detergent concentrations. In contrast, a steady increase in fluorescence intensity is

Figure 3.2. Thermal denaturation of His$_6$-porin as followed by far-UV CD spectropolarimetry. The experiments were carried out as described in Materials and Methods. **A)** 5 μM His$_6$-porin in 3.5 mM SDS. Full spectra were obtained at the following temperatures: 20°C (solid line), 60°C (long dashes), 95°C (short dashes), and following cooling of the sample to 20°C (dotted line). **B)** Unfolding monitored by mean-reside ellipticity at 208 nm. Measurements were taken every 1°C in the experiment described in A). The open circle indicates the value following cooling to 20°C. **C)** Thermal denaturation of 5 μM porin in SDS$_{3.5}$DDM$_{30}$. Spectra were obtained at the following temperatures: 20°C (solid line), 35°C (long dashes), 45°C (short dashes), 55°C (dotted line), 80°C (dash-dot-dash), and following cooling to 20°C (dash-dot-dot-dash). The arrow marked "ppt" indicates the point at which an increase in HT voltage indicates precipitate in the sample. **D)** 5 μM porin in 3.5 mM SDS and 30 mM DDM thermal unfolding monitored at 216 nm: cooled 20°C (triangle), ppt temperature indicated by arrow. Unfolding monitored by mean-reside ellipticity at 208 nm. Measurements were taken every 1°C in the experiment described in C). The open triangle indicates the value following cooling to 20°C.

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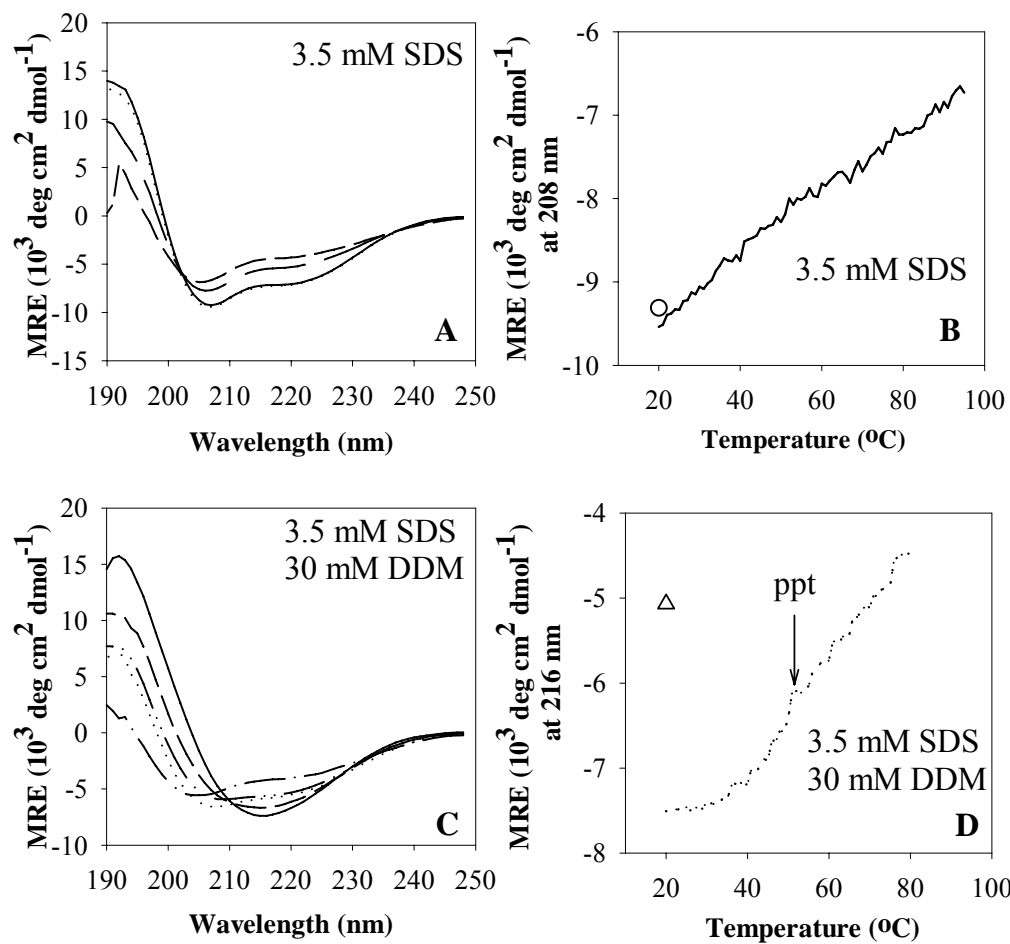
observed upon addition of DDM to the amide compound in SDS.

Addition of OG to the SDS-solubilized model compounds had a more modest effect on λ_{max}Trp for N-Ac-W-NH$_2$ (Table 3.2). A shift in λ_{max}Trp of N-Ac-W-OEth from Class III (347 nm) to Class II (342 nm) is observed only upon addition of high concentrations of OG (100 mM). In contrast, N-Ac-W-NH$_2$ remains mainly in the aqueous environment and only a small blue-shift, within the limits of Class III, is observed. In general, the fluorescence intensities for both compounds increase with the addition of 12.5 mM OG, but remain constant in the presence of up to 100 mM OG. Combinations of SDS and the two zwitterionic detergents, DPC and LDAO were also examined (Table 3.2). Only one DPC/SDS combination was used, because of the limitations of this system for porin studies (see above). Trp fluorescence of N-Ac-W-OEth is blue-shifted to Class II (337-338 nm) in both SDS$_{3.5}$/LDAO$_{40}$ and SDS$_{3.5}$/DPC$_{10}$, while that of the amide is shifted slightly less (to 344 nm). Thus, both compounds are interacting more often with these mixed micelles than with SDS alone. However, the fluorescence intensity is more similar to that in DPC or LDAO alone, than in SDS, indicating relative absence of quenching.

Both model compounds interact more with DPC than with SDS micelles (Table 3.2). For example, in DPC N-Ac-W-NH$_2$ displays Class II fluorescence, and that of N-Ac-W-OEth is intermediate between Classes I and II (Chapter 2). The addition of DDM or OG to DPC-solubilized model compounds does not alter λ_{max}Trp of N-Ac-W-Oeth (337-338 nm), and causes a slight blue-shift to that of N-Ac-W-NH$_2$ in DPC$_{20}$DDM$_{30}$, but its fluorescence remains near Class II (338-342 nm). In both DPC$_{20}$DDM mixtures, no change in intensity of N-Ac-W-OEth fluorescence is observed, suggesting that the interactions of this model compound with the DPC/DDM mixed micelles are similar to those in DPC alone. In

Table 3.2. Summary of fluorescence data collected at excitation wavelengths of 280 nm (Tyr) and 296 (Trp) nm. His$_6$-porin and amino model compounds were solubilized in various mixed detergents. Maximum intensity (Int$_{max}$) values were corrected for differences in concentration.

	His$_6$-porin Trp		BOC-Y-OMe Tyr		N-Ac-Y-NH$_2$ Tyr		N-Ac-W-OEth Trp		N-Ac-W-NH$_2$ Trp		N-Ac-W-OEth + BOC-Y-OMe Tyr*	N-Ac-W-OEth + BOC-Y-OMe Trp		N-Ac-W-NH$_2$ + N-Ac-Y-NH$_2$ Tyr*	N-Ac-W-NH$_2$ + N-Ac-Y-NH$_2$ Trp	
	λ$_{max}$	Int$_{max}$	λ$_{max}$	Int$_{max}$	λ$_{max}$	Int$_{max}$	λ$_{max}$	Int$_{max}$	λ$_{max}$	Int$_{max}$	λ$_{max}$	λ$_{max}$	Int$_{max}$	λ$_{max}$	λ$_{max}$	Int$_{max}$
Sample in 3.5 mM SDS with																
no addition	336	0.03	299	0.12	301	0.13	347	0.04	351	0.11	301	346	0.06	301	350	0.12
7.5 mM DDM	337	0.03	302	0.12	299	0.15	341	0.07	348	0.12	303	341	0.05	299	348	0.16
15 mM DDM	330	0.11	306	0.16	300	0.14	337	0.13	345	0.13	305	337	0.11	302	345	0.15
30 mM DDM	322	0.08	303	0.15	299	0.22	343	0.08	340	0.17	302	336	0.10	300	339	0.22
12.5 mM OG	339	0.05	304	0.14	298	0.17	347	0.09	349	0.15	304	347	0.09	299	350	0.13
25 mM OG	333	0.09	301	0.18	299	0.18	347	0.07	350	0.12	303	346	0.08	301	349	0.16
50 mM OG	330	0.08	301	0.21	302	0.19	345	0.10	352	0.13	302	344	0.12	301	349	0.20
100 mM OG	330	0.07	302	0.12	304	0.12	342	0.07	347	0.16	300	341	0.07	301	347	0.16
10 mM DPC	336	0.10	302	0.15	298	0.17	338	0.13	344	0.20	301	338	0.14	299	342	0.27
40 mM LDAO	332	0.14	302	0.04	303	0.12	337	0.15	344	0.25	300	338	0.22	303	344	0.20
Sample in 20 mM DPC with																
no addition	337	0.06	301	0.16	302	0.20	337	0.22	342	0.25	302	337	0.22	301	344	0.24
7.5 mM DDM	335	0.05	303	0.18	301	0.09	337	0.21	343	0.09	303	338	0.17	302	340	0.10
30 mM DDM	327	0.17	306	0.26	302	0.12	338	0.22	338	0.09	306	337	0.23	302	337	0.11
12.5 mM OG	333	0.09	302	0.13	300	0.15	338	0.11	342	0.11	301	340	0.13	302	343	0.12
50 mM OG	334	0.07	305	0.11	302	0.14	339	0.11	342	0.12	304	338	0.11	303	342	0.11
100 mM OG	334	0.07	302	0.10	302	0.06	338	0.09	342	0.11	301	335	0.10	303	342	0.09
50 mM LDAO	331	0.09	302	0.05	301	0.06	335	0.11	341	0.19	303	335	0.14	301	340	0.16

* Tyr fluoresence intensity could not be determined in mixtures of Trp and Tyr

contrast, fluorescence intensity of N-Ac-W-NH$_2$ in the DPC$_{20}$DDM mixtures was about half

of that in DPC alone, indicating quenching in the mixed system. Similarly, both model

compounds display decreased fluorescence in the DPC$_{20}$OG mixtures. Compared to that in

DPC alone, the fluorescence intensity of N-Ac-W-OEth in DPC$_{20}$LDAO$_{50}$ is about half, and a

smaller reduction is observed for the amide compound. These observations suggest

quenching by the headgroups in the mixed micelles, which interestingly does not occur when

either model compound is dissolved in the secondary detergent alone (Chapter 2, data not

shown for OG and DDM).

3.4.3 Fluorescence of tyrosine model compounds in mixed-detergent systems

In terms of tyrosine fluorescence, intensity changes are more informative because

only small shifts in λ_{max}Tyr occur, which are smaller than the +/- 3 nm sensitivity of the

spectrofluorometer used. As seen in Table 3.2, λ_{max}Tyr ranges from 298-304 nm. The

fluorescence intensity, however, provides clues about the degree of exposure of the model

compounds to the aqueous environment. As shown previously (Chapter 2), in SDS both N-

Ac-Y-NH$_2$ and BOC-Y-OMe have similar fluorescence characteristics, reflecting the fact that

the neither compound interacts extensively with the interior of the micelle. These

characteristics are unchanged for the BOC-Y-OMe in all of the mixed SDS/DDM micelles

tested, and for N-Ac-Y-NH$_2$ in the lower concentrations of DDM. However, the intensity at

λ_{max}Tyr of N-Ac-Y-NH$_2$ in SDS$_{3.5}$DDM$_{30}$ is twice that in SDS alone, suggesting that this

compound spends slightly more time in the hydrophobic interior of the micelle.

For both compounds, intensity at λ_{max}Tyr is slightly higher (0.14-0.21 nm) in mixed

SDS micelles formed in solutions containing up to 50 mM OG. However, these enhanced

interactions are not detected in SDS$_{3.5}$OG$_{100}$, where the intensity is similar to that in SDS

alone (0.12). In $SDS_{3.5}DPC_{10}$ micelles, fluorescence of BOC-Y-OMe and NAc-Y-NH$_2$ (0.15-0.17) is increased slightly in comparison to SDS alone (0.12-0.13). In $SDS_{3.5}LDAO_{40}$, however, the intensity of BOC-Y-OMe fluorescence is about one-third of that in SDS alone, while that of the amide is similar in both SDS and SDS/LDAO. This suggests that BOC-Y-OMe is in a quenching environment.

In DPC alone, both BOC-Y-OMe and N-Ac-Y-NH$_2$ have similar fluorescence characteristics (Table 3.2). Addition of $DPC_{20}DDM_{30}$, but not $DPC_{20}DDM_{7.5}$ increases fluorescence intensity of BOC-Y-OMe and decreases that of the N-Ac-Y-NH$_2$. Both model compounds display decreased fluorescence intensity in DPC/OG mixtures. Slight reductions of the fluorescence intensities of BOC-Y-OMe in all DPC/OG concentrations is seen, while a continued decrease in N-Ac-Y-NH$_2$ is observed as OG concentration increases. Very strong quenching is observed for N-Ac-Y-NH$_2$ in the $DPC_{20}OG_{100}$. Similar to BOC-Y-OMe in $SDS_{3.5}LDAO_{40}$, low fluorescence of both Tyr model compounds in $DPC_{20}LDAO_{50}$ occurs. Likely the LDAO headgroups are quenching BOC-Y-OMe fluorescence, as is observed in this detergent alone (Chapter 2).

3.4.4 Tryptophan fluorescence of His$_6$-porin in detergent

With the background information provided by the model compound studies, the Trp fluorescence of His$_6$-porin was investigated following excitation at 296 nm. In 3.5 mM SDS, His$_6$-porin fluorescence is intermediate between classes I and II, indicating that the Trp residues are partially exposed to the aqueous environment. The low intensity of the Trp fluorescence suggests quenching by the SDS headgroups as seen for hydrophobic model compounds (Chapter 2). These attributes are unchanged in $SDS_{3.5}DDM_{7.5}$. In $SDS_{3.5}DDM_{15}$, a dramatic blue-shift is observed (330 nm, Class I), indicating movement of the Trp residues

to more hydrophobic environments where they are hydrogen bonding in 2:1 exiplexes (Burstein *et al.* 1973). The blue-shift in λ_{max}Trp is more dramatic in $SDS_{3.5}DDM_{30}$ (322 nm, intermediate between Class I and Class S fluorescence) and the Trp residues reside in an environment of increased hydrophobicity.

In terms of fluorescence intensity at λ_{max}Trp, no difference is observed between that of His_6-porin in $SDS_{3.5}$ and in $SDS_{3.5}DDM_{7.5}$, in agreement with the consistency in λ_{max}Trp and the secondary structure composition. However, the fluorescence intensity in $SDS_{3.5}DDM_{15}$ and $SDS_{3.5}DDM_{30}$, is two-to-three fold greater in intensity, compared to that in SDS alone. Combined with the corresponding alterations in secondary structure (Table 3.1), this observation suggests that the arrangement of the protein-detergent complex in these mixtures differs significantly from that in SDS alone, and that the Trp residues no longer encounter a quenching environment similar to that in SDS alone.

Generally, similar trends were observed in the SDS/OG system, but a higher mole fraction of OG was required to induce the conformational shift. λ_{max}Trp blue-shifted from 339 nm (Class I/II) in $SDS_{3.5}OG_{12.5}$ to 330 nm (Class I) in $SDS_{3.5}OG_{50}$ or $SDS_{3.5}OG_{100}$. Accompanying the blue shift is a three-fold increase in intensity in $SDS_{3.5}OG_{25}$, but the magnitude of the increase was less in the mixtures with higher OG concentrations. In agreement with the far-UV CD analysis (Table 3.1), the structures promoted by $SDS_{3.5}OG_{50}$ and $SDS_{3.5}OG_{100}$ are indistinguishable. Therefore, the consequences of the addition of the non-ionic detergents DDM and OG to SDS-solubilized His_6-porin are similar.

In contrast, addition of DPC to SDS-solubilized porin does not affect λ_{max}Trp; however, a three-fold increase in intensity is observed. Therefore, the very α-helix rich structure promoted by this detergent combination does not alter the overall hydrophobicity of

the Trp environments, but does remove the quenching effect of SDS. Quenching is even lower in the $SDS_{3.5}LDAO_{40}$ mixture, and in this case, the Trp residues are exposed to a slightly more hydrophobic environment than in SDS alone, but λ_{max}Trp remains intermediate between classes I and II.

Like SDS, DPC promotes a conformation of His_6-porin with significant α-helical content (Chapter 2). Addition of 7.5 mM DDM to porin in DPC_{20} did not change Trp fluorescence significantly, but a blue-shift to 327 nm (Class I) and a three-fold increase in intensity was observed in $DPC_{20}DDM_{30}$. Given that quenching is not observed for porin in DPC alone (Chapter 2), these results suggest that the more hydrophobic environment inside the mixed micelles is responsible for the increase in fluorescence intensity. In contrast, in three DPC/OG mixtures, λ_{max}Trp of porin is only slightly blue-shifted to 333-334 nm (Class I), and there is little change in fluorescence intensity. Thus the increased levels of β-strand in porin dissolved in DPC_{20}/OG versus that in DPC_{20} likely do not reflect differences in the folded state of the regions containing the Trp residues. Finally, there is a blue-shift in λ_{max}Trp in $DPC_{20}LDAO_{50}$ (331 nm, Class I); the accompanying increase in fluorescence intensity is small, indicating small changes in the phobicity of the Trp environment.

3.4.5 Tyrosine fluorescence of His_6-porin in detergent

As described for the model compounds, the intensity of Tyr fluorescence is the most informative factor. However, due to the large blue-shifts in Trp fluorescence in the SDS/DDM mixtures, it is difficult to precisely assign the intensity of Tyr fluorescence following excitation at 275 nm (Fig. 3.3). Nonetheless, several general trends can be noted. The spectrum of the protein in $SDS_{3.5}DDM_{7.5}$ or in $SDS_{3.5}OG_{12.5}$ broadens considerably, suggesting a significant contribution of Tyr fluorescence and that the addition of small

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amounts of DDM or OG disrupts the protein/SDS micelle in such a way that at least some of the Tyr residues are exposed to more hydrophobic environments. At higher concentrations of DDM or OG, the overlap between Tyr fluorescence and the blue-shifted Trp fluorescence is too extensive to separate and properly analyze.

In $SDS_{3.5}LDAO_{40}$, Tyr fluorescence is quenched, as was observed for the model compounds in this mixture (Table 3.2). The clearly visible shoulder in the spectrum of $SDS_{3.5}DPC_{10}$-solubilized His_6-porin indicates a massive increase Tyr fluorescence compared to that in SDS alone; a difference of similar magnitude is not observed for the model compounds, indicating the folded state of the protein is responsible for the increased Tyr fluorescence in SDS/DPC micelles.

Blue-shifted Trp fluorescence also limits the analysis of His_6-porin in DPC/DDM and DPC/OG mixtures; in both systems the intensity near 300 nm is significantly higher than that in the SDS systems. In contrast, in $DPC_{20}LDAO_{50}$, quenching of the Tyr fluorescence of the protein is observed (Fig. 3.2F), and again likely reflects interactions with the LDAO headgroups.

3.4.6 SDUV analysis of tyrosine exposure in detergent-solubilized His_6-porin

Due to limited solubility, there were only three systems that were amenable to analysis by UV absorption: SDS/DDM, SDS/DPC and DPC/LDAO (Table 3.3). First, the model compounds were analyzed to examine interactions of the amino acids with the detergents. The second derivative plots of UV absorbance spectra were used to calculate *r* values; in general, high *r* values indicate exposure to solvent, while lower values are indicative of interactions with hydrophobic environments (Ragone *et al.* 1984). For

Figure 3.3. Fluorescence of His_6-porin in mixed detergent systems following excitation at 275 nm. Fluorescence of 0.42 μM His_6-porin in detergent was measured as indicated in Materials and Methods. The protein was dissolved in the following detergent mixtures: **A)** 3.5 mM SDS (dash-dot-dot-dash), $SDS_{3.5}DDM_{7.5}$ (dotted line), $SDS_{3.5}DDM_{15}$ (dashes), $SDS_{3.5}DDM_{30}$ (solid line). **B)** 3.5 mM SDS (dash-dot-dot-dash), $SDS_{3.5}OG_{12.5}$ (dotted line), $SDS_{3.5}OG_{25}$ (dashed line), $SDS_{3.5}OG_{50}$ 50 mM OG (solid line), and $SDS_{3.5}OG_{100}$ 100 (dash-dot-dash) **C)** 3.5 mM SDS (dash-dot-dot-dash), $SDS_{3.5}DPC_{10}$ (dotted line), $SDS_{3.5}LDAO_{40}$ (dashed line) **D)** 20 mM DPC (dash-dot-dot-dash), $DPC_{20}DDM_{7.5}$ (dotted line), $DPC_{20}DDM_{30}$ (dashed line) **E)** 20 mM DPC (dash-dot-dot-dash), $DPC_{20}OG_{12.5}$ (dotted line), $DPC_{20}OG_{50}$ (dashed line), $DPC_{20}OG_{100}$ (solid line) **F)** 20 mM DPC (dash-dot-dot-dash), $DPC_{20}LDAO_{50}$ (dotted line).
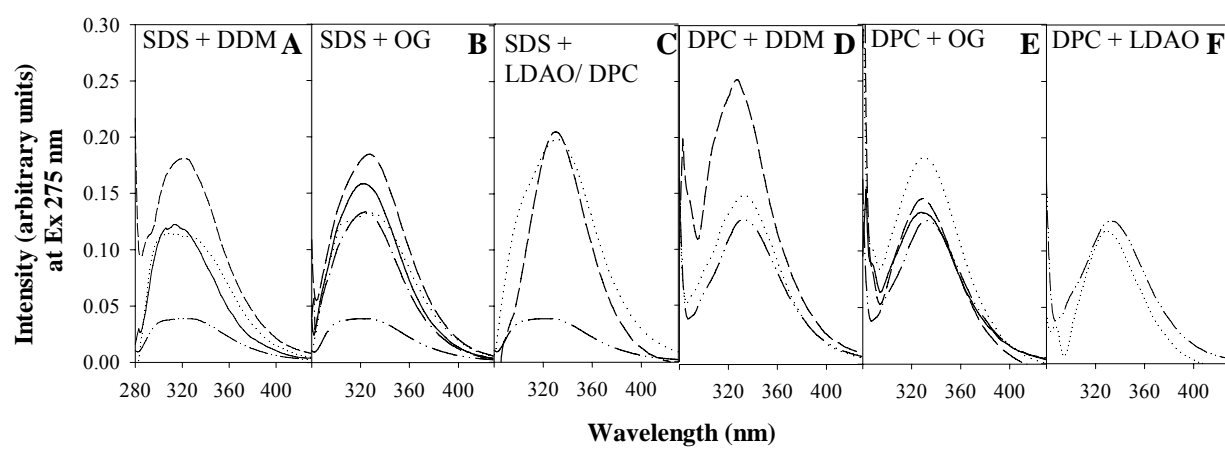
example, in SDS, r calculated for the more hydrophobic Tyr compound, BOC-Y-OMe, is lower (1.51) than that of the amide compound, N-Ac-Y-NH$_2$ (2.24). In SDS$_{3.5}$/DDM$_{7.5}$, the r value calculated for BOC-Y-OMe (1.82) is slightly higher that that in SDS alone, but the values in mixtures with DDM concentrations of 15-20 mM are similar to that in SDS alone. In SDS$_{3.5}$DDM$_{30}$, Tyr exposure drops significantly, indicating that the interactions of the hydrophobic BOC-Y-OMe are more extensive with these DDM/SDS micelles than in SDS$_{3.5}$. As expected, solvent exposure of the hydrophilic NAc-Y-NH$_2$ compound is higher than for BOC-Y-OMe in SDS/DDM mixtures. Compared to those obtained for N-Ac-Y-NH$_2$ in SDS, lower r values are observed in SDS$_{3.5}$DDM$_{15}$ and SDS$_{3.5}$DDM$_{20}$, but not in SDS$_{3.5}$DDM$_{30}$ and SDS$_{3.5}$DDM$_{60}$. Finally, in SDS$_{3.5}$DPC$_{10}$, Tyr exposure is reduced for both compounds compared to SDS. The amide compound does not interact appreciably with DPC or DPC/LDAO micelles, as r values are high (2.5-2.7). In contrast, very low r values are calculated for BOC-Y-OMe and Tyr exposure is negligible in DPC$_{20}$LDAO$_{50}$. The striking differences between the SDS and DPC based systems likely reflect the strong ionic character of the SDS headgroups.

As noted previously, Tyr exposure is high (77%) in SDS-solubilized porin (Chapter 2). In comparison to that in SDS$_{3.5}$, there is a large difference between the environments seen by Tyr residues in porin solubilized in SDS$_{3.5}$DDM$_{7.5}$; on average only 22% (~2) of the Tyr residues are exposed to the aqueous environment in the latter system. This observation confirms the prediction that increased Tyr fluorescence intensity in the SDS/DDM system (Table 3.2) reflects a higher degree of interaction between the Tyr residues and the detergent micelles. At higher DDM concentrations (15-30 mM) in SDS, Tyr exposure is higher (50%),

Table 3.3. SDUV analysis of His_6-porin and model compounds. r and Y_{exp} values were calculated as described in Materials and Methods. For the amide model compounds and His_6-porin, each experiment was repeated at least twice; averages are reported; standard deviations were less than 22% for each listed value. For the hydrophobic compounds, the data reported are from a single experiment.

	r			
Detergents	**N-Ac-W-OEth + BOC-Y-OMe**	**N-Ac-W-NH$_2$ + N-AcY-NH$_2$**	**His$_6$-porin**	**Y$_{exp}$ (H)**
3.5 mM SDS*	1.51	2.24	1.39	0.77
7.5 mM DDM + 3.5 mM SDS	1.82	2.48	0.85	0.22
15 mM DDM + 3.5 mM SDS	1.53	1.63	1.02	0.42
20 mM DDM + 3.5 mM SDS	1.59	1.80	1.16	0.55
30 mM DDM + 3.5 mM SDS	0.85	2.26	1.18	0.58
60 mM DDM + 3.5 mM SDS	1.17	2.36	1.40	0.79
10 mM DPC + 3.5 mM SDS	1.23	1.95	0.59	0.00
20 mM DPC	0.38	2.53	0.65	0.06
50 mM LDAO + 20 mM DPC	0.03	2.66	0.51	0.00

*Data taken from previous work (Chapter 2, Table 2.4)

in agreement with the apparent loss of Tyr fluorescence suggested by the narrowing of the spectrum following excitation at 275 nm (Fig. 3.3A).

In contrast, addition of DPC to SDS-solubilized porin essentially eliminated Tyr exposure, which suggests that the α-helical structure places these residues within the hydrophobic interior of the protein/detergent complex. Tyr exposure is also very low in DPC alone (0.06) and is nearly zero in the $DPC_{20}LDAO_{50}$ system, suggesting that there may be energy transfer to other groups in this particular folded state. Quenching by LDAO headgroups was not observed for the model compounds and therefore is unlikely to occur for the Tyr residues in LDAO-solubilized His_6-porin. Again these results confirm conclusions made based of Tyr fluorescence of porin.

3.4.7 Near-UV circular dichroism spectropolarimetry

To determine if alterations in tertiary interactions accompany the structural changes detected by the methods described above, near-UV CD spectropolarimetry was used. Only the SDS/DDM, DPC/LDAO and SDS/DPC systems held enough porin for these analyses; the latter system was not pursued as it promotes a high level of α-helix and is therefore not relevant for porin structural analysis. As shown previously, near-UV CD spectra of porin in SDS display strong negative ellipticity in the 260-290 nm range (Chapter 2), which includes signals from Trp (270-290 nm) and Tyr (265-270 nm). Higher concentrations of detergent were required for these studies, but the same molar ratios of the pairs of surfactants were used. For the SDS systems, the protein was first solubilized in 21 mM SDS, followed by addition of DDM to final concentrations of 45 to 270 mM. Increased negative ellipticity in the Tyr (268 nm) and the Trp (~275 nm) regions was observed in $SDS_{21}DDM_{45}$ (Fig. 3.4A), indicative of more extensive tertiary contacts involving these amino acids. In $SDS_{21}DDM_{90}$,

the overall shape of the spectrum is similar to that of the protein in urea (Chapter 2) or LDAO

(Fig. 3.4B), but the negative intensity is intermediate between that in urea and in

$SDS_{21}DDM_{45}$. Remarkably, the ellipticity is positive in $SDS_{21}DDM_{180}$ and $SDS_{21}DDM_{270}$,

suggesting that the contacts surrounding the aromatic residues are significantly different in

these micelles.

As in SDS, the near UV-spectra of His_6-porin in 100 mM DPC display negative

ellipticity. A near-UV CD spectrum of similar overall shape is acquired from His_6-porin in

$DPC_{100}LDAO_{300}$; neither spectrum resembles that of the molten-globule state of the protein in

300 mM LDAO alone (Fig. 3.4B and Chapter 2).

To assess the stability of the tertiary interactions involving aromatic residues, thermal

denaturation was followed using near-UV CD. In SDS, the secondary structure composition

of porin is not altered significantly by heating to 95^oC (Chapter 2). However, heating alters

the near-UV CD spectrum (Fig. 3.5A-B); above 40^oC, the spectra resemble those of molten

globules, with very little ellipticity. When the ellipticity at 268 nm is followed during heating

to 90^oC (Fig. 3.5B), a steady reduction in negative ellipticity is observed. Upon cooling of the

heated sample to 20^oC, some tertiary contacts are established, but the spectrum is not

identical to that of the unheated sample.

During heating, the near-UV CD spectra of the protein in $SDS_{21}DDM_{180}$ show a

steady decrease in ellipticity until 45^oC is reached. At this point the spectrum resembles that

of the protein in 8 M urea (Chapter 2). Protein precipitation is occurring at this temperature,

as detected by an increase in HT voltage (Fig. 3.5D). Absorbance measurements taken after

the completion of the experiment indicate that about one-third of the protein remained in

solution. The near UV spectra of the protein at temperatures above 45^oC therefore represent

Figure 3.4. Near-UV analysis of 33 μM His$_6$-porin in mixed systems. Spectra were obtained as described in Materials and Methods. **A)** SDS/DDM systems. Near-UV CD spectra of porin are as follows: SDS$_{21}$DDM$_{270}$ (filled circles), SDS$_{21}$DDM$_{180}$ (empty triangles), SDS$_{21}$DDM$_{90}$ (solid line), SDS$_{21}$DDM$_{45}$ (empty squares), and 21 mM SDS (filled triangles). **B)** DPC/LDAO systems. Near-UV spectra of His$_6$-porin in the following detergents are presented: 300 mM LDAO (solid line), DPC$_{100}$LDAO$_{300}$ (filled circles), and 100 mM DPC (open triangles).
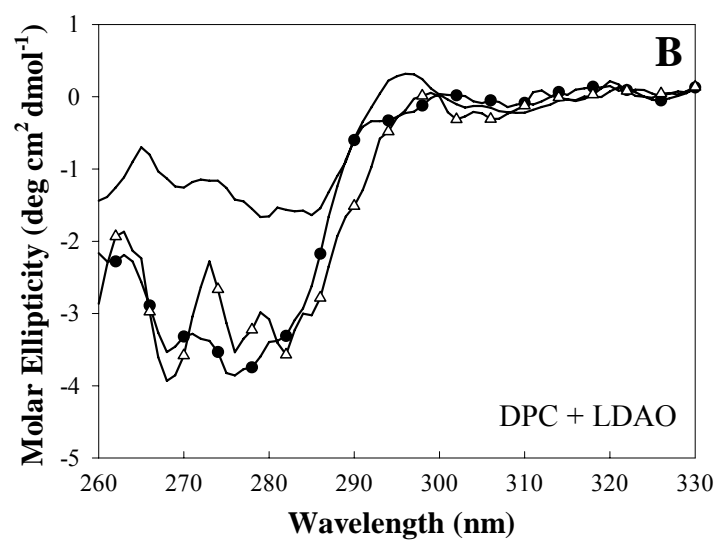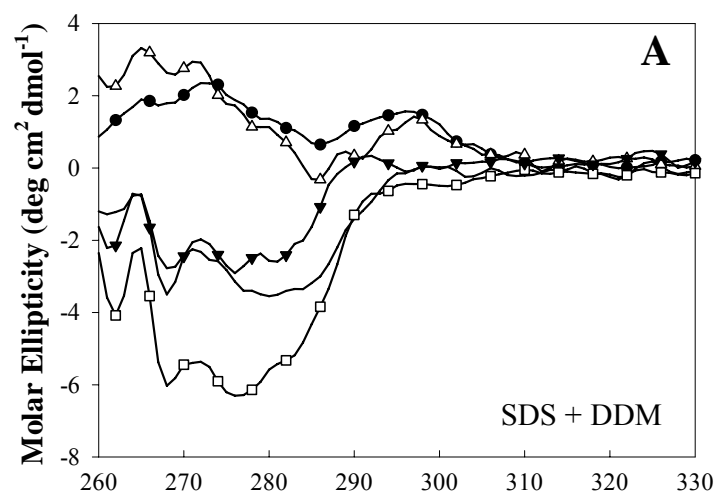
Figure 3.5. Thermal denaturation of His_6-porin in SDS and $SDS_{3.5}DDM_{180}$. Experiments were carried out as described in Materials and Methods. **A)** Near-UV CD spectra obtained following heating of 33 μM His_6-porin dissolved in 21 mM SDS. Results obtained at 20^oC (filled circles), 40^oC (open triangles), 60^oC (solid line), 90^oC (open squares) are presented. The spectrum following cooling from 90oC to 20oC is indicated by filled triangles. **B)** Thermal denaturation during the experiment in A), but monitored at 268 nm by near-UV CD at 1^oC intervals. The filled circle indicates the molar ellipticity following cooling to 20^oC. The dotted line indicates the HT values measured during the experiment. **C)** Near-UV CD spectra obtained following heating of 33 μM His_6-porin dissolved in $SDS_{21}DDM_{180}$. Results obtained at 20^oC (filled circles), 35^oC (open triangles), 45^oC (solid line), 55^oC (open squares) and following cooling of the sample at 90^oC to 20^oC (filled triangles) are presented. **D)** Thermal denaturation during the experiment in **D)**, but monitored at 268 nm by near-UV CD at 1^oC intervals. Symbols are the same as those in B). The arrow labelled "ppt" shows the temperature at which an increase in HT indicates precipitation in the sample.
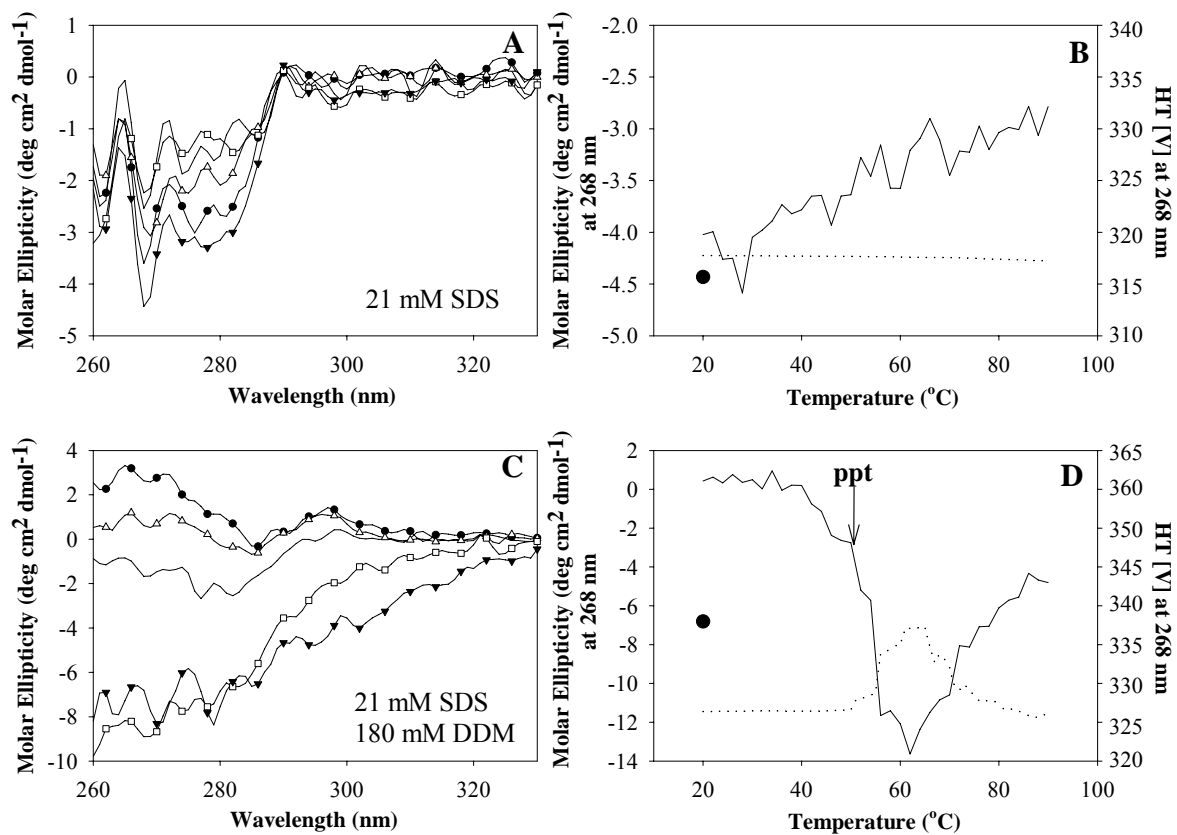
signals from a mixture of soluble and aggregated protein. In these cases, negative ellipticity was observed in both the Trp and Tyr regions (Fig. 3.5C).

3.4.8 Reconstitution of SDS/DDM-His$_6$-porin into liposomes

Since His$_6$-porin can be maintained at a high initial concentration in SDS$_{3.5}$/DDM$_{30}$, where it adopts a secondary structure with high β-strand content, this system was selected for further characterization in artificial membranes. Higher concentrations of DDM could not be used because the final DDM concentrations would have been in excess of its CMC. The SDS$_{3.5}$/DDM$_{30}$ system was also chosen for His$_6$-porin reconstitution into liposomes based on its ability to maintain high initial concentrations of protein in this solution necessary for membrane incorporation.

Liposome swelling assays performed for FT, SON, and INS SDS/DDM-His$_6$-porin-liposomes indicated that only FT proteoliposomes demonstrated swelling activity in response to sucrose (Fig. 3.6). Both INS and SON proteoliposomes had liposome swelling profiles that were almost identical to their respective control SDS/DM-liposomes prepared in the absence of protein (data not shown).

3.4.9 Protease sensitivity of mitochondrial porin and SDS/DDM-solubilized His$_6$-porin reconstituted into liposomes

Protease resistance is often used as a criterion for refolding of bacterial porins, such as OmpG in detergent (Conlan and Bayley 2003) or OmpA following reconstitution into artificial membranes (Dornmair *et al.* 1990, Kleinschmidt and Tamm 1996). It was also used to confirm that *Neurospora* mitochondrial porin was assembled in the outer membrane

Figure 3.6. Liposome swelling assays. SDS/DDM-His$_6$-porin liposomes were prepared by

the freeze-thaw (FT) method and swelling was measured directly (solid line; a) or following

heating to 65°C (dashed lines; b) or 100°C (dotted lines; c) and liposomes lacking His$_6$-porin

were measured as a control (line-dot-line; d). Relative A$_{400\text{ nm}}$ values for each sample were

plotted on the same scale, which permitted individual plots to be distinguishable from each

other.
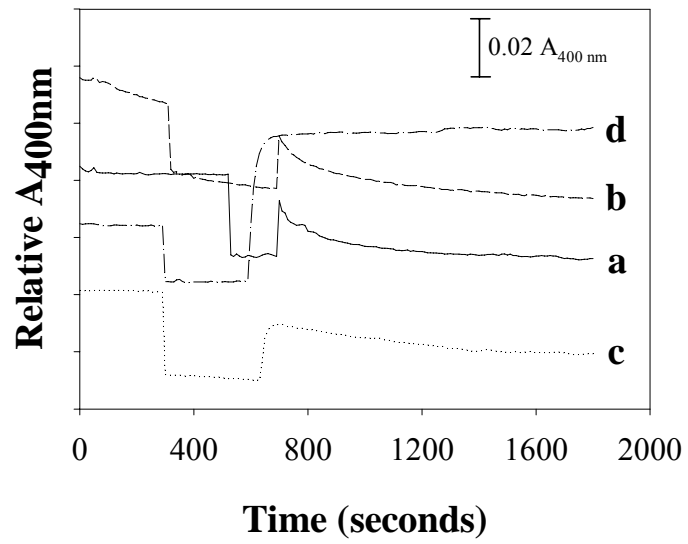
(Freitag *et al.* 1982). Protease sensitivity of native mitochondrial porin, and SDS or SDS$_{3.5}$/DDM$_{30}$-solubilized His$_6$-porin prior to and following reconstituting into liposomes was examined to determine the effects of mixed detergents on porin conformation. Immunoblotting was used to detect the low amounts of His$_6$-porin (< 2 µM) maintained in liposomes and porin in the isolated mitochondria. Trypsin (T) was also detected on the blots as 24 or 25-kDa bands due to sequence similarity between T and the porin peptide (residues 7-20) used to generate antibodies for this study.

Western blots of undigested *N. crassa* mitochondria in 3.5 mM SDS showed the intense 30-kDa porin band and minor 28 kDa, 21 kDa and 20 kDa proteolytic fragments resulting from activity of mitochondrially-associated proteases during isolation and/or the 30-min incubation at room temperature (Fig. 3.7A). All four species are in protease-sensitive conformations in this detergent, as they are not detected following T digestion. Blots of mitochondria in SDS$_{3.5}$/DDM$_{30}$ similarly have an intense band of intact porin at 30 kDa and minor proteolytic fragments (Fig. 3.7A). The full-length protein is highly resistant to digestion with T and very minor proteolytic fragments observed from the undigested sample lane are still detectable (Fig. 3.7A), as is the case for protease-digested *N. crassa* mitochondria in SEM (Chapter 2). Longer digestions did not reduce the intensity of the 30-kDa band (data not shown). A faint 55-kDa band can also be observed following T digestions of mitochondria in SDS$_{3.5}$/DDM$_{30}$, suggestive of a porin dimer.

His$_6$-porin migrates more slowly than mitochondrial porin (30 kDa) and has an apparent molecular mass of 32 kDa. Interestingly, undigested FT SDS/DDM-His$_6$porin-liposomes contained one additional faint 60-kDa species in the sample (Fig. 3.7B), suggesting dimers of the protein are present in the liposomes. Following digestion with

Figure 3.7. Protease susceptibility of porin in mitochondria, liposomes and detergent**. A)**

Western blot analysis of *N. crassa* mitochondria. Mitochondria (2.5 µg protein/well) were

solubilized in 3.5 mM SDS (lanes 1 and 2) or 3.5 mM SDS/30 mM DDM (lanes 3 and 4) and

analyzed following a 30-min digestion in the absence (lanes 1 and 3) or presence (lanes 2

and 4) of 0.75 µg T. **B)** Western blot analysis of FT, INS, and SON proteoliposomes formed

in the presence of 0.9 mM SDS/7 mM DDM and containing 2.5 µg His$_6$-porin. Samples were

undigested (lanes 1, 3 and 5) or digested with 0.075 µg T (lanes 2, 4, 6). **C) U**rea-SDS-PAGE

analysis of 7.5 µg of His$_6$-porin solubilized in of 3.5 mM SDS (lanes 1 and 2), or 3.5 mM

SDS/30 mM DDM (lanes 3 and 4). Samples were treated as described in B). An image of the

Coomassie blue stained gel is presented. The molecular weights of markers in the Sigma low

molecular weight ladder are indicated to the left of each panel. Filled circles indicate

multimers of His$_6$-porin and cross-reacting trypsin is indicated by asterisks.
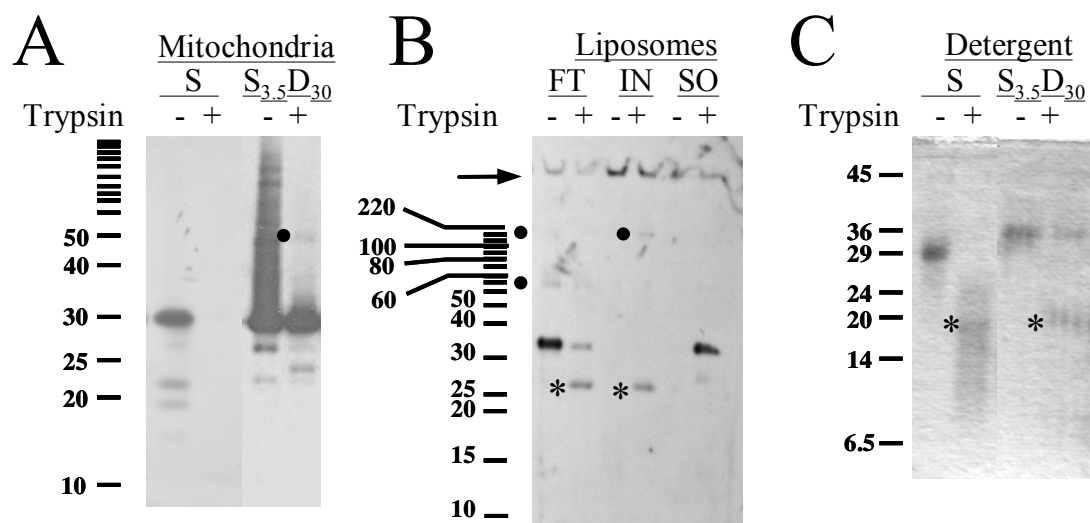
trypsin, the full-length His_6-porin band remained, but at less than half the intensity of the intact band in the undigested sample, and the 60-kDa species was no longer detectable.

Immunoblot analysis of INS and SON SDS/DDM-His_6porin-liposomes was much more difficult due to the high amount of protein retained in the wells of the gel (Fig. 3.7B). Only the lanes containing the SON SDS/DDM-His_6-porin-liposomes showed the 32-kDa His_6-porin band; additional bands at 65 and 90 kDa were not present (Fig. 3.7B). This material was very sensitive to protease. In contrast, almost no His_6-porin was detected in the lanes containing the INS type proteoliposomes, possibly due to its inability to enter the gel.

Since His_6-porin could be maintained at high concentrations in $SDS_{3.5}/DDM_{30}$, the protease sensitivity of this form of the protein was examined directly on SDS-PAGE gels stained with Coomassie blue (Fig. 3.7C). Undigested $SDS_{3.5}/DDM_{30}$-His_6-porin contained a single porin species with an apparent molecular weight slightly larger than that of the native protein solubilized in 3.5 mM SDS (Fig. 3.7C). Bands of 65 kDa or greater were not observed on these gels or upon western blotting (data not shown). Digestion of $SDS_{3.5}/DDM_{30}$-His_6-porin with T resulted in reduced intensity of the 35-kDa His_6porin band compared to the undigested protein band. This differs significantly from His_6-porin in 3.5 mM SDS which is completely digested by trypsin (Fig. 3.7C).

3.5 Discussion

These studies revealed the usefulness of mixed detergent systems for two-step folding of recombinant mitochondrial porins, and of model compounds for interpreting the Tyr and Trp environments in the mixed micelles.

Overall, the SDS/DDM system was most suitable for maintaining high concentrations of His_6-porin in a β-strand rich state supported by tertiary contacts. The usefulness of the other systems examined, with the exception of the DPC/LDAO mixture, was limited by low solubility of the protein. Phase separation of mixed detergent systems containing LDAO, and the relatively high cost, compared to SDS based systems, limits the usefulness of the DPC/LDAO system, which also promoted high levels of β-strand in His_6-porin. Furthermore, addition of LDAO to DPC solubilized His_6-porin did not promote changes to the near-UV CD spectrum, suggesting that the environments surrounding the aromatic residues had not changed.

Addition of different molar ratios of DDM and SDS to porin promoted unique structural conformations, which are summarized in Fig. 3.8. At the low concentrations of DDM in $SDS_{3.5}/DDM_{7.5}$ mixtures, the overall secondary structure content and $\lambda_{max}Trp$ are similar to those in SDS alone. However, rearrangements involving Tyr residues occur, as indicated by the large change in Tyr exposure. These changes are associated with increased negative ellipticity in the Tyr region of the near-UV CD spectrum. Since the α-helix and β-strand content are unchanged from those in SDS alone, these rearrangements are likely occurring within the turns or unordered regions of the protein. Seven of the nine Tyr are clustered in the C-terminal half of the protein (Fig. 3.8B), suggesting that this segment is unordered in the SDS-solubilized protein.

In $SDS_{3.5}DDM_{15}$, large-scale secondary structure changes occur, most notably the increase in β-strand at the expense of α-helical structure. This rearrangement involves placement of Trp residues in hydrophobic environments, as $\lambda_{max}Trp$ is blue-shifted. To date,

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Figure 3.8. Summary of the folded state of His$_6$-porin in SDS/DDM systems. **A)** Composite figure showing the differences in β-strand content (filled circles), λ_{max}Trp (open triangles), Tyr exposure (filled squares) and molar ellipticity at 268 nm (open diamonds). All values were adjusted to the same arbitrary scale; the exact values of each parameter are listed on the x-axis in the same column as the descriptor for that parameter. **B)** One model for the secondary structure of mitochondrial porin. The model is that of (Runke *et al.* 2006), based on deletion analysis and the compilation of data from other experimental systems. Solid filled rectangles indicate predicted β-strands, and the lines joining them represent turns and loops. The N-terminus of the protein is shown on the left (diagonal line unfilled rectangle). Stars and filled ovals indicate the positions of the Tyr and Trp residues in the protein.

A

B
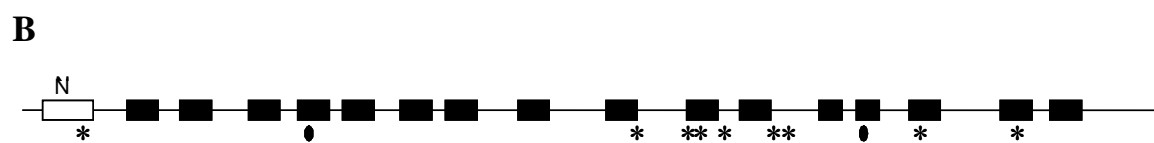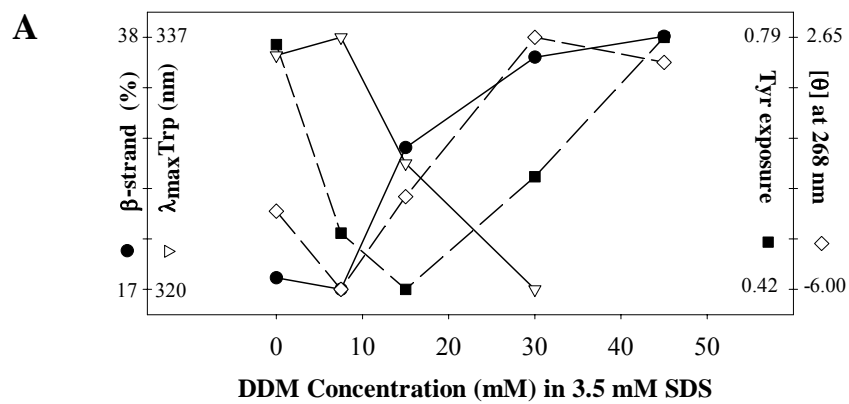
all models of mitochondrial porin structure (reviewed by Bay and Court 2002; Casadio *et al.* 2002) place the two Trp residues in predicted β-strand sequence (Fig. 3.8B). It is tempting to speculate that the regions of the protein flanking the two Trp residues are in α-helical structure in SDS and are arranged in β-strands in $SDS_{3.5}DDM_{15}$. Near-UV CD analysis suggests that the tertiary interactions involving Trp are less extensive in this conformation. Accompanying this change in secondary structure, Tyr exposure and negative ellipticity in the Tyr region of the near-UV CD spectrum are further reduced, suggesting significant changes to the folded state of large segments of the protein (Fig. 3.8B).

Higher DDM concentrations ($SDS_{3.5}DDM_{30}$), promote a higher level of β-strand (35%), with a concomitant decrease in α-helix. A large blue-shift in λ_{max}Trp is observed, indicating that the Trp residues are hydrogen-bonded in a very hydrophobic environment. Tyr exposure in $SDS_{3.5}DDM_{30}$ is intermediate between that in SDS alone and in $SDS_{3.5}DDM_{15}$. These observations suggest structural differences in several regions of the protein as less α-helix and more β-strand form. The fact that these are significant changes is supported by the observation that the near-UV CD spectrum displays positive ellipticity in both the Tyr and Trp regions. Strong positive ellipticity has been reported in the near-UV CD spectra of the native forms of the PorB class 2 (Minetti *et al.* 1997) and class 3 (Minetti *et al.* 1998) porins of *Neisseria*, and for the β-barrel segment of OmpA (Ohnishi and Kameyama 2001). Although the correlation is not absolute, a conversion from positive to negative ellipticity accompanies unfolding of other water-soluble β-strand proteins, such as cardiotoxin analogue III (CTXIII, Kumar *et al.* 1995, Sivaraman *et al.* 1997), CD40L (Matsuura *et al.* 2001) and TNF-α (Narhi *et al.* 1996). In His_6-porin, the positive ellipticity is lost upon heating to 45°C, and protein precipitation occurs, indicating that these interactions are required for stability in

SDS/DDM micelles and therefore they likely represent stable tertiary contacts within the protein. The negative ellipticity in the near-UV CD spectra of SDS-solubilized porin is lost upon heating, but, in contrast, precipitation does not occur, and the contacts can be restored upon cooling to 20°C. Thus, the positive ellipticity seen for porin in SDS-DDM is consistent with a β-barrel structure.

Protease digestions of His$_6$-porin at high DDM concentrations provide further support for well-folded His$_6$-porin in SDS/DDM systems. Both mitochondria and His$_6$-porin solubilized in SDS$_{3.5}$/DDM$_{30}$ demonstrate similar protease resistance to T, which suggests that the recombinant protein in this mixed system may share a similar conformation with mitochondrial porin.

In addition, SDS$_{3.5}$/DDM$_{30}$-His$_6$-porin could be readily reconstituted into liposomes by FT. Swelling assays of FT SDS/DDM-His$_6$-porin-liposomes demonstrated the most rapid osmotic response to sucrose, with a change of -0.02 A$_{400\ nm}$ over 20 min (Fig. 3.6). This response to sucrose is slightly better than the swelling measured for previous FT proteoliposomes prepared in single detergents (Chapter 2). These FT SDS/DDM-proteoliposomes also demonstrated far greater tolerance to heating than those formed in the presence of SDS, LDAO, or DPC (Chapter 2). This suggests that SDS/DDM-His$_6$-porin forms pores that are much more resilient to high temperatures than His$_6$-porin solubilized in single detergents.

If the conformation of His$_6$-porin in SDS$_{3.5}$/DDM$_{30}$ does represent a membrane bound arrangement of mitochondrial porin perhaps its reconstitution into liposomes by FT results in a much more stable pore. This stabilizing influence on pore formation in FT SDS/DDM-liposomes is likely due to the contribution of both ionic and non-ionic detergents within the

membrane. Studies focusing on the affects of mixed detergent-liposome interactions have indicated that SDS/ Triton X-100 (de la Maza and Parra 1996) and SDS/ nonylphenol ethylene oxide (NP(EO)10) mixed detergents (de la Maza and Parra 1996) cause increased solubilization and disruption of PC liposomes resulting in decreased liposome size and reduced individual CMC for each detergent. This behaviour of mixed detergent-liposomes must affect the incorporation of SDS/DDM-solubilized His_6-porin perhaps by facilitating the insertion of the protein into the phospholipid bilayers due to their disruptive abilities.

Taken together, these studies reveal that SDS/DDM promotes a His_6-porin conformation very similar to that for the protein reconstituted into artificial membranes (Chapter 2). The identification of a mixed detergent system for well-folded mitochondrial porin is an important first step toward high resolution structural studies using approaches such as NMR spectroscopy, which has recently been used to analyze the OmpA β-barrel and OmpX solubilized in detergent (Arora *et al.* 2001, Fernández *et al.* 2001 Fernández *et al.* 2001, Fernández *et al.* 2001, Fernández, 2004 #334}).

CHAPTER 4 The influence of sterols on the conformation of recombinant mitochondrial porin in detergents and liposomes

The material presented in chapter 4 was written with the intent to submit to the Journal of Bioenergetics and Biomembranes. I completed all of the experiments outlined in this chapter. The data were analyzed and the manuscript was written with guidance and feedback from my advisor and Dr. J. O'Neil, who are co-authors on the manuscript.

4.1 Abstract

Addition of sterols to detergent-solubilized mitochondrial porins is required for channel formation in artificial membranes. Although native mitochondrial porins co-purify with sterols, it is unclear what role sterols play with respect to mitochondrial porin folding *in vitro*. To investigate these interactions, detergent-solubilized recombinant *Neurospora crassa* His-tagged porin (His_6-porin) was incubated with various sterols (cholesterol, 4-cholesten-3-one, and ergosterol) and the resultant protein conformations were examined by fluorescence spectroscopy and CD spectropolarimetry. Sterols do not alter the secondary structure of porins dissolved in the detergents lauryl dimethylamine oxide (LDAO), dodecylphosphocholine (DPC), and mixtures of sodium dodecylsulfate (SDS) and dodecyl maltopyranoside (DDM). Near UV CD spectropolarimetry and fluorescence spectroscopy indicate that detergent-sterol solubilized porins possess slightly different tertiary interactions as compared to porin dissolved in detergent alone. His_6-porin reconstituted into liposomes in the presence of ergosterol by a freeze-thaw method generates altered osmotic responses to sucrose. Finally, His_6-porin was not capable of spontaneous insertion into ergosterol-liposomes. The subtle differences between the overall conformations in the presence and

absence of sterols suggests that the small changes in tertiary interactions are not sufficient for the formation of an insertion-competent structure and that the role of the sterol is to facilitate insertion into the artificial bilayers.

4.2 Introduction

Mitochondrial porin is the most abundant protein in the outer membrane and is responsible for the bi-directional transport of molecules across the lipid bilayer. *In vitro* analysis of this channel in artificial lipid bilayers has demonstrated pore-forming ability and voltage-dependence similar to those of bacterial porins (Benz 1994). Interactions of porin with many solutes and both soluble and membrane-bound proteins contribute to regulated channel activity that in turn controls mitochondrial metabolism. In particular, the participation of porin in the regulation of apoptosis, supports an important role for this protein in the global control of mitochondrial function in healthy and diseased cells (reviewed by Blachly-Dyson and Forte 2001, Lemasters and Holmuhamedov 2006).

Characterization of pore forming properties of native mitochondrial porin has largely been focused on reconstitution of detergent-solubilized porin into planar artificial membranes (Schein *et al.* 1976, Colombini 1980, Roos *et al.* 1982, Freitag *et al.* 1982, Pfaller *et al.* 1985, Aljamal *et al.* 1993, Troll *et al.* 1992) or liposomes (Colombini 1980, Colombini *et al.* 1987). In artificial planar lipid bilayer (or black lipid bilayer) experiments, mitochondrial porins form conductive channels under applied voltages, where the pores are anion-selective in their high conductance open state, but are converted to a slightly cation-selective partially-closed low conductance state with increasing voltage. These characteristics led to their alternative designation as voltage-dependent anion-selective channels (VDAC, Colombini 1980). In addition to revealing information regarding overall channel function, black lipid bilayer

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experiments have been used in biotinylation assays to determine the relative positions of particular residues within the folded protein (Song *et al.* 1998, Song *et al.* 1998), to characterize channel ion selectivity (Blachly-Dyson *et al.* 1989, Blachly-Dyson *et al.* 1990), to examine the effects of amino acid deletions on pore formation and assembly (Runke *et al.* 2006), and to investigate auto-directed insertion (Zizi *et al.* 1995, Xu and Colombini 1996, Xu and Colombini 1997, Li and Colombini 2002). Liposomes have also been successfully used to examine porin function pertaining to diffusion of interacting molecules (Ca^{2+} Bathori *et al.* 2006, Gincel *et al.* 2001; Koenig's polyanion Colombini *et al.* 1987; reactive oxygen species Madesh and Hajnoczky 2001), or interacting proteins (Bcl-2 family Shimizu *et al.* 1999, Shimizu *et al.* 2000, Shimizu and Tsujimoto 2000; Plasminogen kringle 5 Gonzalez-Gronow *et al.* 2003).

In many of these artificial membrane experiments, in particular those using black lipid bilayers, sterols are required in the artificial membrane or with the solubilizing detergent, suggesting that sterols may influence mitochondrial porin channel formation *in vitro*. An *in vivo* role is suggested by the observations that ergosterol copurifies with *Neurospora crassa* porin (Freitag *et al.* 1982) and five molecules of cholesterol are associated with the rat heart and bovine mitochondrial porins (De Pinto *et al.* 1989). The requirement for sterols during mitochondrial porin insertion into artificial membranes was first demonstrated in black lipid bilayer experiments involving *N. crassa* mitochondrial porin (Pfaller *et al.* 1985). In this study, the protein was converted to a water-soluble form by treatment at high pH to remove any carry-over lipids. It formed conductive voltage-dependent channels only in artificial membranes containing one of several sterols. Similarly, detergent solubilized or water-soluble mitochondrial porins from *Dictyostelium discoideum*, *Paramecium tetraurelia*, and

rat liver only display strong channel forming activity and ion selectivity when pre-incubated

with sterols or in membranes containing sterol (Popp *et al.* 1995). Sterols are also required

for wild-type channel formation by recombinant mitochondrial (Popp *et al.* 1996, Koppel *et

al.* 1998) or plastidal (Popp *et al.* 1997) porins expressed in *E. coli,* and bearing N-terminal

hexahistidinyl-tags (His$_6$-porin). Far UV CD analysis of detergent-solubilized native and

recombinant pea root plastid porins indicated similar high levels of β-strand content both in

the presence or absence of sterol (Popp *et al.* 1997).

In contrast to experiments involving pre-formed membranes, detergent-solubilized *N.

crassa* His$_6$porin can be reconstituted into phospholiposomes lacking sterols by the freeze-

thaw (FT) method (Chapters 2 and 3). Following reconstitution, His$_6$-porin has high β-strand

content and displays positive ellipticity in the near UV CD region, similar to that observed for

folded bacterial porins. His$_6$-porin did not directly insert into liposomes without FT

treatment, but structural examination by CD analysis and fluorescence spectroscopy strongly

suggested that the protein was interacting with liposomes. Therefore, a sterol may be

necessary for either insertion into preformed artificial membranes or for proper folding

therein.

With the establishment of fluorescence and near UV CD as tools for investigating

mitochondrial porins (Chapters 2 and 3), we are able to further investigate the role sterols

play on the folded state of *N. crassa* mitochondrial porin in detergents and artificial

membranes. His$_6$-porin was solubilized in a collection of detergents, SDS/DDM, LDAO, and

DPC in the presence of three different sterols: ergosterol, the native sterol found in *N. crassa*

mitochondrial outer membranes (de Kroon *et al.* 1997), cholesterol, a major sterol common to

mammalian mitochondrial outer membranes (Asworth and Green 1966), and 4-cholesten-3-

one, an oxidized form of cholesterol, to examine the effects of each sterol on the conformation of the protein in solution. Channel forming activity of His_6-porin reconstituted into liposomes containing ergosterol was examined and CD spectropolarimetry, fluorescence spectroscopy and protease digestion were used to determine the effect of sterols on porin structural arrangements.

4.3 Materials and Methods

4.3.1 Lipids, sterols, detergents, and amino acid derivatives

Egg yolk L-α-phosphatidyl choline, egg lecithin L-α-phosphatidic acid, SDS, urea, cholesterol, 4-cholesten-3one (cholestenone), and ergosterol were obtained from Sigma (St. Louis, MO). DPC, DDM, and LDAO were purchased from Anatrace (Maumee, OH).

4.3.2 Expression and purification of His_6-porin

The cloning of the cDNA for *N. crassa* mitochondrial porin into the pQE-9 vector (Qiagen, Toronto, ON), which encodes an N-terminal His_6-tag has been described previously (Popp *et al.* 1996). Protein expression was carried out using the QIA*express E. coli* M15 [pREP4] strain from Qiagen.

The recombinant His_6-porin used in this study was expressed in *E. coli* and purified by immobilized nickel nitrolotriacetic acid (Ni-NTA) in 8 M urea, using the procedure described in (Chapter 2). The yield of His_6-porin was 2-5 mg/L and the protein concentration was determined using the calculated extinction coefficient at 280 nm of 24,050 M^{-1} cm^{-1}. Protein purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was estimated to be at least 95%.

4.3.3 Sterol-detergent solubilization of His$_6$-porin.

Dialyzed His$_6$-porin was acetone-precipitated and the dried pellets were resuspended in aqueous detergent-sterol solutions, buffered in 50 mM sodium phosphate pH 7. Detergent-sterol solutions were prepared in microfuge tubes by evaporation of 0.10 mg sterol (ergosterol, cholesterol, or cholestenone) in 1 ml of chloroform overnight in a fumehood at room temperature (22°-25°C). Each 0.10 mg aliquot of dried sterol was resuspended in 1 ml of a high concentration of detergent (21 mM SDS, 300 mM LDAO, and 100 mM DPC). These samples were vortexed for 10 minutes and inverted overnight at room temperature. The detergent-sterol solutions were centrifuged at 10,000 g for 15 minutes to remove any insoluble material; pellets were not visible after centrifugation. One ml from each high concentration detergent- (0.1 mg/ml) sterol supernatant was added directly to acetone-precipitated His$_6$-porin at high concentrations (0.90-1.80 mg/ml) to prepare near-UV CD samples. For far-UV CD analysis, detergent-sterol supernatants were diluted 1/5 into 50 mM sodium phosphate buffer pH 7 and used to solubilize acetone-precipitated His$_6$-porin at lower initial concentrations (0.15-0.30 mg/ ml). For SDS/ DDM-sterol samples, powdered DDM was added to His$_6$-porin solubilized in 21 mM SDS, 0.1 mg/ml sterol to a final concentration of 180 mM DDM. The detergent-sterol- His$_6$-porin samples were mixed by repeated inversion overnight (14-16 hrs) at room temperature, centrifuged at 10,000 g for 15 minutes, and the optically-cleared supernatants were collected. Samples lacking His$_6$-porin were prepared as described above and used as baselines for spectroscopic analysis or as negative controls for swelling assays.

4.3.4 Liposome stock preparation

His$_6$-porin was reconstituted into small unilamaellar vesicles in the presence of ergosterol using modifications of the procedure described in (Chapter 2). Briefly, a 10:1:0.8 (w/w/w) mixture of egg yolk L-α-phosphatidyl choline, egg lecithin L-α-phosphatidic acid, and ergosterol was dissolved in chloroform and evaporated in a fumehood at room temperature overnight. Dried lipids and sterols were resuspended in 50 mM sodium phosphate buffer at pH 7 or stored at -20°C in a dessicator until needed. The resulting milky suspension was diluted 1:1 with phosphate buffer pH 7 and sonicated on ice until the microtip (Fisher Scientific Sonifier Model 300) could clearly be observed to yield the stock SUV-ergosterol suspension (20 mg phospholipid/ml). Stock SUV suspensions lacking ergosterol were prepared by the same method as described above.

4.3.5 Liposome swelling assays

Liposome swelling assays were based on the methods described by (Chapter 2); all samples were buffered with 50 mM sodium phosphate pH 7. To determine if the inclusion of ergosterol in the detergent (type D) or in the artificial membranes (type L) affects liposome swelling, proteoliposomes were prepared by freeze-thaw (FT) or insertion (INS) methods. Type D experiments were set up by mixing equal volumes of stock SUV lacking ergosterol and 0.5- 2.5 μM His$_6$-porin solubilized in detergents containing 0.01 mg/ml ergosterol. Following mixing, detergent concentrations were 1.25 mM SDS, 1.25 mM SDS/15 mM DDM, 3.75 mM LDAO, and 1 mM DPC. These samples were then subjected to either FT or INS protocols as described in (Chapters 2 and 3). Briefly, freeze-thaw (FT) samples were subjected to three cycles of 1 min in liquid nitrogen followed by thawing at room temperature. The insertion (INS) method was based on that of Surrey *et al.* (Surrey and

Jahnig 1995), and involved incubation of His_6-porin/detergent complexes with preformed

liposomes at room temperature. Following either treatment, the proteoliposomes were placed

on a rotating mixer overnight at room temperature. Following liposome swelling analysis,

samples were centrifuged at 10,000 g for 10 min and the pelleted material was resuspended in

8 M urea for measurement by UV absorption spectroscopy at 280 nm. This allowed

determination of the concentration of insoluble protein in the pellet, which was then used to

calculate the concentration of soluble His_6-porin. Final concentrations of each component

were as follows: 6.6 mg/ml phospholipid, 0.002 mg/ml ergosterol, 0.2-0.8 μM His_6-porin,

and for each detergent examined 0.6 mM SDS, 0.6 mM SDS/ 5 mM DDM, 1.3 mM LDAO,

or 0.3 mM DPC.

Type L (ergosterol-containing) proteoliposomes were prepared by first diluting stock

SUV 1:2 (high ergosterol) or 1:20 (low ergosterol) with stock SUV lacking the sterol. Both

high and low ergosterol-liposomes were mixed 1:1 with detergent-solubilized His_6-porin at

the same concentrations as described for type D proteoliposomes. These samples were

subjected to either FT or INS treatments as described for type D proteoliposomes (above).

The only exception in these treatments was for type L INS samples, which were briefly

sonicated on ice to ensure homogenous ergosterol-liposomes formation before incubation

with detergent- His_6-porin. The lower ergosterol concentration (0.01 mg/ml) in the type L

samples was identical to that in type D; the higher ergosterol concentration was 10-fold as

high. The concentrations of all other components were identical in the types D and L

experiments.

Liposome swelling was measured on an Ultrospec 3100 pro spectrophotometer at an

absorbance of 400 nm. Both type D and L proteoliposome samples prepared as described

above were diluted 1/100 into 50 mM sodium phosphate pH 7 buffer to a final volume of 0.5

ml and measured in a 1 cm quartz cuvette. After 300-400 seconds, liposomes were diluted

with 40 μl of iso-osmotic phosphate buffer pH 7, followed by an addition of 40 μl of

hyperosmotic 1 M sucrose after 600-700 seconds. Liposome re-swelling, as indicated by a

gradual decrease in $A_{400\ nm}$ was followed for 1200 or 1800 seconds after sucrose addition.

4.3.6 Reconstitution of SDS-solubilized His$_6$-porin in ergosterol-containing liposomes for spectroscopic analysis

His$_6$-porin was resuspended in 3.5 mM SDS to 10-35 μM; subsequent dilutions of 1:3

or 1:4 in phosphate buffer resulted in final detergent concentrations near the CMC (0.9-1.0

mM SDS). Ergosterol was examined in these studies since it is the predominant sterol in *N.*

crassa mitochondrial outer membranes. Other detergents were not pursued due to low initial

concentrations (≤ 5 μM) of His$_6$-porin maintained in solution. The SDS-solubilized protein

was diluted 1:4 into type L liposomes (2 mg phospholipid/ml) containing 0.02 mg/ml

ergosterol, buffered in 50 mM sodium phosphate pH 7. Liposome-ergosterol-detergent-His$_6$-

porin mixtures were then subjected to one cycle of freeze-thaw (FT) or the INS method

described above. Type D SDS- His$_6$-porin-ergosterol-liposomes were not examined

spectroscopically since liposome swelling profiles were similar to samples lacking ergosterol

(see results section 4.4).

4.3.7 Fluorescence spectrophotometry

Fluorescence spectroscopic analyses of detergent-sterol-solubilized His$_6$-porin were

performed using a JASCO-810 (FMO-427S) spectropolarimeter/fluorometer. All emission

spectra were measured in a 1-cm path length quartz cuvette after excitation at 280 nm or 296

nm. Detergent-sterol-solubilized His$_6$-porin samples were measured at A$_{280}$ of 0.01, which

corresponds to a protein concentration of 0.42 μM. All His$_6$-porin-liposome samples were

diluted 1:10 in 50 mM sodium phosphate buffer pH 7 to a final lipid concentration of 0.2

mg/ml, 0.002 mg/ml ergosterol, and 1.0-0.2 μM His$_6$-porin. His$_6$-porin-ergosterol-liposome

spectra were normalized to a concentration of 0.42 μM with reference to His$_6$-porin in urea

(Chapter 2).

4.3.8 Circular dichroism (CD) spectropolarimetry

CD spectra were acquired on a JASCO J-810 spectropolarimeter-fluorometer

calibrated with (+)-10-camphorsulfonic acid and purged with N$_2$ at 20 L/min (Manley and

O'Neil 2003). CD spectra of 1.5 μM sterol-detergent-solubilized His$_6$-porin samples and

His$_6$-porin-ergosterol-liposome samples (Type L) containing 0.5-2.0 μM His$_6$-porin were

measured in the far UV region (190-250 nm) using 0.05-0.10-cm path length quartz cuvettes

at 22°C, a scan rate of 10 nm/min and a response time of 8 s. High initial concentrations of

His$_6$-porin in SDS were necessary to maintain 1-2 μM of protein in the SUV samples after

centrifugation. CD spectra were corrected by baseline subtraction and were converted to

mean residue ellipticity (MRE) according to the formula: $[\theta]_M = M\theta / \{(10)(l)(c)(n)\}$ where

$[\theta]_M$ is 10^{-3} deg cm^2 dmol^{-1}, M is the molecular weight of His$_6$-porin (31 402 g/mol), θ is the

measured ellipticity in millidegrees, l is the path length of the cuvette in cm (0.1 cm), c is the

protein concentration in g /L, and n is the number of amino acid residues in the protein (295).

Detergent-sterol-solubilized His$_6$-porin spectra were deconvoluted with the CONTINLL

algorithm (Provencher and Glockner 1981, van Stokkum *et al.* 1990) in the DichroWeb

package (Whitmore and Wallace 2004). Light scattering by the ergosterol-liposome samples

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prevented reliable acquisition of spectra below 200 nm and prohibited deconvolution with secondary structure prediction algorithms.

Temperature was controlled during thermal denaturation experiments of His$_6$-porin-ergosterol-liposomes using the Peltier device in the spectropolarimeter. These experiments were carried out in the far UV region (200-250 nm), with a 1°C / min ramp speed; θ (in mdeg) was monitored at 217 nm at 1°C intervals, and full spectra were collected at 10°C intervals.

Near-UV (245-330 nm) CD spectra of 33 µM His$_6$-porin in 0.02 mg/ml sterol-detergent were measured with a JASCO J-810 spectropolarimeter-fluorometer in a 1-cm path length jacketed quartz cuvette at room temperature, with a scan rate of 2 nm/min, and a response time of 8 s. CD spectra of His$_6$-porin reconstituted into Type L ergosterol-liposomes were measured in a 5-cm path length jacketed quartz cuvette at room temperature with a scan rate of 10 nm/ min, a response time of 8 seconds. Scans were measured in triplicate for averaging to reduce noise. Molar ellipticity of His$_6$-porin in detergent-sterol or sterol-containing liposomes was calculated from the baseline corrected spectra according to the formula: $[\theta] = M\theta / \{(10)(l)(c)\}$ where $[\theta]$ is the molar ellipticity in degrees cm^2 dmol^{-1}, θ is the measured ellipticity in millidegrees, l is 1 cm for detergent-sterol-His$_6$-porin or 5 cm for His$_6$-porin-sterol-liposomes, and c is the protein concentration in g/ L.

4.3.9 Protease digestions of His$_6$-porin reconstituted into ergosterol-liposomes

His$_6$-porin in liposomes was digested with protease after spectroscopic examination. Each liposome sample was digested with 0.15 mg/ml trypsin (T) or chymotrypsin (CT) for 30 minutes at room temperature and contained 0.0075- 0.015 mg/ml (0.25- 0.5 µM) His$_6$-porin, 0.01 mg/ml ergosterol, and 1 mg/ml phospholipid. All protease digestions were arrested by

adding 20 mM PMSF (f.c.) and 3:1 dilution to Laemmli (Laemmli 1970) buffer containing 8

M urea. All reactions were stored at -20°C until loaded onto SDS-PAGE gels.

4.3.10 Western blots of His$_6$-porin-ergosterol-liposomes

All protease digested samples were loaded onto 0.1 % SDS- 3 M urea- 14% PAGE

gels and electrophoresis was performed with the Mini-Protean$^{\circledR}$ II system (Bio-Rad

Laboratories, Hercules, CA). 10 μl of 7.5- 15 μg/ ml His$_6$-porin in 1 mg/ml phospholipids

with or without 0.02 mg/ml ergosterol were loaded into each well. Gels were blotted with a

Trans-Blot$^{\circledR}$ Cell system (Bio-Rad) onto 0.45 μM nitrocellulose membranes (Bio-Rad)

overnight at 4°C and stained with Ponceau S (Sigma) to visualise the Benchmark protein

ladder (Invitrogen, Burlington, ON).

Immunoblotting was carried out at room temperature using rabbit antibodies directed

against residues 7- 20 of *N. crassa* mitochondrial porin (αNcPor-N, generated by R. Lill, at

Universität München). Membranes were immunoblotted according to the procedure

described by (Chapter 2). Membranes were detected by CDP- star (Roche Diagnostics,

Indianapolis, IN) and exposed for 1- 2 hrs on X-Omat Blue XB-1 Scientific Imaging film

(Kodak, Rochester, NY) before image development.

4.4 Results

4.4.1 Liposome swelling assays of His$_6$-porin in the presence of ergosterol

Osmotic swelling assays were performed on His$_6$-porin reconstituted into liposomes

in the presence of ergosterol added in detergent (Type D) or incorporated into the stock SUV

(Type L) to determine the effects of sterol on porin channel formation. Type L liposome

solutions were formed in the presence of either 20 μg/ml (Fig. 4.1A, D, G, and J) or 200

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Figure 4.1. Liposome swelling assays. Detergent-solubilized-His$_6$-porin-liposomes with ergosterol included either in the liposomes or in the detergent were prepared by the freeze-thaw (FT) method (**A,B, D, E, G,H, J, K, M, N, O**) or the insertion (INS) method (**C, F, I, L**). In all FT proteoliposomes, swelling was measured directly (solid line) or following heating to 65°C (dashed lines) or 100°C (dotted lines) and liposomes lacking His$_6$-porin were measured as a control (line-dot-line). In panel A only, a proteoliposome sample lacking ergosterol is shown (line-dot-dot-line). Panels M, N, O display liposome swelling assays for type L liposomes prepared with 10-fold greater ergosterol concentrations (high ergosterol – HE) than in all remaining experiments. For INS proteoliposomes (**C, F, I**) prepared with ergosterol added to the lipid (solid (RT) and dashed lines (30°C)) or to the detergent (dotted (RT) and line-dot-lines (30°C)), swelling was monitored directly (RT) or after overnight incubation at 30°C. For SDS/DDM INS proteoliposomes, swelling of INS proteoliposomes lacking sterol (dot-dash) and FT SDS/DDM proteoliposomes (dotted line) are also shown along with INS type L (solid line) and INS type D liposomes (dashed line). Relative A$_{400\ nm}$ values collected over time for each sample were adjusted and plotted on the same scale which permitted individual plots to be distinguishable from each other.

Figure panels with labels:
- A: FT Type L SDS
- B: FT type D SDS
- C: INS SDS
- D: FT type L LDAO
- E: FT type D LDAO
- F: INS LDAO
- G: FT type L DPC
- H: FT type D DPC
- I: INS DPC

Y-axis: Relative A$_{400\ nm}$; X-axis: Time (seconds); Scale bar: 0.005 A$_{400\ nm}$

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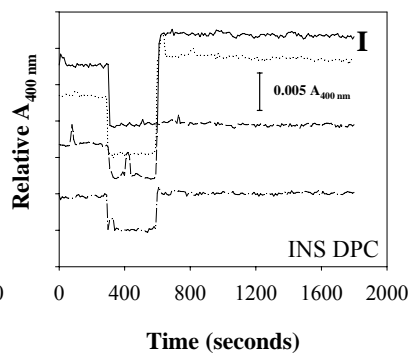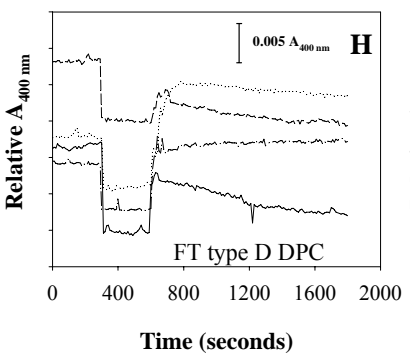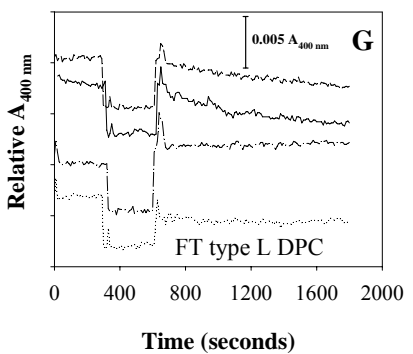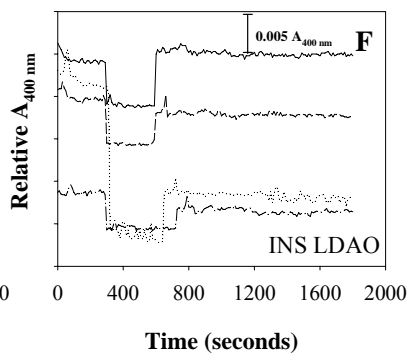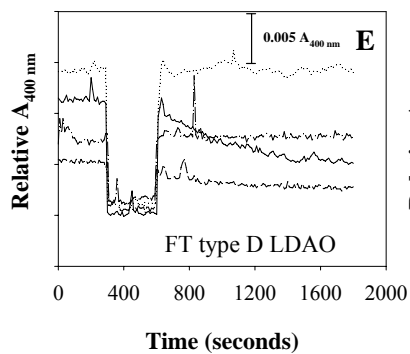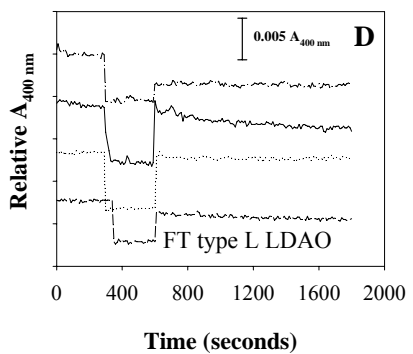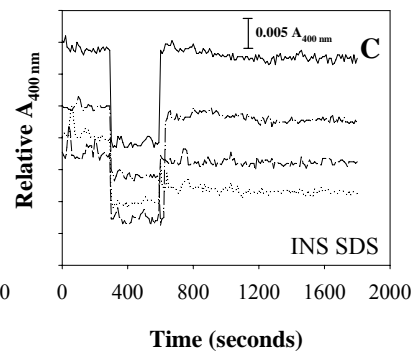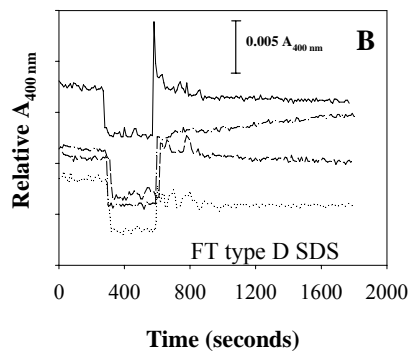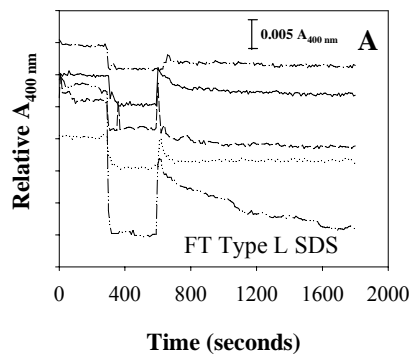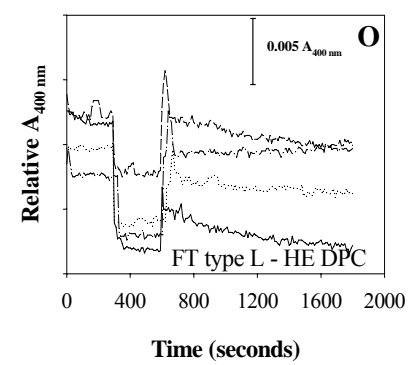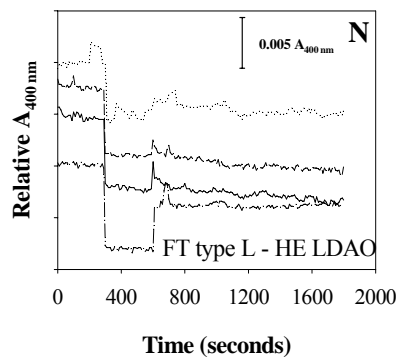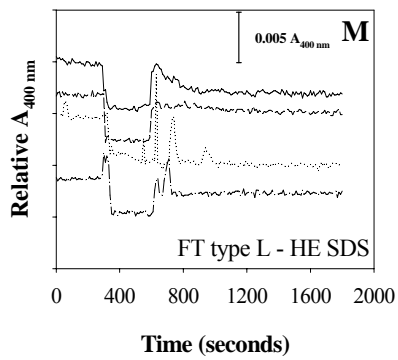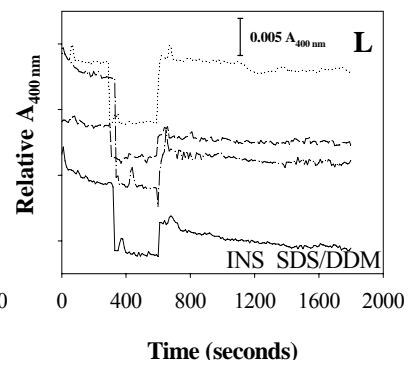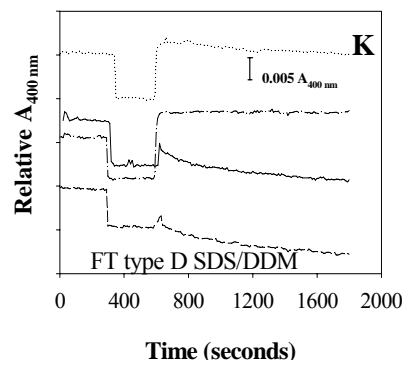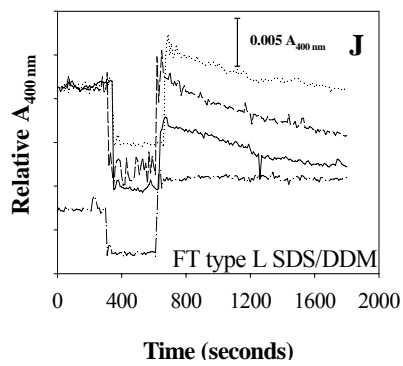
μg/ml ergosterol (Fig. 4.1M, N and O; Table 4.1). Due to the poor solubility of ergosterol in the low detergent concentrations necessary for type D experiments, these solutions were prepared in the presence of 20 μg/ml ergosterol only (Fig. 4.1B, E, H and K; Table 4.1). Osmotic responses were detected as a gradual decrease in $A_{400\ nm}$ following the addition of sucrose to the liposome solution at 600 seconds (Shao *et al.* 1996).

Following FT, in all detergents tested, both types of D and L proteoliposomes demonstrated an osmotic response to sucrose (Fig. 4.1, Table 4.1). The presence of ergosterol increased the noise in the spectra (see Fig. 4.1A) and many of the traces from sterol-containing liposomes displayed a sharp drop in $A_{400\ nm}$ at the beginning of the swelling phase, followed in some cases by a more gradual decline in $A_{400\ nm}$. With the exception of DPC-containing type D liposomes, the rapid drop was not observed in the absence of porin, suggesting that it reflects movement of solution through porin, rather than resulting from altered membrane structure *per se*. The reason then for two-phase reswelling is not clear – it could represent either two populations of liposomes, with different porin content or activity. The slow phase is characteristic of wild-type pore activity (Shao *et al.* 1996, Bathori *et al.* 1993). For these reasons, only the slow reswelling phase will be considered in the following discussion.

The magnitude of the osmotic response to sucrose in FT, ergosterol-containing liposomes was generally less than that in the corresponding sterol-free systems. For example, the associated $A_{400\ nm}$ changes ranged from –0.004 to –0.008 in type D proteoliposomes, and from –0.002 to –0.006 in both high and low ergosterol Type L systems. In contrast, a gradual change of –0.01 A_{400} units was observed for SDS-His$_6$-porin liposomes lacking ergosterol (Fig. 4.1A, see Chapter 2).

Table 4.1. Summary of proteoliposome swelling data. Relative liposome swelling activity was determined for each preparation of detergent-solubilized His_6-porin reconstituted into type L or D containing liposomes, or those without sterol. Assays were carried out as described in Materials and Methods.

Detergent for Porin Solubilization	Ergosterol Content *	Proteo-liposome Type †	Temperature (^{o}C)	Rapid Reswelling Phase ‡	Slow Reswelling Phase ‡
SDS	none§	none	RT**	+	+++
			65	++	++
			100	-	-
	high	L	RT	+	+
			65	-	-
			100	nd††	nd
	low	L	RT	++	-
			65	++	-
			100	+	-
	low	D	RT	+++	-
			65	-	-
			100	-	-
DPC	none	none	RT	-	+++
			65	+	+++
			100	-	+
	high	L	RT	+	+
			65	++	+
			100	+	-
	low	L	RT	+	++
			65	+	+
			100	+	-
	low	D	RT	-	++
			65	-	+
			100	-	-
LDAO	none	none	RT	-	+++
			65	+++	+++
			100	-	+
	high	L	RT	+	-
			65	-	-
			100	-	-
	low	L	RT	-	+
			65	-	-
			100	-	-
	low	D	RT	+	++
			65	-	-
			100	-	-

SDS/DDM	none	none	RT	++++	++++
			65	+++	++++
			100	-	+++
	high	L	RT	is§§	is
			65	is	is
			100	is	is
	low	L	RT	-	++
			65	+	+++
			100	+	++
	low	D	RT	+	++
			65	+	++
			100	-	+

Table 4.1, footnotes

* high, (0.1 mg/ml); low (0.01 mg/ml)

† L, sterol added in liposomes; D, sterol added in detergent

‡ rapid swelling phase, 100 seconds after sucrose addition; slow swelling phase 100-1800 seconds after addition change in A_{400} <0.0020 (-) ; 0.0020-0.0030 (+), 0.0030-0.0060 (++), > 0.0060 (+++)

§ Data for proteoliposomes lacking sterol are taken from Chapters 1 and 2.

** RT, room temperature (22-25°C)

†† nd, not determined, reproducibly high levels of noise in sample

§§ is, insufficient solubility, high concentrations of ergosterol not soluble in SDS/DDM

Maintenance of swelling activity following incubation at increased temperatures (65°C or 100°C) varied, depending on how the sterol was included in the sample, and on the detergent used (Table 4.1). In SDS alone, gradual reswelling was observed at room temperature. This response was partially or completely lost following incubation at 65°C and 100°C, respectively. In contrast, the slow swelling phase was not observed in the presence of sterols, but a much more rapid temperature-sensitive response was seen. Types D or L LDAO-His$_6$-porin liposomes demonstrated the lowest osmotic response to sucrose among all of the detergents tested (Fig. 4.1D and 4.1E). For L-type proteoliposomes, gradual reswelling was weak or not observed at room temperature, and a relatively small shrinkage phase was observed in high ergosterol Type L liposomes (Fig. 4.1N). However, D type LDAO-proteoliposomes maintained slow reswelling behaviour that was temperature sensitive.

The osmotic response of proteoliposomes formed with DPC-solubilized His$_6$-porin was unchanged upon addition of sterol to the detergent, but was strongly diminished when the sterol was initially incorporated into the liposomes (Table 4.1). The slow phase was absent in the Type L samples with high ergosterol concentrations (Fig. 4.1M, N, O), and reduced at lower concentrations (Fig. 4.1A, D, G, J). Responses seen at room temperature remained after 65°C treatment, but were lost at 100°C. Finally, SDS/DDM could only be used for generating Type L and low-ergosterol Type D liposomes, due to solubility issues at high concentrations of ergosterol. Remarkably, enhanced liposome swelling was observed for Type L SDS/DDM proteoliposomes, and this response was partially resistant to heating at 100°C (Fig. 4.1J). Incorporation of sterol to the stock SUVs did not alter osmotic responsiveness in this system (Fig. 4.1C, F, I, L).

Bacteriorhodopsin can insert into preformed liposomes containing cholesterol, but requires freeze-thaw treatment for integration in the absence of the sterol (Scotto and Zakim 1988). To determine if the same trend exists for mitochondrial porin, swelling assays were performed to determine if detergent-solubilized His$_6$-porin possesses the capacity to insert into preformed liposomes in the presence of ergosterol either added to the porin in detergent (type D) or incorporated into phospholipids (type L). All type D and L His$_6$-porin insertion experiments did not produce liposomes with significant osmotic responses, following incubation overnight at either 30°C or 40°C (Fig. 4.1C and D). Higher temperatures were also not effective, as brief heating (10 minutes) of INS ergosterol-proteoliposomes to 65°C or 100°C before swelling assay collection also did not generate osmotically responsive liposomes (Figs. 4.1C and D). Hence, the addition of ergosterol did not influence the inability of His$_6$-porin to insert into liposomes.

4.4.2 Protease digestion of His$_6$-porin in ergosterol-liposomes

Resistance to protease treatment has been used frequently to confirm bacterial porin refolding in detergent (OmpG; Conlan and Bayley 2003) or following reconstitution into artificial membranes (OmpA; Dornmair *et al.* 1990, Kleinschmidt and Tamm 1996). *Neurospora* His$_6$-porin reconstituted into liposomes has similar resistance to high levels of protease as observed for *N. crassa* mitochondrial porin in protease-treated mitochondria (Freitag *et al.* 1982, Chapter 2). To determine if SDS-solubilized His$_6$-porin reconstituted into ergosterol-laden liposomes by FT or INS acquires protease resistance, western blots of trypsin (T) and chymotrypsin (C) treated samples were performed. As observed on previous immunoblots, T and C were also detected as 24 or 25-kDa bands on the western blots due to

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sequence similarity between T and C and the porin peptide (residues 7-20) used to generate antibodies for this study (Chapter 2).

Three porin species with apparent molecular masses of 32, 65, and 90 kDa were detected in undigested FT SDS- His$_6$-porin-ergosterol-liposomes (Type L); they were identical to those observed in FT SDS-His$_6$-porin-liposomes (Fig. 4.2). The 32 kDa band corresponds to intact His$_6$-porin and the two additional bands with molecular masses of approximately 90 and 65 kDa are suggestive of trimers and dimers. Following digestion with both T and C, full-length protein was still detectable in each lane but the putative oligomeric bands were not observed (Fig. 4.2). Accumulation of protein in the wells during electrophoresis prevented quantitation of intact His$_6$-porin bands but nonetheless confirmed that FT SDS-solubilized His$_6$-porin reconstituted into liposomes prepared with ergosterol acquired protease resistance.

Western blot analysis of undigested INS SDS-His$_6$-porin-ergosterol-liposomes did not reveal species with molecular masses of 90 and 65 kDa. Small amounts of the 32-kDa His$_6$-porin remained following digestion with either T or C (Fig. 4.2). Therefore, some of the protein in INS SDS-His$_6$-porin-ergosterol-liposomes acquired protease-resistance similar to INS SDS- His$_6$-porin-liposomes lacking ergosterol.

4.4.3 Characterization of detergent-sterol-solubilized His$_6$-porin and His$_6$-porin-ergosterol-liposomes by far-UV circular dichroism spectropolarimetry

CD spectropolarimetry in the far UV region (190-250 nm) was used to determine the secondary structures of His$_6$-porin in detergents and liposomes containing sterols (Figs. 4.3 and 4.4). To determine if sterols alter secondary structural arrangements of His$_6$-porin solubilized in 3.5 mM SDS, 3.5 mM SDS/30 mM DDM, 50-75 mM LDAO, and 10-20 mM

Figure 4.2. Protease sensitivity of His$_6$-porin reconstituted into ergosterol containing liposomes. His$_6$-porin was solubilized in SDS and incorporated into liposome in the presence or absence of 0.02 mg/ml ergosterol by the freeze-thaw (FT) or insertion (INS) methods as described in Materials and Methods. In total 0.15 μg His$_6$-porin, 0.05 μg ergosterol and 0.01 mg lipid were digested for 30 minutes at room temperature in the absence (uncut) or presence (+) of 0.15 μg trypsin (T) or 0.15 μg chymotrypsin (C) and analyzed by urea-SDS-PAGE. Urea-SDS-PAGE gels were immunoblotted and probed with anti-NcPor-N antibodies. The Benchmark^{TM} protein ladder (Invitrogen) positions are indicated to the left of the panel. Position of wells (arrow), multimers of His$_6$-porin (filled circles), minor degradation products (open circles), and cross reacting trypsin (asterisk) are indicated on the blot.
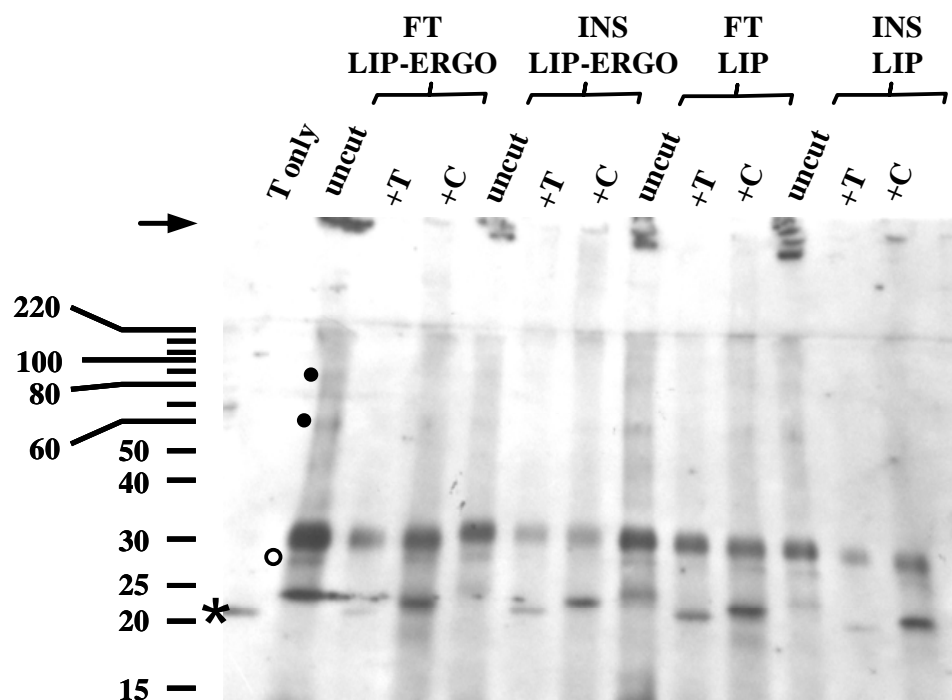
Table 4.2. Analysis of His$_6$-porin solubilized in detergents with sterol. Far- UV CD spectropolarimetry was performed on 1.5 µM porin samples; the resulting spectra were deconvoluted with CONTINLL (Whitmore *et al.* 2004).

Detergent	Sterol	α-helix (%)	β-strand (%)	turn & unordered (%)
50 mM LDAO		16	32	52
50 mM LDAO	cholesterol	17	35	48
75 mM LDAO		19	39	42
75 mM LDAO	ergosterol	9	34	57
75 mM LDAO	cholestenone	16	30	54
10 mM DPC		16	30	54
10 mM DPC	cholesterol	16	28	56
20 mM DPC		20	29	51
20 mM DPC	ergosterol	18	30	52
20 mM DPC	cholestenone	16	31	53
3.5 mM SDS		24	20	56
3.5 mM SDS	ergosterol	23	23	54
3.5 mM SDS	cholesterol	31	17	52
3.5 mM SDS	cholestenone	29	23	48
3.5 mM SDS, 30 mM DDM		18	35	47
3.5 mM SDS, 30 mM DDM	ergosterol	16	34	50
3.5 mM SDS, 30 mM DDM	cholesterol	18	32	50
3.5 mM SDS, 30 mM DDM	cholestenone	17	35	48

Figure 4.3. Far-UV CD spectra of detergent-solubilized His_6-porin in the presence of sterols. In each panel CD spectra of 1.5 µM His_6-porin (solid line) were collected in the presence of ergosterol (dashed line), cholesterol (dot line), and cholestenone (dotted line) and all CD spectra were collected at room temperature. **A**) 3.5 mM SDS. **B**) 3.5 mM SDS, 30 mM DDM. **C**) 50 mM LDAO (line dot lines) and 75 mM LDAO. **D**) 10 mM DPC (solid and line dot lines) and 20 mM DPC (dashed and dotted lines).
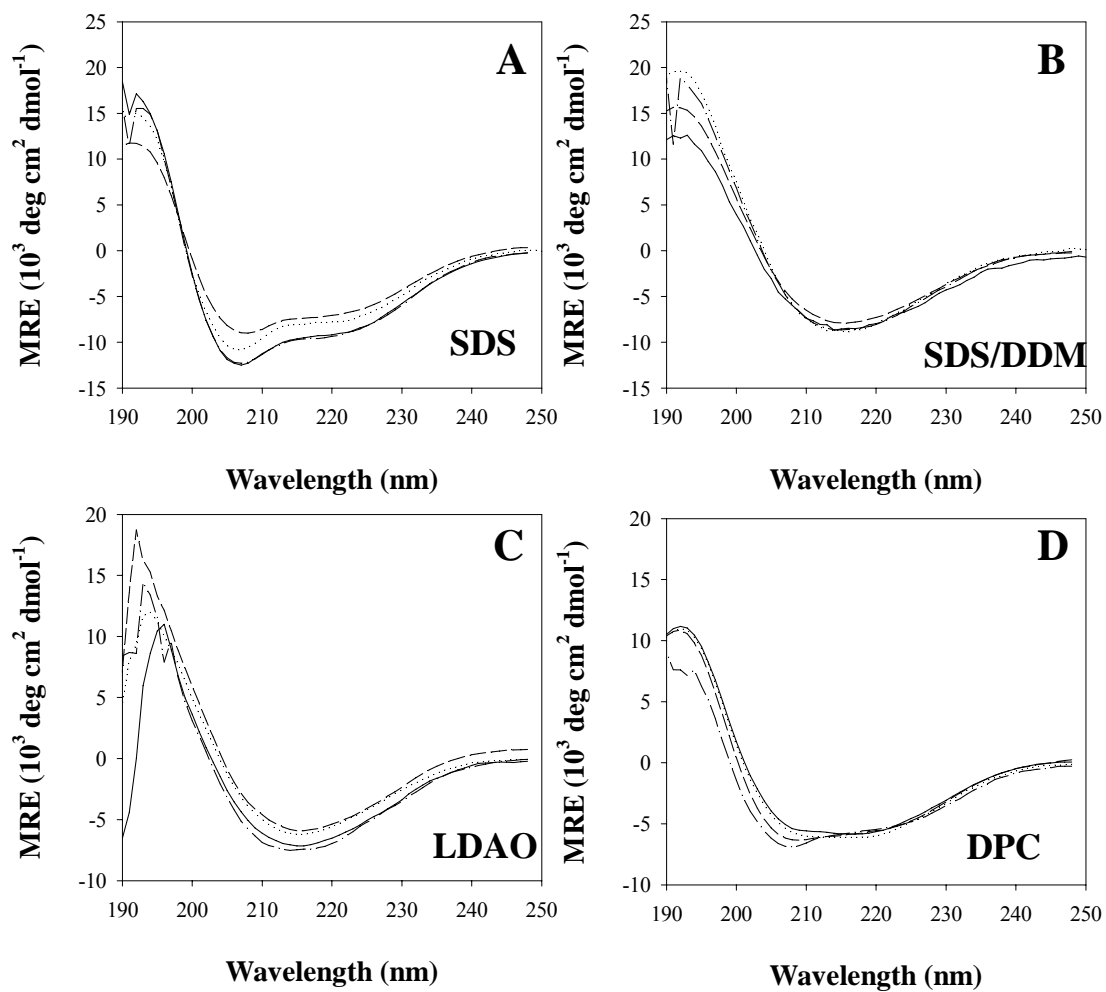
DPC, ergosterol, cholesterol, and cholestenone, were included in each system at a concentration of 0.02 mg/ml. The CD spectrum of His_6-porin solubilized in each detergent in the presence of each sterol is presented in Figure 4.3 and the results obtained by deconvolution with CONTINLL (Whitmore and Wallace 2004) are shown in Table 4.2. The CD spectrum of 3.5 mM SDS-solubilized His_6-porin exhibits the characteristic minimum at 208 nm and shoulder region centred at 220 nm typical for an α-helix rich protein. Far-UV CD spectra of His_6-porin solubilized in SDS containing each of the three sterols were not significantly different, but spectrum flattening was observed in samples containing ergosterol and cholestenone (Fig. 4.3A). Deconvolution of the SDS-His_6-porin-sterol CD spectra revealed that cholesterol and cholestenone containing samples had slight increases in α-helix content (29-31%) whereas samples with or without ergosterol had similar secondary structure (23-24%; Table 4.2).

The addition of 30 mM DDM to 3.5 mM SDS- solubilized His_6-porin results in a CD spectrum with a single sharp λ_{min} of 216 nm, which was present in CD spectra collected in the presence of each sterol (Fig. 4.3B). Differences among the CD spectra of SDS/DDM His_6-porin with and without added sterols occur below 200 nm where increased ellipticity is observed. When spectra of sterol containing SDS/DDM-His_6-porin were deconvoluted, secondary structure content was largely unaltered in comparison to His_6-porin lacking sterol, although differences in the shapes of the spectra suggest perturbation of the system.

The CD spectrum of His_6-porin in 50 or 75 mM LDAO displays a strong minimum centred at 215-216 nm and is characteristic of proteins possessing high β-strand content. The CD spectra of LDAO-His_6-porin in each sterol have similar λ_{min} to the spectrum of LDAO-His_6-porin in the absence of sterol, but at 195 nm, where spectra of His_6-porin in the presence

of sterol have increased ellipticity (Fig. 4.3C). Deconvolution showed that β-strand content is slightly reduced in ergosterol and cholestenone containing samples in comparison to those lacking sterol (Table 4.2). The greatest difference in secondary structure for LDAO-His$_6$-porin is the reduction of α-helix content of LDAO-ergosterol-His$_6$-porin (16% to 9 % α-helix), suggesting either contacts with the protein or slight variations in LDAO micelles encompassing the protein.

CD spectra of individual preparations of His$_6$-porin solubilized in 10 or 20 mM DPC varied from those with a flat broad λ_{min} centred around 214-216 nm to spectra with a λ_{min} of 208 nm characteristic of α-helix rich proteins; this phenomenon is discussed in (Chapter 2). The presence of sterols does not significantly alter the shapes of the CD spectra of DPC-His$_6$-porin (Fig. 4.3D, Table 4.2). Due to the variability in secondary structure content in DPC-His$_6$-porin alone, any sterol interactions with the protein may not be measurable in this detergent system.

SDS-solubilized His$_6$-porin reconstituted into liposomes containing ergosterol (type L) by freeze-thaw and insertion methods were also examined by far UV CD; these liposomes demonstrated swelling activity at increased temperatures and maintained sufficient protein concentrations for spectroscopy. As in previous experiments (Chapter 2 and 3), light scattering by the liposomes prohibited collection at wavelengths below 200 nm and therefore deconvolution of CD spectra for secondary structure estimations.

A direct comparison of SDS-solubilized His$_6$-porin reconstituted into liposomes in the presence and absence of ergosterol demonstrates slightly elevated amounts of noise in the CD spectra of samples possessing sterol in the liposomes (Fig. 4.4A). This increase is likely contributed by the presence of the moderately absorbing ergosterol, which has sufficient π-

bonding to absorb in this region (Arthington-Skaggs *et al.* 1999). The CD spectra of both FT and INS His$_6$-porin-ergosterol-liposomes λ_{min} (FT; 216 nm, INS; 212 nm) indicate slight differences in the secondary structure content in samples lacking sterols (Fig. 4.4A). Differences between λ_{min} of FT proteoliposome spectra collected in the presence and absence of ergosterol are quite small (with ergosterol; 216 nm, no ergosterol; 217 nm) and the minima are sharp, characteristic of protein with high β-strand content, suggesting that ergosterol is not significantly altering the conformation of the protein in the membrane. However, differences are observed when comparing CD spectra of INS proteoliposomes in the presence or absence of ergosterol. INS proteoliposomes prepared with sterols resulted in a CD spectrum with λ_{min} of 211 nm in contrast to that lacking sterols (λ_{min} of 217 nm) (Fig. 4.4A). Based on shape of the CD spectrum and location of the λ_{min} for INS SDS-His$_6$-porin-ergosterol-liposomes, His$_6$-porin is likely richer in α-helix than the β-strand rich conformation observed in INS SDS-His$_6$-porin-liposomes (Chapter 2).

Thermal unfolding experiments of FT SDS-His$_6$-porin-ergosterol-liposomes monitored mean residue ellipticity (MRE) at λ 217 nm, and showed a constant MRE centred around -3 x 10^3 until temperatures exceed 70°C, after which ellipticity steadily increased to -1.5 x 10^3 (Fig. 4.4B). Upon cooling to 20°C an MRE close to that at 20-30°C was observed. Examination of CD spectra (200-250 nm) collected at 10°C intervals demonstrates a progressive broadening and increase in λ_{min} during heating from 20°C (λ_{min} 216 nm) to 60°C (λ_{min} 220 nm) (Fig. 4.4C). CD spectra collected from 60°C to 80°C have similar shapes with spectral broadening at λ_{min} 220 nm indicating that the conformation of protein at these temperatures is not undergoing significant changes. At temperatures of 90°C to 100°C, λ_{min} is 212 nm, suggesting increased α-helix content in His$_6$-porin. Upon cooling the samples to

Figure 4.4. Far-UV CD spectra of His_6-porin in ergosterol-liposomes. **A**) His_6-porin reconstituted into liposomes prepared with (solid and dotted lines) or without ergosterol (short and long dashed lines) by freeze-thawing (FT) (solid and long dashed lines) or by direct insertion (INS) (dotted and short dashed lines). Panels **B**) and **D**) correspond to the thermal denaturation profiles of FT (**B**) and INS (**D**) SDS-His_6-porin reconstituted into ergosterol-containing liposomes monitored at 217 nm from 20-100°C and 20°C cooled endpoint (circle and triangle). Panels **C**) and **D**) correspond to full length (200-250 nm) CD spectra of FT (**C**) and INS (**E**) SDS-His_6-porin reconstituted into ergosterol-liposomes at 20°C (solid line), 60°C (long dashed lines), 100°C (short dashed lines), and 100-20°C cooled (dotted lines).
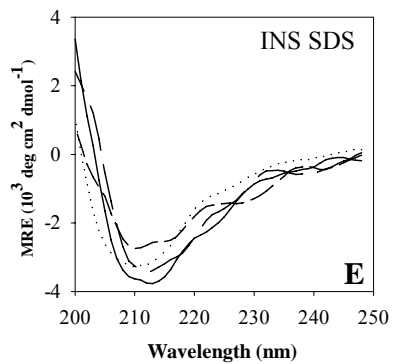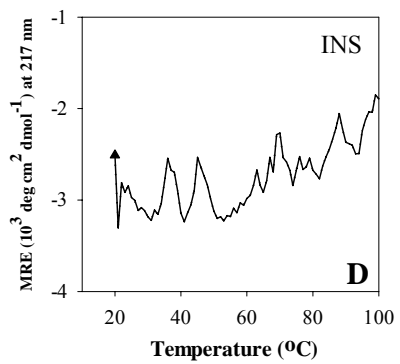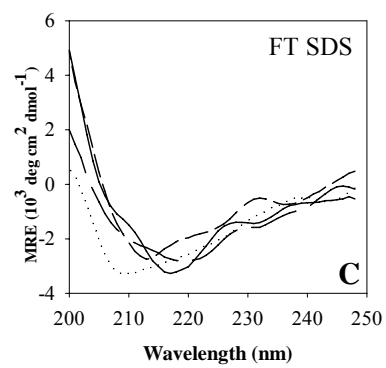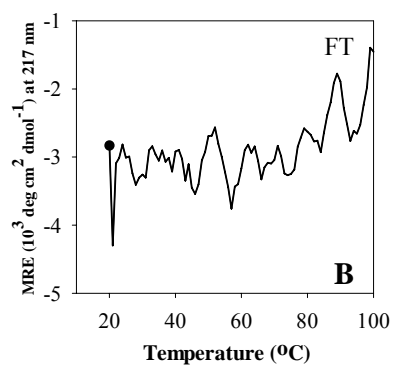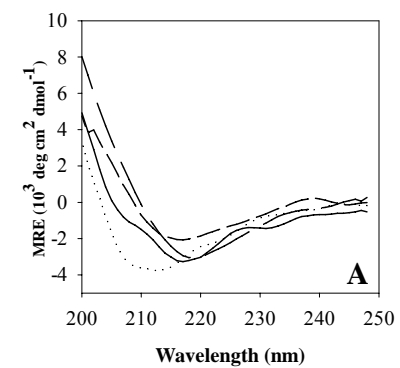
20°C, the resulting CD spectrum has λ_{min} of 209 nm, indicating retention of high α-helix content (Fig. 4.4C). These results are in stark contrast to thermal unfolding CD spectra collected for FT SDS-His$_6$-porin-liposomes which maintain a constant λ_{min} of 217 nm during heating from 20-100°C and subsequent cooling to 20°C (Chapter 2). Clearly, the presence of ergosterol in the phospholipid membrane allows significant structural alterations of the protein at elevated temperatures; however, these altered His$_6$-porin conformations maintain some channel forming ability at 100°C (Fig. 4.1A).

The thermal denaturation profile of INS SDS-His$_6$-porin-ergosterol-liposomes show MRE values at 217 nm that remain unchanged at around -3×10^3 until temperatures reached 60°C (Fig. 4.4D). Heating the protein above 60°C resulted in a small gradual increase in MRE to -2×10^3 and a shift in λ_{min} to 210 nm above 70°C, suggesting that the protein has higher α-helix content than at lower temperatures (Fig. 4.4E). Cooling these liposomes to 20°C resulted in an MRE value of -2.5, and a CD spectrum resembling that at 100°C, suggesting that the protein has adopted a conformation similar to one observed above 60°C. The heating profile of INS SDS-His$_6$-porin-ergosterol-liposomes differs dramatically from INS SDS-His$_6$-porin-liposomes where CD spectra demonstrate a progressive broadening of λ_{min} at 217 nm that shifts to a λ_{min} of 209 nm above 70°C (Chapter 2). Although INS proteoliposomes in the presence and absence of ergosterol have very different initial conformations, both INS proteoliposomes undergo thermally induced changes above 70°C, leading to spectra with λ_{min} at 210 nm. Both INS SDS-His$_6$-porin-liposomes in the presence and absence of ergosterol are incapable of swelling

4.4.4 Near-UV circular dichroism spectropolarimetry of detergent-sterol-solubilized His_6-porin

Near-UV region (260-330 nm) CD spectropolarimetry was used to examine tertiary interactions in His_6-porin. In general, this region provides information on the tertiary arrangements of aromatic residues based on the absorption of tryptophan (270-280 nm) and tyrosine (265-270 nm) residues. Near-UV CD spectra of FT and INS SDS-His_6-porin-liposomes could be collected, but spectra of His_6-porin in sterol-containing liposomes showed no measurable ellipticity (data not shown). This may in part be due the absorption of ergosterol from 240-400 nm (Arthington-Skaggs *et al.* 1999) and light scattering by the liposomes, reducing the signal to noise ratio in spectra of His_6-porin in sterol-containing liposomes.

Increasing detergent concentrations five-fold (21 mM SDS, 21 mM SDS/180 mM DDM, 300 mM LDAO and 100 mM DPC) permitted the solubilization of high concentrations of sterols (0.1 mg/ml) and His_6-porin (≥ 25 µM) for near-UV CD analysis, while maintaining all components at the ratio used for far-UV CD analysis. In general, the addition of sterol to the solubilizing detergents used for His_6-porin resuspension did not result in dramatic changes in the near-UV CD spectra, but small to moderate changes could be observed in some detergent systems (Fig. 4.5).

The CD spectrum of His_6-porin solubilized in 21 mM SDS demonstrates a broad negative ellipticity spanning 265-285 nm, marked by smaller peaks, including one constant peak with λ_{min} of 268 nm. The addition of any sterol to the solubilizing detergent does not appear to significantly alter peak positions in the His_6-porin CD spectrum, but does increase intensity at λ_{min} 268 nm (Fig. 4.5A). Interestingly, this effect is strongest in the 265-

Figure 4.5. Near-UV CD spectra of detergent-solubilized His_6-porin in the presence of sterols. In each panel near-UV region (260-330 nm) CD spectra of 25-33 μM His_6-porin (thick solid line) were collected in the presence of 0.1 mg/ml sterol, specifically: ergosterol (circles), cholesterol (triangles), and cholestenone (squares). All CD spectra were collected at room temperature. **A**) 21 mM SDS. **B**) 21 mM SDS, 180 mM DDM. **C**) 300 mM LDAO. **D**) 100 mM DPC.
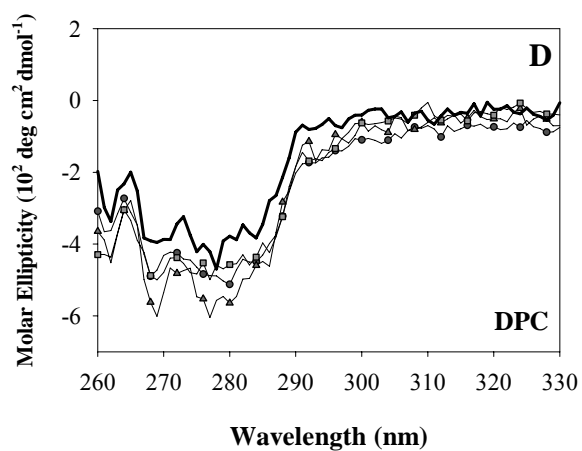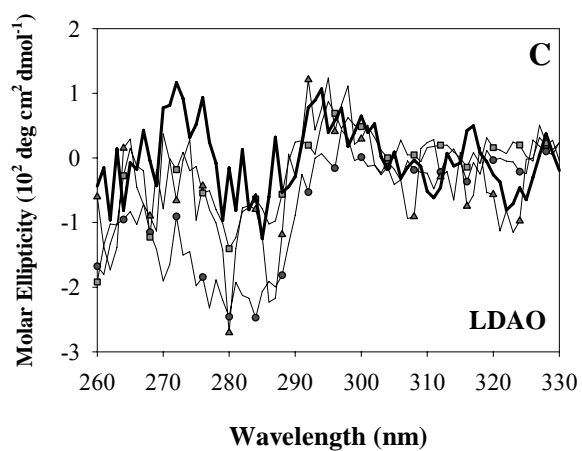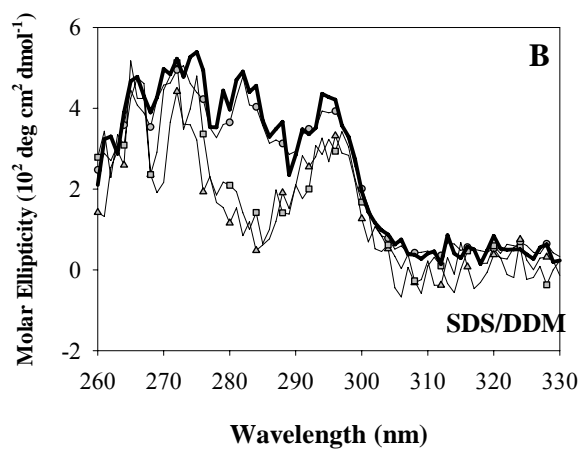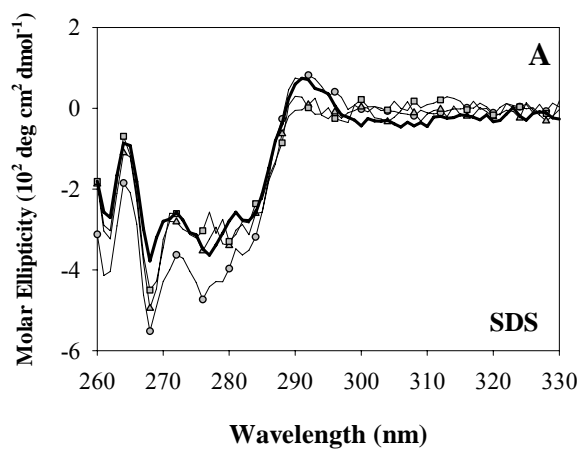
285 region of negative ellipticity in the presence of ergosterol, suggesting that this sterol has some influence on the arrangements of Tyr and Trp residues in the protein.

The near-UV CD spectrum of His_6-porin solubilized in 21 mM SDS/180 mM DDM (Fig. 4.5B) has positive ellipticity at λ_{max} of 275 nm. Positive ellipticity has also been observed for His_6-porin reconstituted into liposomes and suggests that the protein conformation in SDS/DDM is close to that in liposomes (Chapters 2 and 3). The CD spectrum of His_6-porin in SDS/DDM changes very little in the presence of ergosterol. However, a major positive peak near 283 nm in His_6-porin with or without ergosterol is absent from the CD spectra of His_6-porin collected in the presence of cholesterol or cholestenone (Fig. 4.5B). Thus, the presence of the latter sterols appears to alter Trp residue arrangements within His_6-porin.

The CD spectrum of His_6-porin solubilized in 300 mM LDAO closely resembles the spectrum of His_6-porin in 8 M urea (Chapter 2); both near-UV CD spectra fluctuate around zero (Fig. 4.5C). Although LDAO promotes high β-strand content in His_6-porin, tertiary contacts involving aromatic residues are not present and suggest His_6-porin in LDAO is a β-strand rich "molten globule" (Surrey and Jahnig 1995, Kumar *et al.* 1995, Sivaraman *et al.* 1997, Matsuura *et al.* 2001). Of the sterols tested, only ergosterol appears to have some affect on His_6-porin conformation in LDAO, as evidenced by the slight increase in negative ellipticity resulting in a minimum near 280 nm (Fig. 4.5C).

The CD spectrum of His_6-porin solubilized in 100 mM DPC demonstrates broad negative ellipticity that spans 265-285 nm, similar to the CD spectrum of His_6-porin dissolved in 21 mM SDS. As seen for SDS-His_6-porin, the addition of any sterol to the solubilizing detergent does not appear to significantly alter peak positions in the His_6-porin

CD spectrum, but does result in a slight increase in intensity and suggests that each sterol has only slight influences on the arrangements of Tyr and Trp residues in the protein.

Overall, sterols appear to have more influence on the tertiary structure of His_6-porin, as measured by near-UV CD, when the protein has high β-strand content, as in SDS/DDM and LDAO. Ergosterol has less of an effect on His_6-porin structure in SDS/DDM than in SDS, DPC, or LDAO detergent systems.

4.4.5 Tryptophan fluorescence of His_6-porin in detergent-sterol and sterol-liposomes

Fluorescence spectrophotometry is a useful technique to probe the environments surrounding aromatic residues Trp and Tyr in His_6-porin. Due to high amounts of light scattering in sterol-containing solutions at low wavelengths (≤ 310 nm) Tyr fluorescence could not be reliably determined. However, Trp fluorescence could be examined following excitation at 280 or 296 nm in detergent-sterol and ergosterol-liposome systems. In most mitochondrial porin structural models, both Trp residues are located within β-strand spanning regions of the protein (reviewed by Bay and Court 2002, Runke *et al.* 2006), where hydrophobic interactions with the surrounding lipids as well as with adjacent β-strands are expected. In the current studies, there is the added complexity of possible protein-sterol interactions.

First, the effects of sterols on Trp environments in detergent-solubilized porin were examined. Emission spectra of His_6-porin solubilized in SDS or DPC in the presence of either cholesterol or cholestenone are similar and slightly blue-shifted λ_{max} (2-3 nm) from those of the corresponding samples lacking sterols, while the addition of ergosterol led to a larger blue shift (~6 nm) (Figs. 4.6A and 4.6B, Table 4.3). Cholesterol and cholestenone were associated with slightly decreased fluorescence intensity at λ_{max} (Int_{max}), while Int_{max} was unchanged in

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Figure 4.6. Fluorescence analysis of His_6-porin in detergent-sterol systems. Fluorescence spectra were collected following excitation at 280 nm (and 296 nm) of 0.4 μM His_6-porin in one of four detergents containing - 0.01-0.02 mg/ml sterol. In each panel (**A-D**) His_6-porin (solid) alone or in the presence of ergosterol (dashed lines), cholesterol (line-dot-lines), or cholestenone (dotted lines) is shown. **A**) 3.5 mM SDS. **B**) 10 mM DPC (dotted and line-dot-lines) and 20 mM DPC (solid and dashed lines) **C**) 50 mM LDAO. **D**) 3.5 mM SDS/ 30 mM DDM.
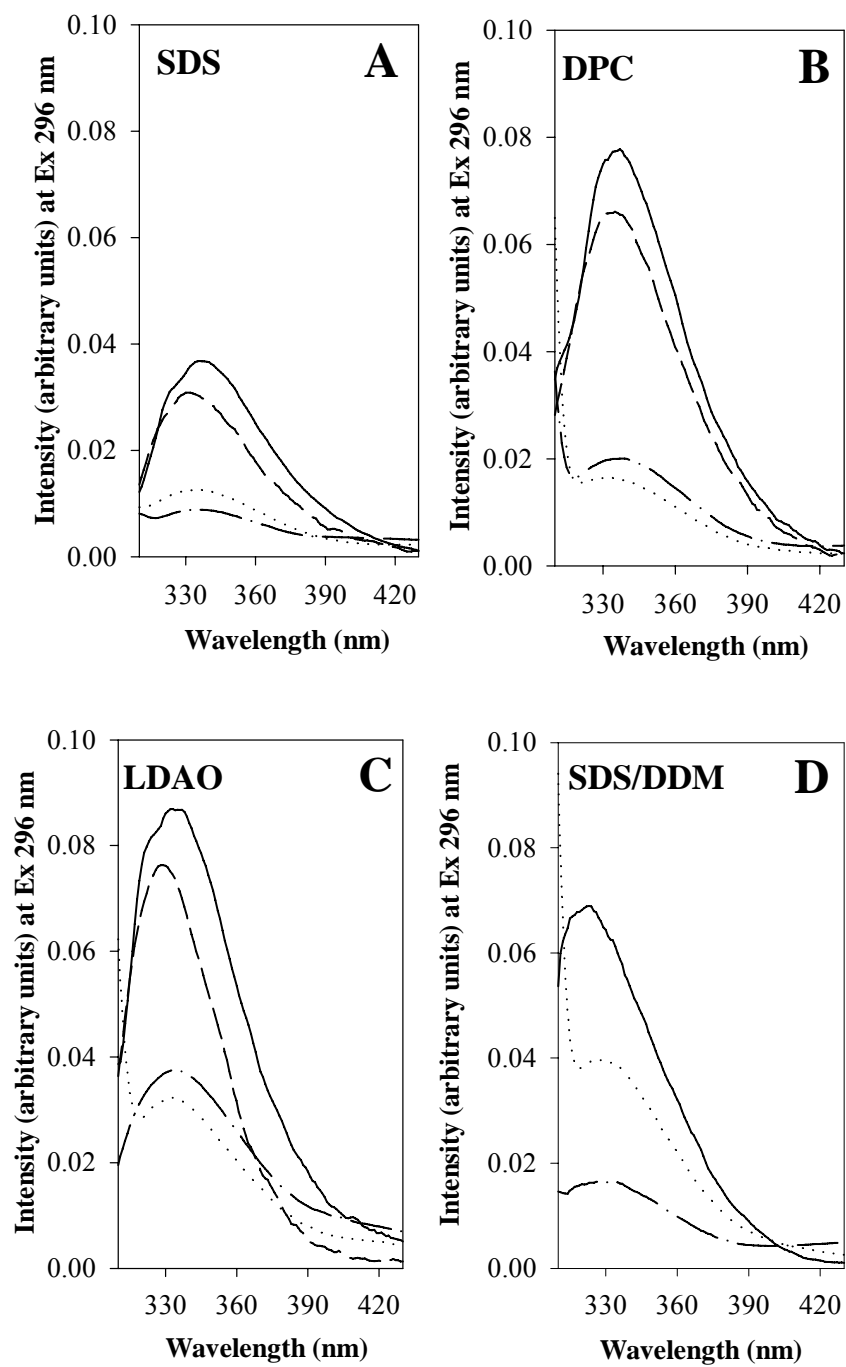
Table 4.3. Summary of normalized fluorescence emisson maxima collected at excitation wavelength of 296 nm of His_6-porin and solubilized in various detergents in the prescence or absence of sterol. Intensity values were corrected for differences in concentration. Note that the intensity of Trp fluorescence could not be determined in His_6-porin (nd).

Detergent/ Liposome	Sterol	Fluorescence Trp (Ex 296 nm)	
		λ_{max}	Intensity$_{max}$
3.5 mM SDS		336	0.04
	0.02 mg/ml ergosterol	327	0.03
	0.01 mg/ml cholesterol	334	0.02
	0.01 mg/ml cholestenone	334	0.01
3.5 mM SDS, 30 mM DDM		322	0.08
	0.02 mg/ml ergosterol	nd*	nd*
	0.01 mg/ml cholesterol	335	0.02
	0.01 mg/ml cholestenone	335	0.04
50 mM LDAO		333	0.09
	0.02 mg/ml ergosterol	328	0.08
	0.01 mg/ml cholesterol	335	0.04
	0.01 mg/ml cholestenone	335	0.03
20 mM DPC		337	0.08
	0.02 mg/ml ergosterol	335	0.07
10 mM DPC		339	0.08
	0.01 mg/ml cholesterol	334	0.02
	0.01 mg/ml cholestenone	333	0.02
FT 0.2 mg/ml liposomes		329	0.04
	0.02 µg/ml ergosterol	330	0.03
INS 0.2 mg/ml liposomes		335	0.09
	0.02 µg/ml ergosterol	328	0.03

* Scattering below 320 nm prohibited reliable data acquisition.
nd, not determined

the presence of ergosterol. Thus, the two types of sterol promote different conformations of the protein; in the presence of the former two sterols one or both Trp residues may be placed close to quenching moieties in environments less hydrophobic than those formed in the presence of ergosterol.

The emission spectra of His_6-porin-LDAO and His_6-porin-SDS/DDM, in the presence of either cholesterol or cholestenone, following excitation at 296 nm have slightly (2 nm) or highly red shifted λ_{max} (13 nm), in comparison to those of the protein in each detergent alone (Figs. 4.6C and 4.6D, Table 4.3). Fluorescence intensity is also reduced, as expected if the Trp residues are located in a region of decreased hydrophobicity, perhaps in the vicinity of quenching moieties. The presence of ergosterol did not influence fluorescence properties of LDAO-His_6-porin. Light scattering was too intense to allow analysis of the effects of this sterol on SDS/DDM-His_6-porin.

Next, the effects of sterols on liposome-embedded His_6-porin were examined. Emission spectra were collected for FT SDS-His_6-porin-ergosterol-liposomes only, as this system is the most efficient at incorporating His_6-porin into liposomes, and ergosterol is the native sterol in fungal mitochondria. Similar trends were observed following excitation at 280 nm (data not shown) or 296 nm (Fig. 4.7A). In contrast to the detergent-based systems, ergosterol has little effect on λ_{max} and leads to only a slight reduction in Int_{max} of spectra of His_6-porin reconstituted into liposomes by the FT method (Fig. 4.7A and Table 4.3). This suggests that ergosterol has less influence on the Trp residue environment in the protein when SDS-His_6-porin is FT into ergosterol-liposomes.

In contrast, the presence of ergosterol in liposomes led to significant changes in the intensity of Trp fluorescence of His_6-porin treated by the INS method. Following excitation at

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Figure 4.7. Fluorescence spectra of His$_6$-porin reconstituted into ergosterol-liposomes. Fluorescence spectra were collected following excitation (Ex) at 275 nm, 280 nm, and 296 nm of 0.1-0.2 µM His$_6$-porin reconstituted into 0.2 mg/ml liposomes in the presence or absence of 0.02 µg/ml ergosterol prepared by freeze-thawing (FT) or insertion (INS) methods. In each panel (**A-C**) FT proteoliposomes in the presence (solid line) or absence (dashed line) of ergosterol and INS proteoliposomes in the presence (dotted line) or absence (line-dot-line) of ergosterol are presented. **A**) Ex 275 nm. **B**) Ex 280 nm. **C**) Ex 296 nm.
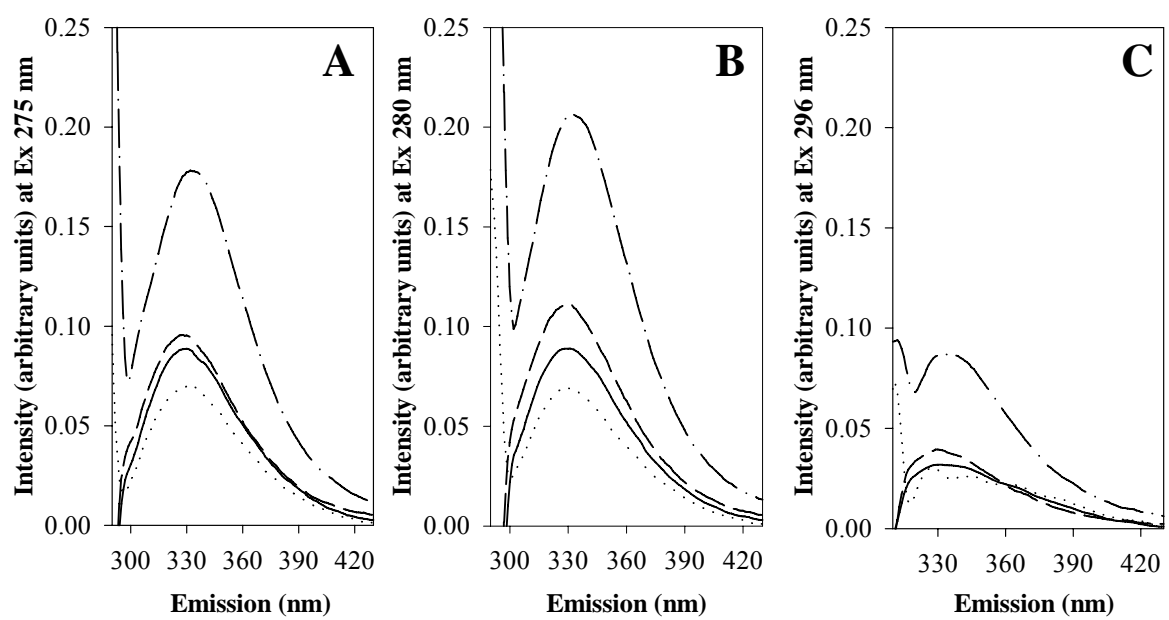
280 nm, the λ_{max} of INS proteoliposomes with and without ergosterol remained similar (~329 nm) but Int_{max} was reduced to about ⅜ of the intensity of INS SDS-His$_6$-porin-liposomes (Fig. 4.7B). The reduced fluorescence intensity of INS ergosterol-proteoliposomes suggests that the environment of the Trp residues is far more quenched than in liposomes lacking sterol and that these residues could be interacting with adjacent charged residues in the protein or associating with SDS detergent/ phospholipid head groups or the sterol. When taken together with the results of far- UV CD analysis, this may suggest that the protein is loosely associated with liposomes, where ergosterol encourages α-helix rich His$_6$-porin arrangements and Trp quenching in the liposomes.

4.5 Discussion

4.5.1 The influence of sterols on His$_6$-porin conformation in detergents

Each detergent system, SDS, SDS/DDM, LDAO, and DPC, examined in this study promotes a unique combination of secondary structure and arrangement of Trp and Tyr residues (Chapters 2 and 3). Although only His$_6$-porin in SDS/DDM has secondary structure and aromatic residue arrangements that are consistent with a folded β-barrel protein (Ohnishi and Kameyama 2001, Chapter 3), the other systems promoted protein arrangements compatible with incorporation into liposomes, making all of these detergents valuable tools to study the influence of sterols on the conformation of His$_6$-porin.

Previous examination of the influence of sterols on eukaryotic porin folding involved far-UV CD analysis of recombinant N-terminally His-tagged pea root plastid porin solubilized in Genapol-X080 (Popp *et al.* 1997). Neither of the plant sterols stigmasterol or β-sitosterol significantly altered secondary structure in detergent. The data presented herein

are similar: far-UV CD analysis of recombinant *N. crassa* mitochondrial porin (His$_6$-porin) in various detergent-sterol mixtures did not demonstrate any significant changes in the estimated protein secondary structure content. However, the use of other techniques, namely near-UV CD spectropolarimetry and fluorescence spectrophotometry revealed changes in the environments surrounding the aromatic residues within these systems and thereby indicating that sterols do have detergent-specific effects on the conformation of His$_6$-porin.

In SDS, fluorescence revealed that the Trp residues in His$_6$-porin are exposed to increasingly hydrophobic environments in the presence of sterols. Both cholesterol and cholestenone also led to quenching of Trp fluorescence, possibly due to promoting interactions between Trp and charged side-chains or detergent head groups, or directly quenching fluorescence. The largest difference between the near-UV CD spectra obtained in SDS in the presence and absence of these sterols, is the intensification of the peak near 268 nm, supporting a change in the Tyr environment(s). Ergosterol has a different influence on His$_6$-porin conformation in SDS, as evidenced by blue-shifted but unquenched Trp fluorescence, and increased intensity of Trp (276 nm), Tyr (268 nm) and Phe (263 nm) derived peaks in the near-UV spectrum. Therefore, all of the sterols examined in this study influence the folded arrangement of SDS-solubilized His$_6$-porin. These conformational changes promoted by the sterol may also influence channel formation based on previous black lipid bilayer (BLB) experiments of native mitochondrial porin isolated in SDS which only demonstrated channel forming ability in the presence of sterol (Popp *et al.* 1995). Therefore, these small structural changes in sterol-SDS-His$_6$-porin have some influence on protein conformation in SDS but are more significant for porin channel forming ability in BLB.

Sterols appear to have little influence on secondary structure arrangements or aromatic residue interactions within the protein in DPC (Figs. 4.3, 4.5, 4.6). The addition of sterol does lead to more hydrophobic environments surrounding Trp; significant quenching of fluorescence was not observed. Although both SDS and DPC promote α-helix rich conformations, these results suggest that the interactions between the sterols and the detergent-protein micelles are different in each case and that the overall conformation of His$_6$-porin in DPC is far less influenced by the addition of any native or non-native sterols than is the conformation in SDS. These observations also confirm previous reports that DPC promotes a His$_6$-porin conformation that is far less influenced by the addition of a secondary detergent (Chapter 3) or by increasing temperature (Chapter 2) than the SDS system. Taken together, these findings support a unique conformation of His$_6$-porin in DPC, that promotes much more hydrophobic Trp residue environments in the presence of a sterol; Trp residues are likely buried deeper within the acyl chain core of DPC micelles or reside closer to the sterol(s).

The near-UV spectra of LDAO-His$_6$-porin in the presence or absence of cholestenone or cholesterol were very similar to that of His$_6$-porin in 8 M urea (Chapter 2) indicating that these sterols do not promote tertiary contacts involving the aromatic residues of His$_6$-porin (Fig. 4.5C). Some quenching of Trp fluorescence is observed. In contrast, in the presence of ergosterol, the near-UV CD spectrum showed negative ellipticity similar to that in the spectra of SDS or DPC solubilized His$_6$-porin, and Trp fluorescence that was blue-shifted and moderately reduced in intensity. These observations suggest that this sterol promotes stable, albeit weak, interactions involving aromatic residues interactions. It is noteworthy that

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LDAO promotes a β-rich structure, and only interacts with the native fungal sterol – ergosterol.

Previous BLB experiments involving LDAO-solubilized His-tagged yeast and *N. crassa* recombinant mitochondrial porins have demonstrated channel formation in the presence of ergosterol only (Koppel *et al.* 1998). However, it is uncertain if cholesterol and cholestenone would be capable of promoting LDAO-His$_6$-porin channel formation in BLB experiments. Channel formation by *D. discoideum*, *P. tetraurelia*, and rat water-soluble mitochondrial porins occurs in the presence of cholesterol, but each is enhanced when a native sterol was used (Popp *et al.* 1995). Therefore, it is very likely that both cholesterol and cholestenone could promote channel formation of LDAO-His$_6$-porin in BLB experiments but enhanced channel forming ability would require ergosterol.

The SDS/DDM system was of greatest interest because it promotes a conformation of His$_6$-porin most similar to that of the membrane inserted form of the protein (see Chapter 3). Very similar near-UV CD spectra were generated for SDS/DDM-His$_6$-porin in the presence and absence of ergosterol; unfortunately reliable Trp fluorescence could not be obtained for this system. In contrast, both cholesterol and cholestenone led to marked changes in the Trp-derived peaks in the near-UV spectrum of the protein, and blue-shifted Trp fluorescence of greatly reduced intensity was observed. Interestingly, these may represent non-native protein-sterol interactions, since these sterols are not found in *N. crassa*.

Therefore, sterols influence the conformation of His$_6$-porin in a detergent-dependent manner. Further interpretation of these results is precluded by the absence of information regarding the specific sites of sterol association with mitochondrial porins. Nonetheless, these

differences can be examined with respect to the ability of the protein to incorporate into liposomes, and the resulting stability of the membrane-associated forms.

4.5.2 His₆-porin reconstituted into liposomes in the presence of ergosterol

The freeze-thaw method reconstitutes native and recombinant mitochondrial porins into liposomes to generate osmotically responsive pores (for examples see Chapter 2, Shimizu *et al.* 1999 or Bathori *et al.* 1993) in the absence of sterols, a process that was first described for α-helical membrane proteins such as bacteriorhodopsin (Scotto and Zakim 1988). Given that native mitochondrial porin inserts into the sterol-containing outer membrane, we wanted to test the effects of sterol on His₆-porin incorporation into liposomes, with particular attention to the effects in different detergents. With the exception of type D LDAO-His₆-porin liposomes, a swelling response was observed in all FT detergent-His₆-porin-liposomes prepared in the presence of ergosterol, whether it was added to the detergent (type D) or to the SUV stock (type L) (Fig. 4.1). Generally, the osmotic response was of a lower magnitude, but much more rapid than that in sterol-free systems (Chapter 2 and see Fig. 4.1A). This may suggest that a relatively small number of very responsive pores occur within these ergosterol-containing liposomes or more likely, the incorporation of sterol into the liposomes may have reduced the capacity for swelling by promoting altered membrane organization (see below). Notably, the effects differed depending on whether the sterol was added to the detergent, or incorporated into the SUV stock. For LDAO and SDS, the response was of greater magnitude when the sterol was in the stock SUV; in fact, swelling was often not observed for LDAO-ergosterol solubilized His₆-porin. These observations fit with the lack of significant changes to LDAO-His₆-porin arrangement detected by the various spectroscopic methods. Although the addition of sterol to LDAO-solubilized protein does not

promote an insertion-competent state of His_6-porin, the sterol enhances either the channels'

ability to incorporate into the membrane, or the state of the channel therein. In contrast, in

SDS/DDM, the swelling response was greater if the sterol was added to the protein-detergent

solution, which is intriguing given that the significant changes were not observed in the far

and near UV CD spectra obtained in this system. It is conceivable that ergosterol stabilizes

the tertiary contacts detected in SDS/DDM-His_6-porin – and perhaps these contacts survive

dilution during proteoliposome formation, where the detergent concentrations are at or below

the CMC.

Differences between type D and low ergosterol type L FT proteoliposome swelling

profiles were observed after heat treatment at $65^{\circ}C$ and $100^{\circ}C$; the associated structural

changes were followed for the low ergosterol type L SDS system. As shown previously

(Chapter 2), heating of both SDS- and SDS-ergosterol-proteoliposomes to $65^{\circ}C$ does not alter

the overall β-strand character of the CD spectra, but does reduce swelling significantly at

$65^{\circ}C$ (see Table 4.1). This general trend in reduced swelling at $65^{\circ}C$ was also observed if

ergosterol was present in the SDS-protein solution (Type D). In striking contrast, low

ergosterol type L FT detergent-His_6-porin-liposomes retained swelling activity following

heating to $65^{\circ}C$ or $100^{\circ}C$. Above $70^{\circ}C$, far UV CD spectra of type L FT SDS-ergo-

proteoliposomes has transitions from β-strand to α-helix character and upon cooling from

$100^{\circ}C$, the CD spectrum resembles α-helix character unlike SDS-proteoliposomes which

retain overall β-strand character from $70-100^{\circ}C$ heating and cooling (Chapter 2). This

suggests that the sterol in the membrane does not act effectively as a chaperone, but perhaps

allows folding of at least a subpopulation of the protein into conformations that create

osmotically responsive liposomes. Caution must be taken in interpreting the refolding data

however, as the CD spectra were collected over a total of 210 min of heating, while the samples for swelling were only heated for 10 min – it remains possible that irreversible changes to folding occurred during long-term heating.

Incorporation of sterol into liposomes reduced the osmotic responses of proteoliposomes formed with SDS-, DPC- and LDAO-solubilized porin. In contrast, the swelling activity of SDS/DDM-His$_6$-porin liposomes was increased in the presence of sterol. These observations may reflect the membrane rigidifying effects of sterols on lipid bilayers. Increasing the concentration of cholesterol has been shown to result in more densely packed, ordered phospholipid membranes, in comparison to the more fluid membranes at lower sterol concentrations (Kakorin *et al.* 2005). Comparative molecular dynamics modeling experiments performed for lipid bilayers containing cholesterol or ergosterol at 25 mol % of sterol indicated that ergosterol resulted in higher rigidification of phospholipid bilayers than cholesterol (Czub and Baginski 2006). In this study, 2 mol % sterol was used for low ergosterol type L and type D proteoliposomes; this concentration was sufficient for a large impact on osmotic responsiveness. The high ergosterol type L proteoliposomes (20 mol%) were closer to those used by Czub *et al.* 2006 and Kakorin *et al.* 2005. Hence, increased membrane rigidity in high ergosterol FT proteoliposomes may explain the enhanced loss of swelling after 100°C treatment of SDS- and DPC-His$_6$-porin-liposomes in the presence of high, but not low ergosterol concentrations. *In vivo* sterols occur within membranes at 30-50 mol % (Simons and Ikonen 2000), therefore the behaviour of high ergosterol FT proteoliposomes at high temperatures may have more relevance to porin channel activity *in vivo*.

Finally, addition of sterol to either the detergent-solubilized protein or the stock SUV does not encourage or facilitate direct insertion (INS) of His$_6$-porin into liposomes. Even at elevated temperatures of 30°C or 40°C, His$_6$-porin insertion into type D or L liposomes did not lead to proteoliposomes with swelling activity. However, different interactions with the liposomes were established, as higher α-helical content was demonstrated in His$_6$-porin following interaction with ergosterol-containing liposomes (Fig. 4.4). The α-helical nature of the protein was retained during heating to 100°C, indicating that increased temperature did not drive insertion into the membrane.

In DPC, LDAO and SDS/DDM systems, swelling of type D proteoliposomes was similar to that in the absence of sterols (Table 4.1). One possible explanation for this observation may be found in studies of detergent-solubilized sterol incorporation into lipid vesicles (Proulx *et al.* 1984). These experiments have primarily focused on the use of bile salts to deliver sterols to the necessary membranes. Both ionic and non-ionic detergents have demonstrated a limited ability to incorporate sterols into phospholipid containing membranes by partially disruptive mechanisms (Proulx *et al.* 1984). This suggests that sterols added to the solubilizing detergent can incorporate sterol into phospholipid membranes, but that this process results in very low overall sterol concentrations within vesicles. Based on these experiments, it is likely that type D proteoliposomes contained very little sterol, and were therefore less rigid than low ergosterol type L proteoliposomes.

The lack of insertion into liposomes is in contrast to the direct insertion of OmpA into liposomes at 30°C (Surrey and Jahnig 1995), and the insertion of α-helical proteins such as bacteriorhodopsin and UDP glucuronosyltransferase into sterol-containing liposomes in the absence of freeze-thaw treatment (Scotto and Zakim 1988). The most likely explanations

involve the energetic requirements for mitochondrial porin insertion into artificial membranes and the membrane curvature of the target membranes. In black lipid bilayer experiments recombinant and native mitochondrial porin insertion occurs only at low applied voltages in the presence of sterol. Under these conditions, the actual mechanism of initial porin insertion into the membrane is unknown, but likely involves a specific conformation of porin inserting into the artificial membrane at a site where the energy barrier of insertion can be overcome under transmembrane potential (Zizi *et al.* 1995, Xu and Colombini 1996, Xu and Colombini 1997; Li and Colombini 2002). For mitochondrial porins, these initial porin insertion sites may be focused in or around sterols (Popp *et al.* 1995, Popp *et al.* 1997), as has been suggested for other β-barrel forming integral membrane proteins such as members of the cholesterol binding pore forming toxin (CBT) family (reviewed by Gilbert 2002) and the α-helix rich integral membrane proteins (Scotto and Zakim 1988). However, as is the case for CBT family proteins, porin insertion into artificial membranes can occur in the absence of sterol (Popp *et al.* 1995).

The second major difference between the SUV experiments described herein and the planar bilayer studies is the curvature of the respective membranes. Planar artificial membranes during black lipid bilayer experiments are in stark contrast to the highly curved lipid bilayers of SUVs and LUVs. Membrane curvature and thickness as well as membrane elasticity influence the insertion of OmpA into LUVs and SUVs and the kinetics of the process (Kleinschmidt and Tamm 2002, Pocanschi *et al.* 2006, Marsh *et al.* 2006). High insertion rates of OmpA were observed in liposomes with thicker membranes (C_{14}-C_{18} chains) and high membrane curvature (SUVs), although insertion into C_{12} LUVs was also achieved (Kleinschmidt and Tamm 2002). The addition of sterols, such as cholesterol or

ergosterol, to phosphatidyl choline bilayer membranes alone also increases membrane rigidity, resulting in thicker and more densely packed phospholipids (Kakorin *et al.* 2005, Czub and Baginski 2006). In our experiments, His_6-porin incubated with SUVs prepared with phosphatidyl choline with C_{12} acyl chains in the presence or absence of ergosterol was incapable of producing pores responsive to sucrose (Chapters 2 and 3). Therefore, ergosterol alone cannot promote His_6-porin insertion directly into liposomes and conversely, may hinder His_6-porin insertion into liposomes due to the membrane rigidifying effects on the ergosterol-containing membranes.

The relevance of these discussions toward understanding the *in vivo* assembly of mitochondrial porin is complicated by the uncertainty regarding the presence of a transmembrane potential across the mitochondrial outer membrane (Lemeshko and Lemeshko 2000, Lemeshko and Lemeshko 2004, Lemeshko and Lemeshko 2004) and by the fact that native pathway for insertion of porin into the mitochondrial outer membrane involves two transmembrane complexes, the translocase of the mitochondrial outer membrane (TOM), and the topogenesis of outer membrane β-barrel protein (TOB) complex, as well as soluble intermembrane space components (reviewed in Paschen *et al.* 2005). It has been argued that the voltage applied in black lipid bilayer studies represents the transmembrane potential that would be present across the mitochondrial outer membrane *in vivo* (as reviewed by Benz 1994, Mannella 1998), Lemeshko and Lemeshko 2004; Lemeshko and Lemeshko 2004). *In vivo,* the energetic constraints are effected by protein-protein interactions, as well as those between protein and lipid/sterol.

Clearly, sterols have some influence on His_6-porin conformations both in detergent and in artificial membranes. In detergents, sterols have a greater influence on tertiary

arrangements of aromatic residues, primarily Trp, than on the secondary structure of His_6-porin. Their presence is not sufficient for insertion into lipids, suggesting that future studies of the assembly of porin into mitochondrial membranes should focus on *in organello* systems.

5.1 Conclusions

In summary, ten major conclusions can be drawn from the experiments described in this thesis and they are as follows:

i.   Single detergent systems do not fold His$_6$-porin into a β-barrel structure reminiscent of bacterial porins.

ii. His$_6$-porin solubilized in single detergents produces osmotically responsive channels when reconstituted into liposomes by freeze-thaw

iii. Reconstitution of detergent-solubilized His$_6$-porin into liposomes by freeze-thaw results in tertiary arrangements similar to those observed for bacterial porins, supporting a β-barrel conformation.

iv. His$_6$-porin solubilized in detergents or in urea cannot insert into pre-formed phosphatidyl choline based liposomes as described for OmpA (Surrey and Jahnig 1995).

v.   Mixed detergent systems, in particular SDS/DDM, can solubilize high concentrations of His$_6$-porin and promote secondary and tertiary structure arrangements similar to those in liposomes.

vi. SDS/DDM-solubilized His$_6$-porin can be reconstituted into liposomes to form osmotically responsive channels.

vii. His$_6$-porin solubilized in both mixed and single detergent systems that promote high β-strand content share similar protease resistance profiles with native mitochondrial porin.

viii.   Porin in proteoliposomes prepared by freeze-thaw, insertion or sonication all possess some protease resistance, but spectroscopic analysis indicates that the each of these protease-resistant forms has a unique structural arrangement

ix.   The presence of sterol in detergents does not drastically alter the secondary structure content of His$_6$-porin, but has some minor effects on tertiary arrangements.

x.   Sterol-containing FT proteoliposomes possess reduced swelling activity relative to sterol-free systems, indicating that sterols did not enhance pore forming activity.

In summary these studies determined that:

i) The folded state of His$_6$-porin in several single detergent systems was not a β-barrel conformation based their tertiary interactions (Fig. 5.1A-C).

ii) The mixed detergent system SDS/ DDM at a 1:9 ratio was capable of promoting a β-rich folded state of His$_6$-porin with similar tertiary arrangements as His$_6$-porin reconstituted into liposomes (Fig. 5.1D-G).

iii)Sterols alter tertiary interactions within His$_6$-porin in both detergent and lipid-based systems and do not promote direct insertion of the protein into liposomes (Fig. 5.1H).

Furthermore, these studies were essential to further the current understanding of mitochondrial porin structure *in vitro* by examining porin arrangements in a variety of membrane mimetic systems. Although the spectroscopic characterization of His$_6$-porin is not itself novel, the focus on porin tertiary arrangement by a combination of spectroscopic techniques has permitted a much more detailed examination of the folded states of porin that extends beyond existing secondary structure analysis. In addition, a large set of aromatic

amino acid compounds were examined in both single and mixed detergent systems by UV absorbance spectroscopy and fluorescence spectrophotometry and will serve as an extremely useful reference set for future *in vitro* spectroscopic analysis of porin, as well as other membrane proteins.

Therefore, these studies represent the first thorough characterization of mitochondrial porin folding states in detergents and in artificial membranes. These experiments on detergent solubilized His_6-porin indicate that only particular mixed detergent systems enable denatured porin to re-fold into a tertiary conformation similar to porin within artificial membranes. They also revealed that sterols are not essential for promoting porin channel formation in liposomes, but do alter some tertiary interactions in detergent-solubilized protein. The major achievement of this work is the first identification of a system capable of solubilizing high concentrations of well folded protein essential for the development of a high resolution structure.

5.2 Future Directions

Logically, the next step toward the *in vitro* characterization of mitochondrial porin structure would be to further analyze the structure of His_6-porin in the SDS/DDM system. Examination of His_6-porin structure by transverse relaxation optimized spectroscopy (TROSY) NMR, used successfully to determine the structural arrangements of other bacterial porins (Arora *et al.* 2001, Fernández *et al.* 2001, Fernández *et al.* 2004), could enable the acquisition of a high resolution porin structural data essential for refining transmembrane models. This detergent system may also serve as a useful starting point for crystallization trials, with the hopes of obtaining crystals of sufficient quality for resolution the conformation of porin by X-ray diffraction.

Future experiments regarding the arrangement of porin across the outer membrane, and its oligomer state could involve fluorescently labelled or green fluorescent protein (GFP) tagged mitochondrial porin both *in vivo* and *in vitro*. A technique referred to as fluorescence resonance energy transfer (FRET) used previously by (Zalk *et al.* 2005) to examine VDAC oligomerization *in vitro*, would be useful for these studies. For example, FRET could be used to determine the orientation of porin inserted within artificial membranes, to identify the location of the N-terminus relative to the barrel or to the membrane (using fluorescently labelled lipids), and to resolve the oligomeric status of the channel in both the outer membrane and in artificial membranes.

These studies have only begun to explore *in vitro* the folding and insertion of mitochondrial porins. Based on the experiments described herein, the folding of mitochondrial porins may differ from the folding and insertion pathway of bacterial OmpA (refer to section 1.8 and reviews by Kleinschmidt 2003, Tamm *et al.* 2004, Kleinschmidt 2006). To expand His_6-porin-liposome studies, time-resolved fluorescence quenching (TDFQ) techniques developed by L. Tamm and J. Kleinschmidt may be useful. Also combining TDFQ experiments with the techniques used to examine synthetic peptide insertion into liposomes as described in (Thundimadathil *et al.* 2005, Thundimadathil *et al.* 2006, Thundimadathil *et al.* 2006, Thundimadathil *et al.* 2005) could be used to study porin insertion and possibly develop a model for the folding mechanism of mitochondrial porin.

In the end, only a three-dimensional structure will set the stage for directed *in vitro* and *in vivo* studies into the mechanism of VDAC gating and into its interactions with proteins in the outer membrane, cytosol, and intermembrane space.

Figure 5.1 Cartoon diagram summarizing the various conformational states of His$_6$-porin in detergents and liposomes. In panels A to H, detergent solubilized His$_6$-porin structure represented by green α-helices and red rectangular β-strand secondary ribbon structures that are separated by red interconnecting lines (loops and β-turns) and are shown within a detergent micelle represented by a circular arrangement of blue filled circles (polar head group) attached to a zig zagging line (hydrocarbon tail). Aromatic amino acids Trp (a blue filled circle labelled with a white "W") and Tyr (a yellow filled circle labelled with a black "Y") are indicated on the His$_6$-porin ribbon diagram that suggests a the possible aromatic teriary arrangement within the protein in the indicated detergent. The phospholipid bilayers of a liposome are represented by yellow filled circles (polar head group) attached to two zig-zagged lines (hydrocarbon tails) arranged in a half circle on panels G and H. Sterols in panel H are indicated as black 4 hexameric rings within the phospholipid bilayer. Denatured His$_6$-porin (red line) in 8 M urea is shown on panel A. In panels B and C, the conformation of His$_6$-porin in single detergent systems LDAO (B) and DPC (C) is indicated. Panels D to F, represent the conformational changes to SDS solubilized His$_6$-porin (D) upon addition of DDM to 5 DDM: 1 SDS molar ratio (E) and to a 9 DDM: 1 SDS molar ratio (F). The cartoon diagram in panel G compares His$_6$-porin conformation in the mixed 1 SDS: 9 DDM detergent system to the conformation of His$_6$-porin reconstituted into liposomes. In panel H, the ability of all detergent solubilized His$_6$-porin preparations to form osmotically responsive pores within liposomes by direct insertion or freeze-thaw is summarized. A crossed red arrow indicates a lack of pore formation while an uncrossed green arrow represents osmotic swelling activity of the proteoliposome.

A His$_6$-porin in 8 M urea

2 W residues
9 Y residues

B His$_6$-porin in 50 mM LDAO

2 exposed Y (SDUV)

C His$_6$-porin in DPC

Higher α–helix content (by far-UV CD)

W LDAO and DPC: residues are in a hydrophobic environment (Fluorescence)

D His$_6$-porin in 3.5 mM SDS

1 exposed W (Fluorescence)

6 exposed Y (SDUV)

E His$_6$-porin in 3.5 mM SDS/ 15 mM DDM

4-5 exposed Y (SDUV)

F His$_6$-porin in 3.5 mM SDS/ 30 mM DDM

G

Both have positive ellipticity by near-UV CD and similar highly hydrophobic Trp environments (Fluorescence)

Both have high overall β-strand character by far-UV CD

His$_6$-porin in 3.5 mM SDS/ 30 mM DDM

His$_6$-porin in liposome

H

Detergent solubilized His$_6$-porin

Direct insertion

Freeze-thaw incorporation

+/- sterols

6. APPENDIX   Protein and gene structures of mitochondrial porins

Some of the material presented in this appendix has been recently submitted to BioMed Central Evolutionary Biology. My major contributions are presented as Sections 6.1 to 6.3, Table 6.1, and Figures 6.2 and 6.3 that were taken, with permission from the coauthors, from the manuscript: The Evolutionary History of Mitochondrial Porins, by Young, M. J., Bay, D. C., Hausner, G. and D. A. Court.

6.1 Introduction

The understanding of mitochondrial porin function has been complicated over the last ten years by the identification of multiple porin isoforms in many cell types. The first porin isoforms isolated were extracted from potato mitochondria (POM34 and POM36), and both formed pores in black lipid bilayers (Heins *et al.* 1994). In *Saccharomyces,* a genetic screen revealed a second isoform that, in high copy number, complemented a strain in which the known VDAC gene, *POR1* was disrupted (Blachly-Dyson *et al.* 1997). Since then, advances in molecular techniques, and the accumulation of large amounts of sequence data from genome sequence projects and expressed sequence tag (EST) libraries, have led to an abundance of information regarding porin isoforms (see Table 6.1 and references therein). The number of porin variants can range from a single isoform in fungi such as *Neurospora*, to five potential isoforms in *Lotus japonicus* (Wandrey *et al.* 2004). In some cases, as in *L. japonicus*, gene sequences obtained from a cDNA library are the only evidence of the presence of isoforms, but these sequences could also represent different alleles at the same locus. Generally, the genes encoding porin isoforms are located on different chromosomes (for example, in mice Sampson *et al.* 1997; see Fig. 6.2), with the exception of *Drosophila* spp. where they are arranged in tandem repeats (Graham and Craigen 2005).

Unfortunately, there are only a few cases where electrophysiological analyses have been performed for the various porin isoforms (for more detailed explanations of electrophysiological analyses refer to Chapter 1). For example, in BLB experiments in addition to complementation experiments using the yeast *ΔPOR1* strain (Table 6.1), revealed that organisms, such as *Drosophila melanogaster*, express at least one porin isoform with the characteristics of the originally described VDAC (Blachly-Dyson *et al.* 1997). This functional conservation is even more striking given the relatively low levels of primary sequence conservation (Fig. 6.1). In contrast, some porin variants were not capable of forming characteristic channels. This may be due to specialization of porin function for developmental regulation or for tissue-specific differential expression which has been suggested for human (Rahmani *et al.* 1998), mouse (Buettner *et al.* 2000) and plant (Elkeles *et al.* 1995, Al Bitar *et al.* 2002, Al Bitar *et al.* 2003) porin isoforms. Further evidence that supports isoform specialization comes from cell lines and knock-out mice lacking porin isoforms singly or in combination (Xu *et al.* 1999). For mammals in particular, VDAC1 has been reported to have NADH-ferricyanide activity within both the plasma membrane and the outer mitochondrial membrane (Baker *et al.* 2004) and VDAC2 has demonstrated pro-apoptotic BAK protein inactivation (Cheng *et al.* 2003). VDAC3 has a variety of spliceoforms that are expressed within specific tissues (Anflous *et al.* 1998, Sampson *et al.* 1998, Decker and Craigen 2000) and in mice the loss of VDAC3 is associated with infertility and sperm immotility (Sampson *et al.* 2001).

Table 6.1. Characteristics of mitochondrial porin isoforms.

Organism	Porin isoform	Pore size (nS)[a,b]	Ion selectivity[c]	Gating	Complements Δpor1 yeast	Reference
Saccharomyces cerevisiae	Por1p or VDAC1 (NP_014343)	4.1	anion	yes	n/a[h]	Ryerse *et al*. 1997; Ludwig *et al*. 1988
	Por2p or YVDAC2 (NP_012152)	none	n/d	n/d	yes – at high copy	Blachly-Dyson *et al*. 1997
Drosophila melanogaster	DVDAC or porin (AAL47980.1)	4.1[d] 4.5[e]	anion[d,e]	yes[d,e]	yes	Komarov *et al*. 2004
	CG17137 or Porin2-PA (NP_609462)	(variable) 0.5-8[d] 4.5[e]	variable[d] cation[e]	variable[d] no[e]	yes	Komarov *et al*. 2004[d], Aiello *et al*. 2004[e]
	CG17140 (AAF53019)	1.38	anion	required 110 mV[f]	no	Komarov *et al*. 2004
	CG17139 (NP_609462)	none	n/d[g]	n/d	no	Komarov *et al*. 2004
Mus musculus	VDAC1 (Q60932)	4.3	anion	yes	yes	Xu *et al*. 1999
	VDAC2 (NP_03582)	3.8	anion	yes	yes	Xu *et al*. 1999
	VDAC3 (NP_35826)	highly variable	n/d	n/d	partial	Xu *et al*. 1999
Homo sapiens	VDAC1 (NP_003365)	4.1	anion	yes	yes[i]	Blachly-Dyson *et al*. 1993
	VDAC2 (NP_003366)	4	anion	yes	yes	Blachly-Dyson *et al*. 1993
	VDAC3 (NP_005653)	n/d	n/d	n/d	n/d	Blachly-Dyson *et al*. 1993
Triticum aestivum	VDAC1 (P46274)	3.8[d] - 4.1	anion	yes	yes	Elkeles *et al*. 1997 or Blumenthal *et al*. 1993
	VDAC2 (S59546)	4.0[d]	anion	yes	yes	Elkeles *et al*. 1997
	VDAC3 (S59547)	4.0[d]	anion	yes	partial	Elkeles *et al*. 1997
Lotus japonicus	VDAC1.1 (AAQ87019)	n/d	n/d	n/d	yes	Wandrey *et al*. 2004
	VDAC1.2 (AAQ87020)	n/d	n/d	n/d	yes	Wandrey *et al*. 2004
	VDAC2.1 (AAQ87022)	n/d	n/d	n/d	yes	Wandrey *et al*. 2004
	VDAC3.1 (AAQ87023)	n/d	n/d	n/d	yes	Wandrey *et al*. 2004
	VDAC1.3 (AAQ87021)	n/d	n/d	n/d	no	Wandrey *et al*. 2004

see footnotes on following page

Table 6.1, footnotes

[a] unless otherwise stated, prepared from mitochondrial membranes

[b] in 1 M KCl or 1 M NaCl

[c] pAnion/pCation = 1.5-1.8

[d] isolated following heterologous expression in *Saccharomyces*

[e] recombinant *D. melanogaster* VDAC2, expressed in *E. coli* and refolded in 1% Genapol X-080 in the presence of 0.5% (w/v) cholesterol

[f] the midpoint for gating is usually around 50 mV

[g] n/d, not determined

[h] n/a, not applicable

[i] Complementation was performed with chimeric constructs in which the HVDAC1 and HVDAC2 genes were fused to the 11th and 22nd codons, respectively, of the yeast *POR1* gene (Blachly-Dyson *et al*.1993).

The purpose of this appendix is to briefly highlight some of my work on the structural similarities and dissimilarities of mitochondrial porins and their genes, from different organisms as well as within families of isoforms. To accomplish this, mitochondrial porin protein sequences retrieved primarily from National Cell Biology Institute (NCBI) GenBank and The Institute for Genomic Research (TIGR), were aligned using the web server PRALINE. From these alignments, conserved residues and porin motif distribution within mitochondrial porins from fungi, plants, and animals were identified. These alignments also permitted potential TM β-strand comparisons using the prediction algorithm SSPRO available from PRALINE. Finally, the genetic organization of mitochondrial porins was also examined using genetic data collected from the NCBI genome map viewer. To determine if porin secondary structure is conserved in parallel to gene organization, the gene structures of representatives, including isoforms, from the animals, plants and fungi have been compared.

6.2. Materials and Methods

6.2.1 Database searches for porin protein and gene sequences

A variety of databases were searched in order to obtain VDAC sequences. GenBank was searched via blastp and tblastx against previously published VDAC sequences from related organisms. Porin sequences were also extracted from the genome sites that included: Stramenopiles: [*Phytophthora* sp., *Thalassiosira pseudonana* (Diatom)]: http://genome.jgi-psf.org/euk_cur1.html; this site also provided access to the following genomes *Fugo, Chlamydomonas, Ciona, Populus*; and for fungi: http://www.fgsc.net/outlink.html; http://genome.jgi-psf.org/whiterot1/whiterot1.home.html (*Phanerochaete chrysosporium*); http://mips.gsf.de/projects/fungi; http://www.broad.mit.edu/annotation/fungi/fgi/

Additional fungal, plants, protozoan, animals genomes were accessed via the TIGR sites: http://www.tigr.org/db.shtml and http://www.tigr.org/plantProjects.shtml. Overall, over 280 potential/putative VDAC homologs were recovered from various databases; however, the final data set comprised a total of 244 sequences.

Additionally, gene sequences used to prepare Fig. 6.3 were collected from the NCBI genome map viewer web server [http://www.ncbi.nlm.nih.gov/mapview/]. VDAC sequences were retrieved based on gene annotation and/or GenBank accession numbers obtained from the initial protein sequence blastp searches.

6.2.2. PRALINE Alignment of porin sequences

The retrieved mitochondrial porin sequences were aligned with the online PRALINE multiple sequence alignment program [http://ibivu.cs.vu.nl/programs/pralinewww, (Simossis and Heringa 2003, Simossis and Heringa 2005)] and this program was utilized for its secondary structure prediction capabilities (Heringa 2000, Heringa 2002, Simossis and Heringa 2005). This multiple sequence alignment program was chosen based on its ability perform global progressive alignments, optimal for proteins with high to medium sequence similarity, coupled with a choice of seven different secondary structure prediction methods.

Predictions were performed using Version 2 of SSPRO (Pollastri *et al.* 2002) following multiple alignment. Similar results (data not shown) were obtained with YASPIN (Lin *et al.* 2005); some predicted β-strands were one or two residues longer in the YASPIN predictions, but otherwise their locations were the same. SSPRO predicts protein secondary structure in three structural classes (α-helix, β-strand and unordered/ turns) using a bidirectional recurrent neural network (Pollastri *et al.* 2002) where as YASPIN predicts secondary structure into 7 structural elements using hidden neural networks. Based on

analysis by EVA [http://cubic.bioc.columbia.edu/eva/], the SSPRO algorithm results in a lower level of "confused" α and β-strand predictions than does YASPIN (BAD 3.0 vs 6.3), while the latter program generates higher percentages of correctly predicted strands compared to observed strands (QE%E60 72% for YASPIN vs 61% for SSPRO).

The conservation of amino acid sequence motifs was assessed on the aligned sequences using the column composition function associated with PRALINE. The following groups of amino acids were considered chemically similar: (S,T), (I,LV), (C,M), (K,R), (D,E), (F,Y,W), and (A,G).

6.3 Results and Discussion

6.3.1 VDAC alignments using PRALINE and secondary structure predictions

Although the precise transmembrane topology of mitochondrial porin is not known, secondary structural predictions are useful in determining whether there have been constraints on porin sequence features and structures during their evolution. To this end, SSPRO was used to predict the secondary structure porin sequences aligned by PRALINE (see Materials and Methods). In general, these predictions revealed remarkably similar patterns for all of the retrieved porin sequences. This similarity extends to the overall secondary structure position and lengths within the annotated isoforms of each of the three crown groups (plants animals and fungi) (Fig. 6.1).

Figure 6.1. PRALINE alignment and secondary structure predictions of eukaryotic porin sequences using SSPRO. Amino acid sequences from **A**) fungi, **B**) animals, and **C**) plants were aligned using PRALINE, as described in Materials and Methods. Amino acids predicted to reside in α-helices (red) or β-strands (green) by SSPRO are indicated. Chemically-similar residues in all four representative sequences are shown in bold. The GLK motif (for example residues 87-89 of Osat1) and the existing eukaryotic porin motif (PS00558, for example residues 216-238 of Osat1) are indicated on each of the three panels by blue boxes.

A

Multiple sequence alignment (residues 110–200)

```
                                                        ....|....110....|....120....|....130....|....140....|....150
Aspergillus nidulans                                    ITQYLPAKQS K-GAKLNLHF KQPNLHARAF FDL--LNGP- SANFDAVLG-
Aspergillus fumigatus                                   LTQYLPSKQS K-GAKLNLYF KQPNLHARAF FDL--LNGP- SANFDAVLG-
Neurospora crassa                                       IFSFLPATNA R-GAKFNLHF KQSNFHGRAF FDL--LKGP- TANIDAIVG-
Magnaporthe grisea                                      LFGFLPATSA T-AAKFNLHF KQSNVHGRAF FDL--LKGP- TANVDAVIG-
Gibberella zeae                                         LFNFLPATAA K-GAKFNLHF KQPGFHGRAF FDL--LKGP- VANVDAVVG-
Yarrowia lipolytica                                     ATSFVPSSGA K-NAKLSLFF KQPSFQARAF FDL--LKGP- TVTSDATLG-
Coccidioides posadasii                                  LTQYLPYSNS K-GAKLNLHF KQPNLHARAF FDL--LKGP- TANFDAVLG-
Mycosphaerella graminicola                              QNLWNPSKAG SAAQKLNLAF KNPNVHSRAF LNYGTAKGNI DAVVDVTAG-
Candida albicans                                        DTSVVPNGAR N--AKLNFFY QQSAVNARLF FDL--LKGP- IATADLVVA-
Clavispora lusitaniae                                   DTSVVPNGAK N--AKLNFFY QQSAVNARLF FDL--LKGP- IATADLTVA-
Debaryomyces hansenii                                   VTSVIPNGPR N--AKLNFFY QQNAVNARLF FDL--LKGP- IALADLSVG-
Pichia guilliermondii                                   VTSVVPNGAR N--AKLNFFY QQNQVNARLF FDL--LKGP- IATADLAVA-
Phanerochaete chrysosporium                             NTSLAANGSG K-SALITTTY KQPGLHTRAF LDL--FKGP- TFTADTVIG-
Cryptococcus neoformans var. neoformans                 ATTLNPAKAS K-SAILTAIY KQPSLHTRAT VDL--FKGP- TFTADTVVG-
Antrodia cinnamomea                                     NTALTPDSGA K-TALLTSIY KQPGLHSRAF LDL--FKGP- TFTADTVLG-
Ustilago maydis[a]                                      VSTLVPEKQT K-NALFASTY KQPGLHTRQH LDL--FKGP- TFTADAVLG-
Schizosaccharomyces pombe                               NTTFSPATAA K-TAILNLEH QHPLIHTHAS VNA--LERK- -FLGDFTVG-
Saccharomyces cerevisiae POR1                           ITSLTPGVAK S--AVLNTTF TQPFFTARGA FDL-CLKSP- TFVGDLTMA-
Saccharomyces paradoxus POR1                            ITSLTPGVAK S--AVLNTTF TQPFFTARGA FDL-CLKSP- TFVGDLTMA-
Saccharomyces mikatae POR1                              ITSLTPGVAK S--AVLNTTF AQPFFTARGA FDL-CLKSP- TFVGDLTMA-
Saccharomyces kudriavzevi POR1                          ITSLIPGVAK S--AVLNTTF VQPFFTARGS FDL-CLKSP- TFVGDLTMA-
Saccharomyces bayanus POR1                              VTSFIPNGAK T--AQLNLSF VQPFFAAKGV FDL--FKTP- SFVGNLTLA-
Saccharomyces castellii POR1                            MTSFVPNISK V--ANLNLSF VQPFFTARGA FDL--LKGP- GFVGDMTLAH
Candida glabrata POR1                                   VTSCVPGTSN A--AKLNLSF VQPAFTARGF FDL--IKGP- SFVGDLTLA-
Eremothecium gossypii                                   ITSGVPGVSK A--AKLNVNF VQPLFAARGF FDL--LKGP- SFVGDLTLA-
Kluyveromyces lactis                                    NASLTPQSIK N--AKFNLSY AKKSLTARSS IDV--LQPK- DFVGSVTLG-
Saccharomyces paradoxus POR2                            NAFLTPQSIK N--AKFNLSY AQKSFAARTS IDI--LQPK- DFVGSVTLG-
Saccharomyces bayanus POR2                              NASLTPQAIK N--AKINLSY AQKSFTTRSS VDI--LQPK- DFVGSLTVG-
Saccharomyces cerevisiae POR2                           NACMTPQAIK N--AKVNLSF AQEFFTTRAS VDL--LHTK- DFVGNVTLA-
Candida glabrata POR2                                   TTNWVPGTVN IKSSRLGFNY FNALMNSKMS VDL--FNPT- KVVGSMTMG-
Ajellomyces capsulatus                                  LTQYLPNSQS K-GVKLNLYF KQPSFHARAF FDL--LKGP- SASFDAVLG-
Botryotinia fuckeliana                                  KIEINDSIAK G--LKAEILS FAPTTQKKGA KVN--LHFK- QPNFHGRAF
Saccharomyces kudriavzevi POR2                          NACLTPQAIK N--AKLNLSY AQQSFSARSS MDI--LQLK- DFVGSISLG-
Blastocladiella emersonii                               LGSLLPHIGQ K-NAKFSAEY KQDAVATRSS IDL--FKGP- IATADIAFN-
Metarhizium anisopliae                                  TSAAIEGKYT D--KPSGLTL TQTWNTANAL DTK--LEVND TLAKGLKLE-
Coprinopsis cinerea                                     ITGDLEAKYT D--KAHGLTV TQAWTTSNVL RNQ--VELEN QLAKGLKLD-
Hebeloma cylindrosporum                                 IHGDIEXKWT D--KVHGLTL TQTWTTSNVL RNQ--VELDN LMAKGLKLD-

                                                        ....|....160....|....170....|....180....|....190....|....200
Aspergillus nidulans                                    HEGFLVGAEG GYD-VQKAAI TKYSAAVGYS -VPQYSAAIQ AANNLTVFSA
Aspergillus fumigatus                                   HEGFLVGAEG GYD-VQKAAI TKYSAAVGYS -VPQYTAAIT AGNNLTVFSA
Neurospora crassa                                       HEGFLAGASA GYD-VQKAAI TGYSAAVGYH -APTYSAAIT ATDNLSVFSA
Magnaporthe grisea                                      HEGFLAGLSG GYN-VQKAAI TTYSAAVGYS -APQYTAAVT ATDNASVFAA
Gibberella zeae                                         HEGFLAGASA GYD-ANKAAI TAYSAAVGYA -APQYSAAIT ASDNLSVFSA
Yarrowia lipolytica                                     HDGFIAGAEV GYD-VSDAKV TKYSAAVGYT -APVYSTSIL ATNSLSVFSA
Coccidioides posadasii                                  HEGFLVGAEG GYD-VQKAAI TKYSAAVAYS -LPEYSAAIT ATNNLTLFSA
Mycosphaerella graminicola                              SDGFLVGGEA GYD-VQKAAI TRYSLGLGYQ -TPNYTASIV GTQNLSIIAA
Candida albicans                                        HDGFTAGAEL GYD-ISSAKV NKYSVGVGYA -NLNYGLAAT ATSNLSVFSA
Clavispora lusitaniae                                   HDGFTAGAEL GYD-IASAKL NKYSVGVGYA -NPLYTVGVT STSNLSVFTA
Debaryomyces hansenii                                   HDGFTAGAEL GYD-IASAKV NRYSLGVGYA -KPLYTVAAT ATNNLSIFSA
Pichia guilliermondii                                   HDGFTAGAEL GYD-INDATV NKYSVGVGYA -HPLYNVAAT ATSNLSVFTA
Phanerochaete chrysosporium                             RDGFLLGAEA SYN-VTEGKV TRYAGAVGFS -APEYAVTLQ GLNNLNLFSA
Cryptococcus neoformans var. neoformans                 RDGFLVGAEA SYD-VLSGAI TRYAGAVGFS -APEYAVTLH GLGNLSTFAA
Antrodia cinnamomea                                     RDGFLAGAEA SYD-VKAGKL SRYNLGAGFH -APDYAVTFL GLGNLSTYSA
Ustilago maydis[a]                                      HEGFLAGAEF GYD-VQKGNV SNYAATIGYL -ASPLSVALQ ASNNLSVFRA
Schizosaccharomyces pombe                               HEGIVGGAEF GYD-ISAGSI SRYAMALSYF -AKDYSLGAT LNNE-QITTV
Saccharomyces cerevisiae POR1                           HEGIVGGAEF GYD-ISAGSI SRYAMALSYF -AKDYSLGAT LNNE-QITTV
Saccharomyces paradoxus POR1                            HEGVVGGAEF GYD-ISAGSI SRYAMALSYF -AKDYSLGAT LNNE-QITTV
Saccharomyces mikatae POR1                              HEGVVGGAEF GYD-ISAGSI SRYAMALSYF -AKDYSLGAT LNND-QITTV
Saccharomyces kudriavzevi POR1                          HEGVVGGAEF GYD-ISAGSI SRYAMALSYF -AKDYSLGAT LNND-QITTV
Saccharomyces bayanus POR1                              HEGLVGGAEF GYD-ITGGTI SRYALALGYS -ARDYTLGIS INEA-QITSA
Saccharomyces castellii POR1                            ESGVVAGAQF GYD-IAAGSL SKYAIALGYA -AQDYHLGLN INND-QVTTA
Candida glabrata POR1                                   HKGFVGGAEV GYD-IAAATV SRYAVALGYR -TGLYSVALG VNNA-QITTA
Eremothecium gossypii                                   HEGFVGGAEV GYD-ISAGAV SRYALALGYN ASGAYSVGLS VNNA-QLITA
Kluyveromyces lactis                                    HRGFVGGTEI AYD-IAGGLL ARYAMSLGYL -SRDYSFILS TNNR-QCATA
Saccharomyces paradoxus POR2                            HRGFVGGTDI AYD-TAAGLC ARYAMSIGYL -AREYSFILS TNNR-QCATA
Saccharomyces bayanus POR2                              HGRFVGGSEV AYD-LLGRSL ARYAMSIGYL -SRDYSFVLS TNNR-QCAIA
Saccharomyces cerevisiae POR2                           HDGFVGGTEI AYD-IPGGAL ARYAVALGYL -ASDYSFVLS TNNK-QFTSA
Candida glabrata POR2                                   YGKMVGGSEV TCD-IAQNKF TRYALSMGIY -AGKRNLTFL INDA-HVMTL
Ajellomyces capsulatus                                  HEGFLVGAEG GYD-VQKAAI TKYSAAIAYC -VPEYSAAVT AQNNLSLFSA
Botryotinia fuckeliana                                  FDLLKGPTAT VDA-VIGQDG FLAGATAGYD -VQKAAITSY SAAVGYTAPT
Saccharomyces kudriavzevi POR2                          RDGFVGGTEI AYD-IPSGSL ARYAMSLGYL -ARDYSFVLS TNNR-QCASA
Blastocladiella emersonii                               HDGFLAGAET AYD-LATGHV SKYNVAFGYA -TREYSVALT GANKFDTFAG
Metarhizium anisopliae                                  GLFNFLPATA GKGAKFNLFF KQPGFHGRAF FDLLKGPVAN VDAV--VGHE
Coprinopsis cinerea                                     LTTSLAPEKG AKTAVLGGVF KQSGLHTRAV LDLFKGPTFT TDAV--VGRD
Hebeloma cylindrosporum                                 LATSLTPDKG SKSAILNAAY KQSGLHTRAA LDVFKGPTFT AXTV--LGRD
```

Multiple sequence alignment (residues ~205–300).

Block 1 (positions ~205–250):

```
                                              210        220        230        240        250
Aspergillus nidulans                 SYYHRVNA-- QVEAGAKATW DSKTGNSV-G LEVASKYRLD PSSFAK-V--
Aspergillus fumigatus                SYYHRVNQ-- QVEAGAKATW DSKAGNSV-G LEVASKYRLD PSSFAKSA--
Neurospora crassa                    SYYHKVNS-- QVEAGSKATW NSKTGNTV-G LEVATKYRID PVSFVK-V--
Magnaporthe grisea                   SYYHKVNS-- LVKAGAKASW NSKIGNSV-G LEVATKYRID PLSFVK-G--
Gibberella zeae                      SYYHKVNS-- QVEAGAKATW NSKTGNAV-G LEVASKYRID PVSFTK-V--
Yarrowia lipolytica                  AYFHRVNP-- ATEVGAKATW DSKAAAAPVG LEIGAKHTLD ANSFAK-A--
Coccidioides posadasii               SYYHRVNS-- QVEAGAKATW DSKAGNTV-G LEVASKYRLD PSSFAK-A--
Mycosphaerella graminicola           SYYQRVNT-- SVEFGAKAGY DVNA-QKASG LEIASKYKLD PLSFAK-A--
Candida albicans                     AYFHKVSP-- LVQVGAKATW DSIKSSNV-N VEFATKYALD NTSFIK-A--
Clavispora lusitaniae                AYYHKVSP-- LVEAGAKATW DSVKSSAV-N VEFATKYKLD ATTFVK-A--
Debaryomyces hansenii                AYYHKVSP-- LVEAGAKATW DSVKSSNV-N VEFATKYKLD STAFVK-A--
Pichia guilliermondii                SYYHKVSP-- LVEAGAKATW DSVKSSAV-N VEFATKYKLD STAFVK-A--
Phanerochaete chrysosporium          SYYHRVSP-- DVEAGAKATY DTKGTTQNVH LEVGAKAYLD NAAFVK-A--
Cryptococcus neoformans var.         SYYHKSK--  DVEAGAKAVY DTKSTAGNVS LEVGAKYLD  NAAFVK-A--
  neoformans
Antrodia cinnamomea                  SYYHRVNP-- DVEAGAKAIY DTKATTAGVS LEVGAKAYLD TAAFVK-A--
Ustilago maydis                      SYYHRVNA-- DTEAGARATY DSKSPNSNVN LEVGTKTYLD NAAFVK-A--
Schizosaccharomyces pombe            SYYHRVSS-- DVEAGGNVTW DAASTANAIT LELASKYALD KDTFVK-G--
Saccharomyces cerevisiae POR1        DFFQNVNA-- FLQVGAKATM NCKLPNSNVN IEFATRYLPD ASSQVK-A--
Saccharomyces paradoxus POR1         DFFQNVNA-- FLQVGAKATM NCKLPNSNVN IEFATRYLPD ASSQVK-A--
Saccharomyces mikatae POR1           DFFQNVNA-- FLQVGAKATM NCKLPNSNVN IEFATRYLPD ASSQVK-A--
Saccharomyces kudriavzevi POR1       DFFQNVNA-- FLQVGAKATM NCKLPNSNVN IEFATRYLPD ASSQVK-A--
Saccharomyces bayanus POR1           DFFQNVNA-- FLQVGAKATM NRKLPNANVN IEFATRYLPD ASSQVK-A--
Saccharomyces castellii POR1         SFYQSISK-- ILQVGAKATL N-PKKGSNVN IEFATKYNPD ATSQVK-A--
Candida glabrata POR1                TFFQNVSP-- QLQIGSRATL NPRQTVSNVN IEFATKYLPD PNSQVK-A--
Eremothecium gossypii                SFFQRVNP-- ALEVGAKAAL N-PAAGSKVN IEFATRYALD LTAQVK-A--
Kluyveromyces lactis                 SFFQKVSP-- ILQVGAKAAL N-PQAGSNVN IEFASKYALD AVSQVK-A--
Saccharomyces paradoxus POR2         TFFQNVNR-- FLQVGTKATL Q-SKENPNMN VEFVTRYVPD PISQVK-A--
Saccharomyces bayanus POR2           SFFQNVNR-- YLQVGTKATL Q-SKTSSNMN IEFVTRYVPD SISQVK-A--
Saccharomyces cerevisiae POR2        SFFQNVNR-- HLQVGAKATV Q-SKATPSMN IEFVTKYLPD STSQVK-A--
Candida glabrata POR2                TLYQKLSP-- QFEAAAKTTT RFNESNKT-S VEVATAYR-H NSAQYK-L--
Ajellomyces capsulatus               SYYHRVSP-- QVEACAKATW DSKAGNSV-G LEVGSKYRLD PSSFAK-V--
Botryotinia fuckeliana               YSAAITAA-- DNLSVFSASY YHKVNTQV-E AGAKATWDS K NTNSVG-L--
Saccharomyces kudriavzevi POR2       SFFQNINR-- YLQVGAKATV --QSKTRI-- -----I---- PPQW------
Blastocladiella emersonii            SYYHKVNK-- D--------- ---------- ---------- ----------
Metarhizium anisopliae               GFLAGASAGY DVNKAAVTAY SAAVGYAAPQ YSAAVTATDN LSVFAASYYH
Coprinopsis cinerea                  GFLAGAEASY NVTEGKVTRY AAALGYNAPE YAVTLHGLNN LSTFTASYYH
Hebeloma cylindrosporum              GFLVGAEAAY NVTGGNITRY AAAVGYNAPE YAVTLHGLNN FKTFSASYYH
```

Block 2 (positions ~250–300):

```
                                              260        270        280        290        300
Aspergillus nidulans                 KIN---DRGI AALAYNVLLR PGVTLGLGAS F--DTQNLNQ ----------
Aspergillus fumigatus                KIN---DRGI AALAYNVLLR PGVTLGLGAS F--DTQNLNQ ----------
Neurospora crassa                    KIN---DRGV AAIAYNVLLR EGVTLGVGAS F--DTQKLDQ ----------
Magnaporthe grisea                   KIN---DRGV AAVAYTTLLR PGVTFGIGAS F--DTQKLDQ ----------
Gibberella zeae                      KIN---DRGI AALAYNVLLR PGVTLGLGGS F--DTQKLDQ ----------
Yarrowia lipolytica                  KIN---NQGI AALAYSVGLK PGVRVGVGAS I--DTQRLEQ ----------
Coccidioides posadasii               KIN---DRGI AALAYNVLLR PGVTLGLGAS V--DTQNLNQ ----------
Mycosphaerella graminicola           KIN---DRGI AALAYSTKLN AGTTLGLGLS L--DTNKLNE ----------
Candida albicans                     KIA---DSGL TALSYTQELR PGVKLGLGAS F--DALKLAE ----------
Clavispora lusitaniae                KIA---DSGL TAVSYSQELR PGVRLGLGAS F--DALKLAE ----------
Debaryomyces hansenii                KIA---DSGL TALAYSQELR PGVRLGLGAS F--DALKLAE ----------
Pichia guilliermondii                KIA---DSGL AALSYSQLLR PGVTLGLGAS F--DALKLAE ----------
Phanerochaete chrysosporium          KIN---NAGI LGLGYTQALR PGVKASFGLA L--DTQRLNE --VG------
Cryptococcus neoformans var.         KIN---NAGV LSLGYTQALR PGVKASAGVS V--DTTRLNE --PQ------
  neoformans
Antrodia cinnamomea                  KIN---NSGI LALGYTQSLR PGVRASFGVA L--DTQRLND VTPS-G----
Ustilago maydis                      KIN---NAGI LCLGYTQALR PGVKASFGLA L--DTFKLSD --SA------
Schizosaccharomyces pombe            KIN---SAGV ATLSYFQTVR PGVTVGLGLQ L--DTQRLGQ ----------
Saccharomyces cerevisiae POR1        KVS---DSGI VTLAYKQLLR PGVTLGVGSS F--DALKLSE ----------
Saccharomyces paradoxus POR1         KVS---DSGI VTLAYKQLLR PGVTLGVGSS F--DALKLSE ----------
Saccharomyces mikatae POR1           KVS---DSGI VTLAYKQLLR PGVTLGVGSS F--DALKLSE ----------
Saccharomyces kudriavzevi POR1       KVS---DSGI VTLAYKQLLR PGVTLGVGSS F--DALKLSE ----------
Saccharomyces bayanus POR1           KVS---DSGI VTLAYKQLLR PGVTLGVGSS F--DALKLSE ----------
Saccharomyces castellii POR1         KIT---DAGL MTLSYKQLLK PGITLGVGTS L--DALKLNE ----------
Candida glabrata POR1                KIS---DAGI VALSYKQALR PGVTLGVGTS F--DALKLSE ----------
Eremothecium gossypii                KIA---DSGI VALSYKQLLR PGVELGVGAS F--DALKLAE ----------
Kluyveromyces lactis                 KVA---DSGI VALSYQQILR PGVTLGIGAS F--DALKLAE ----------
Saccharomyces paradoxus POR2         KIA---DSGL ATLSYKRDLN KDTSLGVGMS F--NALQLTD ----------
Saccharomyces bayanus POR2           KIA---DSGL TTLSYKRNLN KDISLGVGMS F--NALQLTE ----------
Saccharomyces cerevisiae POR2        KIT---DSGL ATLSYKQNLK KDISLGMGMS F--NALELAD ----------
Candida glabrata POR2                KLN---DAGL ACLSYKVPFQ KNISLGLGIS M--DVCNPT- ----------
Ajellomyces capsulatus               KVN---DRGI AALAYNVLLR PGVTLGLGAS M--DTQKLSQ ----------
Botryotinia fuckeliana               EVA---SKYR LDPLSFAKVK INDRGVAAVA Y--NVVLRPG ATLG-LGASF
Saccharomyces kudriavzevi POR2       --R---DS-- -------I S  ---------- ---------- ----------
Blastocladiella emersonii            ---------- ---------- ---------- ---------- ----------
Metarhizium anisopliae               KVNSQVEAGA KATWNSK-TG NAVGLEVASK YRIDPVSFAK VXINDRGIAA
Coprinopsis cinerea                  RINRDVQAGA KAVYDPKATH GGVALEVGSK VYLDAAAFVK AKINNSGVIA
Hebeloma cylindrosporum              RVSRDVEAGA KAIYDSKSTH G--------- ---------- ----------
```

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Multiple sequence alignment (positions 1–100)

Block 1 (positions 1–50):

```
                                              10         20         30         40         50
Homo sapiens VDAC1            - - - M A V P P T Y A D L G K S A R D V F T K G Y G F G L I  K L - D L K T K - - - S E N G - - L - E
Mus musculus VDAC1           - - - M A V P P T Y A D L G K S A R D V F T K G Y G F G L I  K L - D L K T K - - - S E N G - - L - E
Rattus norvegicus VDAC1      - - - M A V S P T Y T D L A K S A R D V F T K D Y G F G L I  K L - D L K T K - - - S E N R - - L - E
Xenopus laevis VDAC1         - - - M A I P P A Y V D L G K S A R D V F T K G Y G F G F I  K L - D L K T K - - - S E N G - - L - E
Xenopus tropicalis           - - - M A I P P A Y A D L G K S A R D I F T K G Y G F G F I  K L - D L K T K - - - S E N G - - L - E
Fundulus heteroclitus VDAC1  - - - L G R A P S Y V D L G K S A R D V F T K G Y G F G L I  K L - D L K T K - - - S D N G - - L - E
Oryzias latipes VDAC1        - - - M A V P P T Y A D L G K S A R D V F T K G Y G F G L I  K L - D L K T K - - - S E N G - - L - E
Danio rerio VDAC1            - - - M A V P P T Y A D L G K S A R D V F T K G Y G F G L I  K L - D L K T R - - - S E N G - - L - E
Petromyzon marinus           - - - M A V P P T F V D L G K S A R D V F S K G F G F G L V  K L - D L K T K - - - T S S G - - V - E
Tetraodon nigroviridis       - - - M T V P P A Y A D L G K S A K D I F N K G Y G F G L V  K L - D V K T K - - - S S T G - - V - E
Oryctolagus cuniculus VDAC3  - - - M C N T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L R T K - - - S C S G - - V - E
Sus scrofa VDAC3             - - - M C N T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L R T K - - - S C S G - - V - E
Bos taurus VDAC3             - - - M C N T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L R T K - - - S C S G - - V - E
Mus musculus VDAC3           - - - M C N T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L K T K - - - S C S G - - V - E
Homo sapiens VDAC3           - - - M C N T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L K T K - - - S C S G - - V - E
Rattus norvegicus VDAC3      - - - M C S T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L K T K - - - S C S G - - V - E
Pongo pygmaeus VDAC3         - - - M C N T P T Y C D L G K A A K D V F N K G Y G F G M V  K I - D L K T K - - - S C S G - - V M E
Gallus gallus VDAC3          - - - M A V P P S Y S D L G K A A R D V F N K G Y G F G M V  K L - E L K T K - - - S S S G - - V - E
Pimephales promelas          - - - M A V P P A Y A D L G K A A K D V F N K G Y G F G M V  K L - D V K T K - - - S A N G - - V - E
Argopecten irradians         - - - - M A P P S Y S D L G K A A K D L S D K G Y N F G L Y  K L - E C K T K - - - S S S G - - V - E
Spisula solidissima          - - - - M A P P A Y G D L G K A A K D L F S K G F N Y G S H  K L - E V K S K - - - T D S G - - V - K
Daphnia magna                - - - - M A P P V Y A D L G K S S R D V F G K G Y H F S L L  K L - E C K T K - - - T S N G - - V - E
Apis mellifera               - - - - - A P P S Y N D L G K S A R D L F S S G Y H F G L I  K L - D V K T K - - - T K S G - - V - E
Homalodisca coagulata        - - - - M A P P T Y G D L G K S A R D V F G K G Y H F G L W  K L - D V K T K - - - T S S G - - V - E
Anopheles gambiae            - - - - M A P P S Y S D L G K Q A R D V F N K G Y H F G L W  K L - D V K T K - - - T N S G - - V - E
Drosophila melanogaster      - - - - M A P P S Y S D L G K Q A R D I F S K G Y N F G L W  K L - D L K T K - - - T S S G - - I - E
Drosophila pseudoobscura PORB - - - - M A P P S Y S D L G K Q A R D I F S K G Y N F G L W  K L - D L K T K - - - T P S G - - I - E
Aedes aegypti                - - - - M A P P A Y A D L G K Q A R D V F N K G Y H F G L W  K L - D V K T K - - - T N S G - - V - E
Rhipicephalus appendiculatus - - - - M A P P C Y A D L G K Q A R D L F N K N Y H F G V V  K L - D C K S T - - - T Q T G - - V - E
Ciona intestinalis           - - M P V P P Q Y T D L G K S A K D L F E K G F G Y G F A  K V - D L K T K - - - T S T G - - V - E
Strongylocentrotus purpuratus - - - - - - - S Y S D L G K A A R D I F G K G F G F G F V  K L - D A K T T - - - T S N N - - V - G
Drosophila pseudoobscura PORA - - - - M A P P S Y P D L G K L A R D L F R R G Y H P G V W  Q L - D C K S M - - - S S P G - - I - E
Drosophila melanogaster      - - - A A K T P T Y P D L G K L A R D L F K R G Y H P G I W  Q I - D C K T L - - - T N S G - - I - E
Monodelphis domestica VDAC3  - - - M C T T P T Y C D L G K A A K D V F N K G Y G - - - -  - E F S - - - - - T S G H - - - - - S
Molgula tectiformis          - - - - K S - - - - - - - A R D L F D K G Y N Y G S N  K V - D V K S K - - - S I D G - - L - E
Macaca mulatta VDAC1         - - - M A V P P K F A D L G K S A R D V F T K G Y G F G L I  E L - D L K T K - - - E K N G - - L - E
Hydra magnipapillata         - - - - T R P D - - - - - - - - - - L I Q K N F H F G V F  K L - E A K T K - - - A K N G - - V - E
Schistosoma japonicum        - - - - M V P P S F S D L G K D A R D L F K K F Y F G V Y  N I - H C E T - - - - K K N N - - I - E
Drosophila melanogaster Porin B - - E G E M P T Y F H V G A L A K D C L I N G F K I G A W  Q M - H C S T R - - - T D N D - - F - Y
Drosophila pseudoobscura     - - - E G E M P T Y F H V G G A A K E C L I R G Y K F G V W  H L - R C S S K - - - M D N G - - V - V
Drosophila melanogaster Porin A - - E G E M P S Y F H V G L L A K M C L I H G Y T I G R W  K L - Q C T S K - - - T E K D - - F - Y
Fugu rubripes                - - - - - - - - - - - - - - - - - - - - E F - - - - - - - - - - T S T G - - - - -
Fugu rubripes                - - - - - - - - - - - - - - - - - - - - E F - - - - - - - - - - A T S G - - - - -
Xenopus tropicalis VDAC1     - - - M A V P P S Y A D L G K S A R D I F N K G Y G F G L V  K L - D V K T K - - - S A T G - - V - E
Xenopus tropicalis           - - - M A V P P S Y A D L G K S A R D I F N K G Y G F G L V  K L - D V K T K - - - S A T G - - V - E
Amblyomma variegatum         - - - - M A P P C Y A D L G K Q A R D L F N K N Y H F G V V  K L - D C K S T - - - T Q T G - - V - E
Branchiostoma floridae       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Pratylenchus penetrans       - - - - L G - - - - - - - - K A A R E L F T K G Y N F G S M  R V - D A C S R - - - S G E N H E V - E
Meloidogyne incognita        - - - - M A P P Q F T D F G K S T K D L F T K G Y T H G L L  K L - D V K S K - - - S G P N - - V - E
Lymnaea stagnalis            - - - - L S P P S F S D L G K S A R D V F S K G F N W G F F  K L - D A K T K - - - T Q N N - - V - E
Necator americanus           - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Ancylostoma ceylanicum       - - - - M A P P T F A D L G K S A K D L F N K G Y N H G F L  K V - D S T T K - - - A G D S K E V - E
Nippostrongylus brasiliensis - - - - M A P P T F A D L G K S A K D L F N K G Y T H G F L  K V - D A T T R - - - A G D N K E V - E
Parastrongyloides trichosuri - - - R T V F N - - - - - - - - - - - - K G Y F P G Y L  K V - E T T S N - - - S G D N K E V - E
Tribolium castaneum          - - - - M A P P P - - - - - - - - - - - Y S D L G K K  A K - D V F G - - - - K G Y H - - F - G
Ambystoma tigrinum           - - - - M A I P P T - - - - - - - - - - Y V D L G K A  A R - D V F A - - - - K G Y G - - F - G
Spermophilus lateralis       - - - - - - - - - - - - - - - - - - - Y A D L G K S  A R - D V F T - - - - K G Y G - - F - G
Ixodes scapularis            - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
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Block 2 (positions 51–100):

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                                              60         70         80         90        100
Ancylostoma caninum          - S H - - - - N L G S G K L G G N L D V K Y K I P A Y G L T L T E K W N T E N Q L G - T I I E V N E
Caenorhabditis elegans VDAC2 F K S A A S H N I G S G K L G G N L D V K Y K I P Q Y G I T L T E K W N T E N Q L G - T V I E V N E
Caenorhabditis briggsae      F K S N A S H N L G S G K L G G N L D V K Y K I P Q Y G I T L T E K W N T E N Q L G - T V V E I N E
Caenorhabditis elegans       F K S A A S H N I G S G K L G G N L D V K Y K I P Q Y G I T L T E K W N T E N Q L G - T V I E V N E
Ascaris suum                 F K T G A V H T I A S G R M A G N L D V K Y K I P S Y G I T L T E K W N T D N M L G - T I I E V Q D
Haemonchus contortus         F K T S A A H N L G S G K L G G N L D V K Y K I P S Y G L T L T E K W N T E N Q L G - T I I E V N E
Monodelphis domestica VDAC2  F S S S G S S N T D T G K V S G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Macaca mulatta VDAC2         F S T S G S S N T D T G K V T G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Bos taurus VDAC2             F S T S G S S N T D T G K V T G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Rattus norvegicus VDAC2      F S T S G S S N T D T G K V S G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Mus musculus VDAC2           F S T S G S S N T D T G K V S G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Homo sapiens VDAC2           F S T S G S S N T D T G K V T G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Sus scrofa VDAC2             F S T S G S S N T D T G K V T G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Homo sapiens VDAC2           F S T S G S S N T D T G K V T G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
Gallus gallus VDAC2          F T T S G S S N T D T G K V N G S L E T K Y K W A E Y G L T F T E K W N T D N T L G - T E I A I E D
Meleagris gallopavo VDAC2    F T T S G S S N T D T G K V N G S L E T K Y K W A E Y G L T F T E K W N T D N T L G - T E I A I E D
Pan troglodytes VDAC2        F S T S G S S N T D T G K V T G T L E T K Y K W C E Y G L T F T E K W N T D N T L G - T E I A I E D
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250

Multiple sequence alignment (residues ~60–100)

Species	Sequence
Gasterosteus aculeatus	FKTTGSSNTD TSKVAGNLET KYKRAEYGLT FTEKWNTDNT LG-TEITVED
Fugu rubripes	FKTSGSSNTD TSKVAGSLET KYKRPEYGLT FTEKWNTDNT LG-TEITVED
Fugu rubripes	FKTSGSSNTD TSKVAGSLET KYKRPEYGLT FTEKWNTDNT LG-TEITVED
Tetraodon nigroviridis	FKTSGSSNTD TSKVAGSLET KYKRPEYGLT FTEKWNTDNT LG-TEITVED
Ictalurus punctatus	FKTSGSSNTD TSKVVGSLET KYKRSEYGLT FTEKWNTDNT LG-TEITIED
Oncorhynchus mykiss	FKTAGSTNTD TSKVAGSLET KYKRSEYGLT FTEKWNTDNT LG-TEITVED
Oryzias latipes VDAC2	FKTSGSSNTD TSKVSGTLET KYKRAEYGLT FTEKWNTDNT LG-TEITIED
Danio rerio	FKTSGSSNTD TSKVAGSLET KYKRSEYGLT FTEKWNTDNT LG-TEINIED
Xenopus laevis VDAC2	FTTSGTSNTD SGKVNGSLET KYKWGEYGLT FTEKWNTDNT LG-TEIAIED
Fugu rubripes	FKTSGSSNID NSKVTGTLET KYKWAEYGLT FTEKWSTENT LG-TEVCVED
Fugu rubripes	FATSGSSNTD TGKSGGHLET KYKMKELGLN FSQKWNTNNT LT-TEVTMED
Squalus acanthias	FTTSGSSNTD TGKATGSLET KYKLKEYGLT FTEKWNTDNN LA-TEITIED
Danio rerio	FTTGGSSNTD TGKAAGNLET KYKVKELGLS LNQKWNTDNV LT-TEVTLED
Salmo salar	FATSGSSNTD TGKAAGNLET KYKVKELGLS FTQKWNTDNT LT-TEVSMED
Monodelphis domestica VDAC1	FTSSGSANTE TSKVSGSLET KYKWSEYGLT FIEKWNTDNT LG-TEITVED
Gallus gallus VDAC1	FTSSGSANSE TSKVSGSLET KYRWVEYGLM FTEKWNTDNT LG-TEITLED
Rattus norvegicus VDAC1	FTSSGSANTE TTKVTGSLET KYRWTEYGLT FTEKWNTDNT LG-SEITVED
Bos taurus VDAC1	FTSSGSANTE TTKVTGSLET KYRWTEYGLT FTEKWNTDNT LG-TEITVED
Sus scrofa VDAC1	FTSSGSANTE TTKVTGSLET KYRWTEYGLT FTEKWNTDNT LG-TEITVED
Homo sapiens VDAC1	FTSSGSANTE TTKVTGSLET KYRWTEYGLT FTEKWNTDNT LG-TEITVED
Mus musculus VDAC1	FTSSGSANTE TTKVNGSLET KYRWTEYGLT FTEKWNTDNT LG-TEITVED
Rattus norvegicus VDAC1	FTSSVSANTE TTKVNCSLET KYRWTEYGVT FTEKWNTDNT LG-TETTVED
Xenopus laevis VDAC1	FTSSGSANAE TSKVSGNLET KYKWAEYGLT FTEKWNTDNT LG-TEITVED
Xenopus tropicalis	FTSSGSANAE TNKVAGSLET KYKWAEYGLT FTEKWNTDNT LG-TEITVED
Fundulus heteroclitus VDAC1	FTSTCSGNTE TSKVAGSLET KYKWAEHGLT FTEKWNTDNT LG-TEITVED
Oryzias latipes VDAC1	FTSTGSANTE TSKVAGSLET KYKWAEHGLT FTEKWNTDNT LG-TEITVED
Danio rerio VDAC1	FKSSGSANTE TSKVAGTLET KYKWAEHGLT FTEKWNTDNT LG-TEITLED
Petromyzon marinus	FSTSGSSNSE TGKVNASLET KYKWADYGLN LTEKWSTDNT LA-TEISVED
Tetraodon nigroviridis	FKTSGSSNID NSKVTGNLET KYKWAEYGLT FTEKWSTENT LG-TEVCFED
Oryctolagus cuniculus VDAC3	FSTSGHAYTD TGKASGNLET KYKVCNYGLT FTQKWNTDNT LG-TEISLEN
Sus scrofa VDAC3	FSTSGHAYTD TGKASGNLET KYKICDHGLT FTQKWNTDNT LG-TEISLEN
Bos taurus VDAC3	FSTSGHAYTD TGKASGNLET KYKICNYGLT FTQKWNTDNT LG-TEISWEN
Mus musculus VDAC3	FSTSGHAYTD TGKASGNLET KYKVCNYGLT FTQKWNTDNT LG-TEISWEN
Homo sapiens VDAC3	FSTSGHAYTD TGKASGNLET KYKVCNYGLT FTQKWNTDNT LG-TEISWEN
Rattus norvegicus VDAC3	FSTSGHAYTD TGKASGNLET KYKVCNYGLI FTQKWNTDNT LG-TEISWEN
Pongo pygmaeus VDAC3	FSTSGHAYTD TGKASGNLET KYKVRNYGLT FTQKWNTDNT LG-TEISWEN
Gallus gallus VDAC3	FTATGSSNTD TGKALGNLET KYKIKDYGLT FIQKWNTDNT LG-TEVSMED
Pimephales promelas	FKTCGTSNMD SNKVSGNLET KYKWAEYGLT FTEKWNTDNT LG-TEITVED
Argopecten irradians	FTTKGSSHD SGKVSGSLET KYKLPEHGLT FVEKWNTDNV LA-TEITIED
Spisula solidissima	FTTKGSHNTD TGRVSGELTT EYKCSDYGTT FKETWNTDNV LS-TEFTIED
Daphnia magna	FTTGGSSNLD SGKVVGNLET KYKVPEYGLT FTEKWNTDNT LG-TEIAIQD
Apis mellifera	FSSGGVSNQD TGKVFGSLET KYNIDDYGLK FSEKWNTDNT LA-TDITFAD
Homalodisca coagulata	FSTGGVSNQE TGKVFGTLET KYKLKDYGVT FTEKWNTDNV LV-TEVS-AQ
Anopheles gambiae	FSTSGHSNQD TGKVFGSLET KYKVKEYGLN FSEKWNTDNT LT-SEVSVEN
Drosophila melanogaster	FNTAGHSNQE SGKVFGSLET KYKVKDYGLT LTEKWNTDNT LF-TEVAVQD
Drosophila pseudoobscura PORB	FNTAGHSNQE SGKVFGSLET KYKVKDYGLT LTEKWNTDNT LF-TEVAVQD
Aedes aegypti	FNTSGSSNQD NGKVFGSLET KYKVKEYGLN FSEKWNTDNT LT-SEVSVEN
Rhipicephalus appendiculatus	FNVSGTSLND SGKVNASLET KYKVPEYGLT LKEKWNTDNT LS-TEICTEE
Ciona intestinalis	FTTKGASCNE SGNINGSLET KYKQPKHGLT FTEKWTTDNN LS-TEVAIED
Strongylocentrotus purpuratus	FTVSSASNND TGKVDASLET KYSWKEYGLS FKEKWNTDNT LA-TEVTIED
Drosophila pseudoobscura PORA	FFTTGFASQD HSKVAGSLQS KYKIEDSGLT LTERWNTDSW FF-GEIVQKD
Drosophila melanogaster	FFTTGFASQD NSKVTGSLQS KYKIEDQGLT LTERWNTENW LF-GEIMHRD
Monodelphis domestica VDAC3	Y------TD TGKASGNLET KYKICDYGLT FIQKWNTDNT LG-TEISMEN
Molgula tectiformis	FTTKVSHSRQ TDQMTGSLES KYKRPANGLT FTEKWTTGNN IS-TEVAVEN
Macaca mulatta VDAC1	FTSSGSANSE TTKVTGRLET KYRWTEYGLT FTEK-NTDNT PG-TEVTVED
Hydra magnipapillata	FTAEGSHTTD TGNVAGSLET KFKYADYGLT FSEKWSTDNV IA-TTISIDN
Schistosoma japonicum	YKSNLSDGPR PNKMYFDLQE KLAFPQYGFA ITKKWSSNNV ID-GEIVFED
Drosophila melanogaster Porin B	LNTFGEGYPT MKNVFGGMEV FKEVGN--YS TSLGWFTNND LL-SEIAVRG
Drosophila pseudoobscura	LSSFGEGNPL LTSVFGGLEV FKEFEN--SH TSLTWVTSSN EFRGELGVRG
Drosophila melanogaster Porin A	LSSFGEGYPT WNTVYGGLEA YKESGN--FH ASLAWLSDGD LL-SDLGVHG
Fugu rubripes	-SAN----TE TSKVSGSLET KYKWAEHGLT FTEKWNTDNT LG-TEITLED
Fugu rubripes	-SSN----TD TGKSGGHLET KYKMKELGLN FSQKWNTNNT LT-TEVTMED
Xenopus tropicalis VDAC1	FATSGSSNTD SGKVNGSLET KYKWGEYGLT FTEKWNTDNT LG-TEIAIED
Xenopus tropicalis	FATSGSSNTD SGKVNGSLET KYKWGEYGLT FTEKWNTDNT LG-TEIAIED
Amblyomma variegatum	FNVSGTSLND TGKVNASLET KYKVPEYGLT FKEKWNTDNT LA-TEICSEE
Branchiostoma floridae	-------------------DYGLT FTEKWNTDNT LA-SEIKIED
Pratylenchus penetrans	FSSAAVHTVA TGKLAGNFDV KYRIPSYGVV LTEKWNTENL LG-TIIEIQD
Meloidogyne incognita	FKSEGEHNLS TQKIKNKLEV KYKIPEYGTT ITESLNTANQ LG-SILELNN
Lymnaea stagnalis	FKASLSSNRD SGKVAGSLET KYKWSEYGLT FTEKWNTENV LN-TEIKIED
Necator americanus	-------SG KL--GGNLDV KYKIPSYGLT LTEKWNTENQ LG-TIIEVNE
Ancylostoma ceylanicum	FKTSASHNLG SGKLGGNLDV KYKIPAYGLT LTEKWNTENQ LG-TIIEVNE
Nippostrongylus brasiliensis	FKTSAAHNLG SGKLGGNLDV KYKIPAYGLT LTEKWNTENQ LG-TIIEVNE
Parastrongyloides trichosuri	FKTNASHNIG SGKLGGNLDV KYKIPQYGLT LTEKWNTENQ LG-TVVEINE
Tribolium castaneum	LIKLDCKTKT G--SG-VEF NTGGVSN--Q ESGK------ ---VFGSLET
Ambystoma tigrinum	LIKLDVKTKS E---NG-LEF TSSGSAN--S ETSK------ ---VTGSLET
Spermophilus lateralis	LIKLDLKTKS E----NG-LEF TSSGSAN--T ETTK------ ---VTGSLET
Ixodes scapularis	----------G-VEF NVSGTSL--X DTGK------ ---VNASLET

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                                              110         120         130         140         150
Ancylostoma caninum                QFGRGL-KLT FDSLY APHAG KR----T GKL KADWS LQTAR --I TAEVG LT
Caenorhabditis elegans VDAC2       QFGRGL-KVT LDSLY APHAG KR----S GKV KLDWA --LPT ARVTADV GVT
Caenorhabditis briggsae            QFGRGV-KVT LDSLY APHAG KR----S GKV KLDWA --LPT ARI TADV GVT
Caenorhabditis elegans             QFGRGV-KVT LDSLY APHAG KR----S GKV KLDWA --LPT ARI TADV GVT
Ascaris suum                       QFARGL-KLT LDSSY APQVG KR----T GKV KAEWA --AES AKVTCDL GLD
Haemonchus contortus               QFGRGL-KLT FDSLY APHAG KR----T GKL KADWS --LNT ARI TGEV GIT
Monodelphis domestica VDAC2        QICQGL-KLT FDTTF SPNTG KK----S GKI KSAYK RECIN LGCDI DFDFA
Macaca mulatta VDAC2               QICQGL-KLT FDTTF SPNTG KK----S GKI KSSYK RECIN LGCDVDFDFA
Bos taurus VDAC2                   QICQGL-KLT FDTTF SPNTG KK----S GKI KSSYK RECIN LGCDVDFDFA
Rattus norvegicus VDAC2            QICQGL-KLT FDTTF SPNTG KK----S GKI KSAYK RECIN LGCDVDFDFA
Mus musculus VDAC2                 QICQGL-KLT FDTTF SPNTG KK----S GKI KSAYK RECIN LGCDVDFDFA
Homo sapiens VDAC2                 QICQGL-KLT FDTTF SPNTG KK----S GKI KSSYK RECIN LGCDVDFDFA
Sus scrofa VDAC2                   QICQGL-KLT FDTTF SPNTG KK----S GKI KSSYK RECVN LGCDVDFDFA
Homo sapiens VDAC2                 QICQGL-KLT FDTTF SPNTG KK----S GKI KSSYK RECIN LGCDVDFDFA
Gallus gallus VDAC2                QIAKGL-KLT FDTTF SPNTG KK----S GKI KSAYK RECLN LGCDVDFDFA
Meleagris gallopavo VDAC2          QIAKGL-KLT FDTTF SPNTG KK----S GKI KSAYK RECLN LGCDVDFDFA
Pan troglodytes VDAC2              QICQGL-KLT FDTTF SPNTG KK----S GKI KSSYK RECIN LGCDVDFDFA
Gasterosteus aculeatus             QIAKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REYVN VGCDVDFDFA
Fugu rubripes                      QIAKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REYVN LGCDVDFDFA
Fugu rubripes                      QIAKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REYVN LGCDVDFDFA
Tetraodon nigroviridis             QIAKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REYVN LGCDVDFDFA
Ictalurus punctatus                QIAKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REYVN LGCDVDFDFA
Oncorhynchus mykiss                QIVKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REFVN LGCDVDFDFA
Oryzias latipes VDAC2              QIAKGL-KLT FDTTF SPNTG KK----S GKV KAAYK REYVN LGCDVDFDFA
Danio rerio                        QIAKGL-KLT FDTTF SPNTG KK----S GKV KTAYK REFVN LGCDVDFDFA
Xenopus laevis VDAC2               QIAKGL-KLT FDTTF SPNTG KK----S GKV KAAYK QEYVN LGCDVDFDFA
Fugu rubripes                      QITKGL-KLN FETTF SPNTG KK----S GKI KTAYK REYVN AGLDVDLDFA
Fugu rubripes                      QLAKGL-KLG LDTSF VPNTG KK----S AKL KTSYK RDFVN VGCDLDFDMA
Squalus acanthias                  QLAKGL-KLT FDTTF VPNTG KK----S GKL KTAYK RDYVN LGCDI DFDFA
Danio rerio                        QLAKGL-KLG LDTSF VPNTG KK----S AKL KTGYK REYMN VGCDLDFDLA
Salmo salar                        QLAKGL-KLA LDSSF VPNTG KK----S AKL KTGYK RDFIN LGCDLDFDMA
Monodelphis domestica VDAC1        QLARGL-KLT FDSSF SPNTG KK----N AKI KSGYK REHIN LGCDMDFDIS
Gallus gallus VDAC1                QLARGL-KLT FDSSF SPNTG KK----S AKI KTGYK REHIN MGCDMDFDIA
Rattus norvegicus VDAC1            QLARGL-KLT FDSSF SPNTG KK----N AKI KTGYK REHIN LGCDMDFDIA
Bos taurus VDAC1                   QLARGL-KLT FDSSF SPNTG KK----N AKI KTGYK REHIN LGCDVDFDIA
Sus scrofa VDAC1                   QLARGL-KLT FDSSF SPNTG KK----N AKI KTGYK REHVN LGCDVDFDIA
Homo sapiens VDAC1                 QLARGL-KLT FDSSF SPNTG KK----N AKI KTGYK REHIN LGCDMDFDIA
Mus musculus VDAC1                 QLARGL-KLT FDSSF SPNTG KK----N AKI KTGYK REHIN LGCDVDFDIA
Rattus norvegicus VDAC1            QLARGL-KLT FDSSF SPNTG EK----N AKI KTGYK REHIN LGCDVDFDIT
Xenopus laevis VDAC1               QLAKGL-KLT FDSSF SPNTG KK----N AKV KSAYK REHLN VGCDMDFDIA
Xenopus tropicalis                 QLAKGL-KLT FDSSF SPNTG KK----N AKV KTAYK REHLN IGCDMDFDIA
Fundulus heteroclitus VDAC1        QLLKGL-KLT LDSSF SPNTG KK----G GKI KTAYK CDHIN LGCDVNYDIN
Oryzias latipes VDAC1              QLAKGL-KLT LDSSF SPNTG KK----G GKV KTAYK CDHVN VGCDVNYDIN
Danio rerio VDAC1                  QLTKGL-KLT FDSSF SPNTG KK----S GKI KSSYQ REHIN LGCDVDYDIN
Petromyzon marinus                 QLAKGL-KLT LDTTF APNTG KK----S GKL KSTYK REYLN LGCDVDLDFA
Tetraodon nigroviridis             QITKGL-KLN FETTF SPNTG KK----S GKL KTAYK REYLN AGLDVDLDFA
Oryctolagus cuniculus VDAC3        KLAEGL-KLT LDTIF VPNTG KK----S GKL KASYK RDCFS LGSNVDIDFS
Sus scrofa VDAC3                   KLAEGL-KLT LDTIF VPNTG KK----S GKL KASYK RECFS LGSNVDIDFA
Bos taurus VDAC3                   KLAEGL-KLT LDTIF VPNTG KK----S GKL KASYK RDCFS LGSNVDIDFS
Mus musculus VDAC3                 KLAEGL-KLT LDTIF VPNTG KK----S GKL KASYR RDCFS LGSNVDIDFS
Homo sapiens VDAC3                 KLAEGL-KLT LDTIF VPNTG KK----S GKL KASYK RDCFS VGSNVDIDFS
Rattus norvegicus VDAC3            KLAEGL-KLT VDTIF VPNTG KK----S GKL KASYR RDCFS VGSNVDIDFS
Pongo pygmaeus VDAC3               KLAEGL-KLT LDTIF VPNTG KK----S GKL KASYK RDCFS VGSNVDIDFS
Gallus gallus VDAC3                QLAEGL-KVA LDTIF VPNTG KK----S GKL KTSYR RDYVN LGCNI DIDLS
Pimephales promelas                QITKGL-KLT FDTTF SPNTG KK----S GKV KSAYK REYLN VGCDVDFDFA
Argopecten irradians               QLCKGL-KLA FESSF APQTG KK----N GQI KTAYK MDYLN ASCDVDFDFA
Spisula solidissima                QLAQGL-KLA FDTSF APQTG KK----S GKV KTGYK NDYVN MNCDVDFDFS
Daphnia magna                      KIAQGL-KLT FDSTF APQTG KK----T GVV KAEFK HDTAT LNADVD--LN
Apis mellifera                     KLLKGL-TLG YGCTF SYQTG TK----T GKL KTAYK HDNVS AAADFDLSLS
Homalodisca coagulata              DFMKGV-KVS LDTSF KPQTG DK----S LKA KTEFR NQTVS VNCDLD-FKA
Anopheles gambiae                  QLVKGL-KVS FDGMF VPHTG SK----T GRF KTAYS HDRVR VDADFNVDLS
Drosophila melanogaster            QLLEGL-KLS LEGNF APQSG NK----N GKF KVAYG HENVK ADSDVNI DLK
Drosophila pseudoobscura PORB      QLLEGL-KLS LEGNF APQSG NK----N GKF KVGFG HEYVK ADSDVNI DLK
Aedes aegypti                      QLVKGL-KLS FDGSF APQTG SK----T GRF KTAYS HDKVR VDADFNVDLA
Rhipicephalus appendiculatus       KLARGL-KVA FNANF APQTG KK----S GAL KAAYK LENVH LNGDVDLGPP
Ciona intestinalis                 QIATGM-KLT LCTSF APNTG KK----S GAL KTAYK RDYIN CNLDTDFNFA
Strongylocentrotus purpuratus      QIAQGV-KLT LDTSF SPQTG KK----S GQI KTAYK RDFIN ITTDVDFDFA
Drosophila pseudoobscura PORA      KLAQGL-MLG LEGRF QPSS- -E----D GKF KLGYV --EDH FNFLTDMGIS
Drosophila melanogaster            KLAQGL-MLA VEAKF QPGSN EA----D GKF KMGYA --QDN FNFLADIGLN
Monodelphis domestica VDAC3        KLADGL-KLT LDTIF VPNTG KK----S GKL KASYR RECFS LGGNVDIDFS
Molgula tectiformis                QLCNGS-KVT LCSAF SPDTG KK----S GAL KTEYK RDYFH LNLNTDFDFA
Macaca mulatta VDAC1               QLSHEL-KVT FNSSF SPNTG EK----M LKS RQGTS --RST LTWAETWILT
Hydra magnipapillata               KIAQGV-KVD FDTTF APVTG KK----S AKV KSAYA HENLH ATTDI DFDFA
Schistosoma japonicum              KLVDGL-KQT FQISR DPFKK CF----N ANL INSFR NDHVN SNVEMFFKSA
Drosophila melanogaster Porin B    MNFGSRLYGL LKSTI GTKDE VSF---QTKL KCGLE --RDP VKVELVVPLY
Drosophila pseudoobscura           LVAGGI-AHSI LRSAL SMTDE HQFSI GSSLL KAGFM--RSP FNI EI VVPIV
Drosophila melanogaster Porin A    DGLGGTWSTV LKSMV SYPEG RKF---QCKL KCGFD--RNP GKVEMYI PIY
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Alignment block 1 (positions ~110–150):

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                                        .....  110 .... 120 .... 130 .... 140 .... 150
Pimephales promelas                QITKGL-KLT FDTTFSPNTG KK----SGKV KSAYKREYLN VGCDVDFDFA
Argopecten irradians               QLCKGL-KLA FESSFAPQTG KK----NGQI KTAYKMDYLN ASCDVDFDFA
Spisula solidissima                QLAQGL-KLA FDTSFAPQTG KK----TGVV KTGYKNDYVN MNCDVDFDFS
Daphnia magna                      KIAQGL-KLT FDSTFAPQTG KK----TGVV KAEFKHDTAT LNADVD--LN
Apis mellifera                     KLLKGL-TLG YGCTFSPQTG TK----TGKL KTAYKHDNVS AAADFDLSLS
Homalodisca coagulata              DFMKGV-KVS LDTSFKPQTG DK----SLKA KTEFRNQTVS VNCDLD-FKA
Anopheles gambiae                  QLVKGL-KVS FDGMFVPHTG SK----TGRF KTAYSHDRVR VDADFNVDLS
Drosophila melanogaster            QLLEGL-KLS LEGNFAPQSG NK----NGKF KVAYGHENVK ADSDVNIDLK
Drosophila pseudoobscura PORB      QLLEGL-KLS LEGNFAPQSG NK----NGKF KVGFGHEYVK ADSDVNIDLK
Aedes aegypti                      QLVKGL-KLS FDGSFAPQTG SK----TGRF KTAYSHDKVR VDADVNVDLA
Rhipicephalus appendiculatus       KLARGL-KVA FNANFAPQTG KK----SGAL KAAYKLENVH LNGDVDLGPP
Ciona intestinalis                 QIATGM-KLT LCTSFAPNTG KK----SGAL KTAYKRDYIN CNLDTDFNFA
Strongylocentrotus purpuratus      QIAQGV-KLT LDTSFSPQTG KK----SGQI KTAYKRDFIN ITTDVDFDFA
Drosophila pseudoobscura PORA      KLAQGL-MLG LEGRFQPSS- -E----DGKF KLGYV--EDH FNFLTDMGIS
Drosophila melanogaster            KLAQGL-MLA VEAKFQPGSN EA----DGKF KMGYA--QDN FNFLADIGLN
Monodelphis domestica VDAC3        KLADGL-KLT LDTIFVPNTG KK----SGKL KASYRRECFS LGGNVDIDFS
Molgula tectiformis                QLCNGS-KVT LCSAFSPDTG KK----SGAL KTEYKRDYFH LNLNTDFDFA
Macaca mulatta VDAC1               QLSHEL-KVT FNSSFSPNTG EK----MLKS RQGTS--RST LTWAETWILT
Hydra magnipapillata               KIAQGV-KVD FDTTFAPVTG KK----SAKV KSAYAHENLH ATTDIDFDFA
Schistosoma japonicum              KLVDGL-KQT FQISRDPFKK CF----NANL INSFRNDHVN SNVEMFFKSA
Drosophila melanogaster Porin B    MNFGSRLYGL LKSTIGTKDE VSF---QTKL KCGLE--RDP VKVELVVPLY
Drosophila pseudoobscura           LVAGGIAHSI LRSALSMTDE HQFSIGSSLL KAGFM--RSP FNIEIVVPIV
Drosophila melanogaster Porin A    DGLGGTWSTV LKSMVSYPEG RKF---QCKL KCGFD--RNP GKVEMYIPIY
Fugu rubripes                      QLTKGL-KLT FDSSFSPNTG KK----SGKI KTAYKCDHLN LGCDVNYDIN
Fugu rubripes                      QLAKGL-KLG LDTSFVPNTG KK----SAKL KTSYKRDFVN VGCDLDFDMA
Xenopus tropicalis VDAC1           QIAKGL-KLT FDTTFSPNTG KK----SGKV KAAYKREYVN LGCDVDFDF-
Xenopus tropicalis                 QIAKGL-KLT FDTTFSPNTG KK----SGKV KAAYKREYVN LGCDVDFDF-
Amblyomma variegatum               KLARGL-KVA FHANFAPQTG KK----SGAL RTAYKLE--N VHLNGDVDLG
Branchiostoma floridae             QIAKGL-SLT FDTKFSPSTG KK----SGQI KTAYKMDYLN VGADVDLDFA
Pratylenchus penetrans             RFARGL-KLT LDSWYAPHNG KR----SGRV KAEWANRNI- -TCNLDVGLD
Meloidogyne incognita              QLAKGL-KVT IDTKYTPNSG KR----ECLV KTDFSGDLV- -RLNANLSLV
Lymnaea stagnalis                  QLAEGL-ELT FDTSFAPTTG KK----SGKV KTAYKMDYVH LNCDVDFDFA
Necator americanus                 QFGRGL-KLT FDSVYAPHAG KR----TGKL KADWSLQTA- -RLTAEVGLT
Ancylostoma ceylanicum             QFGRGL-KLT FDSLYAPHAG KR----TGKL KADWSLQTA- -RITAEVGLT
Nippostrongylus brasiliensis       QFGRGL-KLT FESVYAPHAG KR----TGKL KADWSLNTA- -RITADCSLT
Parastrongyloides trichosuri       QFGRGL-KLT FDSVYAPHAG KR----SGKL KADWALPTA- -RITADVALT
Tribolium castaneum                KYKVKEYGLT FSE--KWNT- -------DN TLATEVAIQ- ---DQLL---
Ambystoma tigrinum                 KYKWAEYGLT FTE--KWNT- -------DN TLGTEITIE- ---DQLA---
Spermophilus lateralis             KYRWTEYGLT FTE--KWNT- -------DN TLGTEITVE- ---DQLA---
Ixodes scapularis                  KYKVSDLGLT LKE--KWNT- -------DN TLATEISCD- ---DQLA---
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Alignment block 2 (positions ~160–200):

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                                              .....  160 .... 170 .... 180 .... 190 .... 200
Ancylostoma caninum               -SAP-VI--N AAGVFS-REG WLFGAAASFD TATNKLASTS LAFGHQTNSY
Caenorhabditis elegans VDAC2      -SAP-VI--N AAGVFS-RDG WLIGAAATFD SSSNKLAATS LAFGHSTPQY
Caenorhabditis briggsae           -SAP-VV--N ASGVFA-RDG WLIGAAATFD SQSNKLAATS LAFGHSTPQY
Caenorhabditis elegans            -AAP-VI--N AAGVFA-RDG WLIGAAATFD SSSNKLAATS LAFGHATPQY
Ascaris suum                      -VGP-IL--N VAAVAA-HQG WLLGLQTSFD AASSRLRGTN VAFGRTGPNY
Haemonchus contortus              -AAP-VI--N AAGVFG-RDG WLFGASATFD TSSNKVSTTA LAFGHQTSHY
Monodelphis domestica VDAC2       --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Macaca mulatta VDAC2              --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Bos taurus VDAC2                  --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Rattus norvegicus VDAC2          --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRSN FAVGYRTGDF
Mus musculus VDAC2               --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRSN FAVGYRTGDF
Homo sapiens VDAC2               --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Sus scrofa VDAC2                 --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Homo sapiens VDAC2               --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Gallus gallus VDAC2              --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FSVGYRTGDF
Meleagris gallopavo VDAC2        --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FSVGYKTGDF
Pan troglodytes VDAC2            --GP-AI--H GSAVFG-YEG WLAGYQMTFD SAKSKLTRNN FAVGYRTGDF
Gasterosteus aculeatus          --GP-TI--H GAAVVG-YEG WLAGYQMSFD TAKSKMTQNN FAIGYKTGDF
Fugu rubripes                    --GP-TI--H GAAVVG-YEG WLAGYQMTFD TAKSKMSQSN FAIGYKTGDF
Fugu rubripes                    --GP-TI--H GAAVVG-YEG WLAGYQMTFD TAKSKMSQSN FAIGYKTGDF
Tetraodon nigroviridis          --GP-TI--H GAAVVG-YEG WLAGYQMTFD TAKSKMTQSN FAIGYKTGDF
Ictalurus punctatus             --GP-TI--H AAAVVG-YEG WLAGYQMSFD TAKSKMTQNN FAVGYKTGDF
Oncorhynchus mykiss             --GP-AI--H GAAVVG-YEG WLAGYQMTFD TAKSKMTQNN FAVGYKTGDF
Oryzias latipes VDAC2           --GP-TI--H NGVVG-YEG WLAGYQMTFD TAKSKMIRNN FAIGYKTGDF
Danio rerio                      --GP-TI--H GAAVVG-YEG WLAGYQMSFD TAKSKMTQNN FAVGYKTGDF
Xenopus laevis VDAC2            --GP-AI--H GSAVVG-YEG WLAGYQMTFD SAKSKLTKNN FAVGYKTGDF
Fugu rubripes                    --GP-TI--H GAAVAG-YDG WLAGYQMTID SAKSKMTHSN FSIGYKTGDF
Fugu rubripes                    --GP-TI--Q AAAVLG-YEG WLAGYQLAFD TAKSTLTQNN FAFGYRAGDF
Squalus acanthias               --GP-TI--H SMAVFG-YEG WLVGHQMAFD TAKSKLAQNN FSLGYKAGDF
Danio rerio                      --GP-TV--H AAAVLG-YEG WLAGYQMAFD TAKSKLAQNN FALGYKAGDF
Salmo salar                      --GP-TV--H AAAVLG-YEG WLAGYQMAFD TAKSKLAQNN FALGYKAGDF
Monodelphis domestica VDAC1     --GP-SI--R GAVVLG-YEG WLAGYQMNFE TSKSRVTQSN FAVGYKTDEF
Gallus gallus VDAC1             --GP-SI--R GALVLG-YEG WLAGYQMTFE TAKSRVTQSN FAVGYKTDEF
Rattus norvegicus VDAC1         --GP-SIRGR GALVLG-YEG WLAGYQMNFE TSKSRVTQSN FAVGYKTDEF
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This page contains a multiple sequence alignment figure (VDAC proteins) showing residue positions approximately 155–250.

Columns ~160–200:

Species	Sequence
Bos taurus VDAC1	- - G P - S I - - R G A L V L G - Y E G W L A G Y Q M N F E T A K S R V T Q S N F A V G Y K T D E F
Sus scrofa VDAC1	- - G P - S I - - R G A L V L G - Y E G W L A G Y Q M N F E T A K S R V T Q S N F A V G Y K T D E F
Homo sapiens VDAC1	- - G P - S I - - R G A L V L G - Y E G W L A G Y Q M N F E T A K S R V T Q S N F A V G Y K T D E F
Mus musculus VDAC1	- - G P - S I - - R G A L V L G - Y E G W L A G Y Q M N F E T S K S R V T Q S N F A V G Y K T D E F
Rattus norvegicus VDAC1	- - G P - S I - - R G S L V L G - Y E G W L A G Y Q M N F E T S K S R V T Q S N F A V G Y K T D E F
Xenopus laevis VDAC1	- - G P - S V - - R G A V V F G - Y E G W L A G Y Q M T F E S S K S R V S Q S N F A V G Y K T D E F
Xenopus tropicalis	- - G P - S V - - R G A A V F G - Y E G W L A G Y Q M T F E S S K S R V S Q S N F A V G Y K T D E F
Fundulus heteroclitus VDAC1	- - G T - A V - - H G A A V V G - F E G W L A G Y Q M T F E A A G T R S P R A I F A V G Y K T D E F
Oryzias latipes VDAC1	- - G T - A I - - H G A A V V G - Y E G W L A G Y Q M T F E A G R N K V T Q S N F A V G Y K T D E F
Danio rerio VDAC1	- - G T - A V - - H G A L V L G - L D G W L A G Y Q M T F E A G R N R I T Q S N F A V G Y K T D E F
Petromyzon marinus	- - G P - T V - - N G A A V V G - Y E G W L A G Y Q L A F D T A K S K L T R S N F A I G Y K T A D F
Tetraodon nigroviridis	- - G P - T I - - H G A A V A G - Y E G W L A G Y Q M T I D S A K S K M A H S N F A I G Y K T G D F
Oryctolagus cuniculus VDAC3	- - G P - T I - - Y G W A V L A - F E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K A A D F
Sus scrofa VDAC3	- - G P - T I - - Y G W A V L A - L E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K A A D F
Bos taurus VDAC3	- - G P - T I - - Y G W A V L A - F E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K A A D F
Mus musculus VDAC3	- - G P - T I - - Y G W A V L A - F E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K A A D F
Homo sapiens VDAC3	- - G P - T I - - Y G W A V L A - F E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K A A D F
Rattus norvegicus VDAC3	- - G P - T I - - Y G W A V L A - F E G W L A G Y Q M S F D T A K S K L C Q N N F A L G Y K A E D F
Pongo pygmaeus VDAC3	- - G P - T I - - Y G W A V L A - F E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K A A D F
Gallus gallus VDAC3	- - G P - T I - - Y G W A V L G - Y E G W L A G Y Q M A F D T A K S K L S Q N N F A L G Y K A G D F
Pimephales promelas	- - G P - A V - - H G A A V L A - F E G W L A G Y Q M T F D T A K S K M T R S N F A I G Y K T G D F
Argopecten irradians	- - G P - T V - - H G A A V V G - Y N N F L A G Y Q M S F D T S K S K L S K N N F A F G Y S S D D M
Spisula solidissima	- - G P - T V - - N G A A V L G - Y N G F L A G Y Q M S F D T S K S K L T K N N I S G G Y D A G D F
Daphnia magna	- G G P - T V - - N G A A V C G - Y M G W L A G Y Q M S F D M S K S Q L T R N N F S I A Y A A K D F
Apis mellifera	- T G P - L V - - N A S T V V G - Y Q G W L A G Y Q A C F D T Q R N K L T K N N F A L G Y T A S D F
Homalodisca coagulata	- G A P - V V - - N A A A V L G - Y N G W L A G Y S T K F D S Q K S K I T G N N F A L G Y A A G D F
Anopheles gambiae	- - G P - L V - - N A S G V A A - Y Q G W L A G Y Q V A F D S Q K S K I T A N N F A L G Y S A G D F
Drosophila melanogaster	- - G P - L I - - N A S A V L G - Y N G W L A G Y Q T A F D T Q Q S K L T T N N F A L G Y T T K D F
Drosophila pseudoobscura PORB	- - G P - L I - - N A S A V L G - Y N G W L A G Y Q A A F D T Q Q T K L T T N N F A L G Y T T K D F
Aedes aegypti	- - G P - L V - - N A S G V F N - Y Q G W L A G Y Q V A F D S Q K S K V T A N N F A L G Y S T G D F
Rhipicephalus appendiculatus	- - G P - V A - - H G A A V L H - Y Q G W L A G A Q V S F D T T K N R L S K T N F A V G Y Q A G D F
Ciona intestinalis	- - G P - T L - - Q G A C V F G - Y E G W L A G Y Q F A F D T N K S A L T K N N V A V G Y N G A D F
Strongylocentrotus purpuratus	- - G P - T V - - H G S T V V G - Y E - G L A G F Q V S F D T A E S K L K K S N F A L G Y K T A D F
Drosophila pseudoobscura PORA	- S S P - I M - - N C S L V L G - H K E F L G G L G C T F D L G D T S L S S W K V A L G W S N D K A
Drosophila melanogaster	- S E P - I L - - N C S L V V G - H K E F L G G V G T E F D V G N T E L K G W K V A L G W T N E T A
Monodelphis domestica VDAC3	- - G P - T I - - Y G W A V L A - Y E G W L A G Y Q M S F D T A K S K L S Q N N F A L G Y K G A D F
Molgula tectiformis	- - G P - T L - - Q G A A V V G - F E G W L A G Y Q F A F D T S C S A L T K N N L A L G Y N G S D F
Macaca mulatta VDAC1	L L G P Q S R - - V L W C W V T - R A G W P A T R I L R M Q S P E P R A T V - Q L A T R L M N S S F
Hydra magnipapillata	- - G P - T V - - H G S A V F A - Y K G F H A G Y Q A S F N S A N S K L T S N N V C L A Y K N G D L
Schistosoma japonicum	- - I P - D L - - S P S L V F G - Y Q G Y L V G A D V K L D C T N Q I L Q K A N F A V G Y T V Q D F
Drosophila melanogaster Porin B	- N E P - L F - - L G Y V L V A P V E N W V L G Y R T E Y N F D E K G F D K H A L C L G Y N N G R T
Drosophila pseudoobscura	- K E P - T L - - M G Y V L A A P A E N W I L G Y R T V Y S V A E K A F D K H A F C L G Y N N G S T
Drosophila melanogaster Porin A	- K E P - L L - - M G Y I M M Q P V K N Y L L G Y R T V F N V E D R D F N M H A F C G G Y S N D V T
Fugu rubripes	- - G T - A I - - H G A A V V G - Y E G W L A G Y Q M T F E A G R N R V T Q S N F A V G Y K T D E F
Fugu rubripes	- - G P - T I - - Q A A A V L G - Y E G W L A G Y Q L A F D T A K S T L T Q N N F A F G Y R A G D F
Xenopus tropicalis VDAC1	- A G P - A I - - H G S A V V G - Y E G W L A G Y Q M T F D S A K S K L T K N N F A V G Y K T G D F
Xenopus tropicalis	- A G P - A I - - H G S A V V G - Y E G W L A G Y Q M T F D S A K S K L T K N N F A V G Y K T G D F
Amblyomma variegatum	P P G P - I A - - H G A A V L H - Y Q G W L A G G Q V S F D T T K N R L S R T N F A V G F Q A G D F
Branchiostoma floridae	- - G P - T I - - H G A A V L G - Y E G W L A G Y Q M S F D T A K S K L T R S N F A L G Y K T G D F
Pratylenchus penetrans	- T G P - Q I - - N F S G V T - G L H G W L L G A Q S A F D V S S S Q L K A I S F S F G R I G T D Y
Meloidogyne incognita	- G A P - V L - - E V F G V S K I Y K D W M A G A K A Y D L Q A N E L K A T S L A F L H Q T S D Y
Lymnaea stagnalis	- - G P - T V - - H G A A V L G - Y E G W L A G Y Q M T F D T S K S K L T R S N F G F G Y A G G D F
Necator americanus	- S A P - V V - - N A A G V F S - R E G W L F G A A A S F D T A T N K L A S T S L A F G H Q T S N Y
Ancylostoma ceylanicum	- S A P - V I - - N A A G V F S - R E G W L F G A A A S F D T A T N K L A S T S L A F G H Q T N S Y
Nippostrongylus brasiliensis	- A S P - V I - - N A A G V F S - R E G W L F G A A A T F D T S T N K V A S T S L A F G H Q H G S Y
Parastrongyloides trichosuri	- S T P - V V - - N A A G V F S - R E G W L F G A A A S F D T A S N K L S S Q L A L G Y G H Q T A D Y
Tribolium castaneum	- - - - - - - - K G L K L S - - S D L T F S P Q T G S K S A R V K T A F T N D R V A L N C D V D L
Ambystoma tigrinum	- - - - - - - - K G L K L T - - F D S S F S P N T G K K N A K I K S A Y K R E H I N L G C D M D F
Spermophilus lateralis	- - - - - - - - R G L K L T - - F D S S F S P N T G K K N A K I K T G Y K R E H I N L G C D V D F
Ixodes scapularis	- - - - - - - - K G L K L A - - F N A N F A P Q T G K K S G A L K T A Y K V D N I H V N S D V D L

Columns ~210–250:

Species	Sequence
Ancylostoma caninum	T L H S F V V N - S T D F G A S L F H K V A H N V E I G T I L G W - K V G G S G - A - D F G V A T K
Caenorhabditis elegans VDAC2	T L H S F V I N - S T D F G A S L Y H K V A S N V E V G T Q L G W - K V G G N G - A - D Y A L A T K
Caenorhabditis briggsae	T L H S F V I N - S N D F G A S L Y H K V A S N V E I G T Q V G W - K V G S N G - A - D Y A L A T K
Caenorhabditis elegans	T L H S F V I N - S N D F G A S L Y H K V A P N V E I G T Q L G W - K V G G N G - A - D Y A L A T K
Ascaris suum	T L H S F V N D - G Q E F G A S L Y H R A A H N V E V G A Q L G W - T V G D Q G - A - R F A L A A K
Haemonchus contortus	T L H S F V V N - S T D F G A S L F H R V S P N V E L G T M L G W - K V G G N G - A - D F A V A A K
Monodelphis domestica VDAC2	Q L H T N V N D - G T E F G G S I Y Q K V C E D L D T S V N L A W - T S G T N C - T - R F G I A A K
Macaca mulatta VDAC2	Q L H T N V N D - G T E F G G S I Y Q K V C E D L D T S V N L A W - T S G T N C - T - R F G I A A K
Bos taurus VDAC2	Q L H T N V N D - G T E F G G S I Y Q K V C E D L D T S V N L A W - T S G T N C - T - R F G I A A K
Rattus norvegicus VDAC2	Q L H T N V N N - G T E F G G S I Y Q K V C E D F D T S V N L A W - T S G T N C - T - R F G I A A K
Mus musculus VDAC2	Q L H T N V N N - G T E F G G S I Y Q K V C E D F D T S V N L A W - T S G T N C - T - R F G I A A K
Homo sapiens VDAC2	Q L H T N V N D - G T E F G G S I Y Q K V C E D L D T S V N L A W - T S G T N C - T - R F G I A A K
Sus scrofa VDAC2	Q L H T N V N D - G T E F G G S I Y Q K V C E D L D T S V N L A W - T S G T N C - T - R F G I A A K
Homo sapiens VDAC2	Q L H T N V N D - G T E F G G S I Y Q K V C E D L D T S V N L A W - T S G T N C - T - R F G I A A K

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Multiple sequence alignment (residues ~205–250):

Species	210 . . 220 . . 230 . . 240 . . 250
Gallus gallus VDAC2	QLHTNVND-G SEFGGSIYQK VSDNLETAVN LAW-TAGSNS -T-RFGIAAK
Meleagris gallopavo VDAC2	QLHTNVND-G SEFGGSIYQK VSDNLETAVN LAW-TAGSNS -T-RFGIAAK
Pan troglodytes VDAC2	QLHTNVND-G TEFGGSIYQK VCEDLDTSVN LAW-TSGTNC -T-RFGIAAK
Gasterosteus aculeatus	QLHTNVND-G AEFGGSIYQK VSDQLETSVN LAW-TAGSNS -T-RFGIAAK
Fugu rubripes	QLHTNVND-G AEFGGSIYQK VSDKLETAVN LAW-TAGSNS -T-RFGIAAK
Fugu rubripes	QLHTNVND-G AEFGGSIYQK VSDKLETAVN LAW-TAGSNS -T-RFGIAAK
Tetraodon nigroviridis	QLHTNVND-G AEFGGSIYQK VSDKLETAVN LAW-TAGSNS -T-RFGIAAK
Ictalurus punctatus	QLHTNVND-G AEFGGSIYQK VSDNLETAVN LAW-TAGSNS -T-RFGIAAK
Oncorhynchus mykiss	QLHTNVND-G VQFGGSIYQK VSPALETAVN LAW-TAGSNS -T-SFGIAAK
Oryzias latipes VDAC2	QLHTNVND-G SEFGGSIYQK VSDKLETAVS LAW-TAGSNS -T-RFGIGAK
Danio rerio	QLHTNVND-G SEFGGSIYQK VSDKLETAVN LAW-TAGSNS -T-RFGIAAK
Xenopus laevis VDAC2	QLHTNVNN-G SEFAGSIYQK VSDKMETAVN LAW-TSGNNS -T-RFGIAAK
Fugu rubripes	QLHTNVNN-G SEFGGSIYQK VNDKLETAVN LAW-TAGSNG -T-RFGIAAK
Fugu rubripes	QLHTSVND-G TEFGGSVYQK VNSNLETAVT LAW-TAGSNN -T-RFGVGAK
Squalus acanthias	QLHTHVND-G AEFGGSIYQK VNDKVETAVN LAW-TAGSNN -T-RFGIAAK
Danio rerio	QLHTNVND-G TEFGGSIYQK VNGQLETAVN LAW-TAGSNN -T-RFGIAAK
Salmo salar	QLHTNVND-G TEFGGSIYQK VNCHLETAIN LAW-TAGSNN -T-RFGIGAK
Monodelphis domestica VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNSN -T-RFGIAAK
Gallus gallus VDAC1	QLHTNVND-G TEFGGSIYQK VNEKLETAVN LAW-TAGNSN -T-RFGIAAK
Rattus norvegicus VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNSN -T-RFGIAAK
Bos taurus VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNSN -T-RFGIAAK
Sus scrofa VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNSN -T-RFGIAAK
Homo sapiens VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNSN -T-RFGIAAK
Mus musculus VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNSN -T-RFGIAAK
Rattus norvegicus VDAC1	QLHTNVND-G TEFGGSIYQK VNKKLETAVN LAW-TAGNRN -T-RFGIAAK
Xenopus laevis VDAC1	QLHTNVND-G TDFGGSVYQK VNDKLETAIN LAW-TAGNSN -T-RFGIAAK
Xenopus tropicalis	QLHTNVND-G TEFGGSVYQK VNDKLETAIN LAW-TAGNSN -T-RFGIAAK
Fundulus heteroclitus VDAC1	QLHTNVND-G TEFGGSIYQK VNDKLETAVN LAW-TAGNSN -T-RFGIAAK
Oryzias latipes VDAC1	QLHTNVND-G TEFGGSIYQK VNEQLETAVN LSW-TAGNSN -T-RFGIAAK
Danio rerio VDAC1	QLHTNVND-G TEFGGSIYQK VNDNLETAVN LAW-TAGNSN -T-RFGIAAK
Petromyzon marinus	QLHTNVND-G TEFGGNIYQK VNDKLETAVD LAW-TAGSNT -T-RFGIAAK
Tetraodon nigroviridis	QLHTNIKN-G AEFEGSIYQK VSDELETAVN LAW-TAGSNS -T-RFGIAAK
Oryctolagus cuniculus VDAC3	QLHTHVND-G TEFGGSIYQK VNEKIETSIN LAW-TAGSNN -T-RFGIAAK
Sus scrofa VDAC3	QLHTHVND-G TEFGGSIYQK VNEKIETSIN LAW-TAGSNN -T-RFGIAAK
Bos taurus VDAC3	QLHTHVND-G TEFGGSIYQK VNEKIETSIN LAW-TAGSNN -T-RFGIAAK
Mus musculus VDAC3	QLHTHVND-G TEFGGSIYQK VNERIETSIN LAW-TAGSNN -T-RFGIAAK
Homo sapiens VDAC3	QLHTHVND-G TEFGGSIYQK VNEKIETSIN LAW-TAGSNN -T-RFGIAAK
Rattus norvegicus VDAC3	QLHTHVND-G TEFGGSIYQR VNEKIETSIN LAW-TAGSNN -T-RFGIAAK
Pongo pygmaeus VDAC3	QLHTHVND-G TEFGGSIYQK VNEKIETSIN LAW-TAGSNN -T-RFGIAAK
Gallus gallus VDAC3	QLHTNVND-G TEFGGSIYQK VNNKVETSVN LAW-TAGSNN -T-RFGIAAK
Pimephales promelas	QLHTNVND-G TEFGGSIYQK VNDDLETAVN LSW-TAGNSS -T-RFGIAAK
Argopecten irradians	TLHCNIND-G AEFGGSVYNK VNSKLQTGVN LSW-SAGNSS -T-RLGLAAK
Spisula solidissima	IINTSVND-F TEFGGSVSQT VNKNLETGVQ LSW-TSGSNA -V-RFGIAGK
Daphnia magna	ALHTNVND-G QEFGGALFQK VNPNLETGVQ LAW-TAGSNA -T-RFGIGCK
Apis mellifera	TLHAAVNN-G CDFSGLIYHK VKPELEGAIN LEW-NSSNNV -T-QFGIATK
Homalodisca coagulata	VFHTSVDN-G QEFGGSIYHK VNPNFETAVN LAW-SAGNND -T-RFGIGCK
Anopheles gambiae	VLHTNVND-G REFGGLIYQR CNDRLETAVQ LSW-ASGSNA -T-KFGMGAK
Drosophila melanogaster	VLHTAVND-G QEFGGSIYQR TSDKLDVGVQ LSW-ASGTSN -T-KFAIGAK
Drosophila pseudoobscura PORB	VLHTSVND-G QEFSGSIFQR TSDKLDVGVQ LSW-ASGNSN -T-KFAIGAK
Aedes aegypti	VLHTNVND-G REFGGLIYQR CNDRLETAVQ LSW-ASGSNA -T-KFGLGAK
Rhipicephalus appendiculatus	VLHTNVND-G QEFAGSVFQR VSPQLQTGVQ LAW-TAGTNA -T-RFGLGCV
Ciona intestinalis	QLLTTMND-A SEFGGSIYQS VNKNLATGIQ LSW-SAGQNN -T-KFGVATK
Strongylocentrotus purpuratus	QLHTAVNE-G SDFSGSIYQK VNDSLQTAIN VGW-ASTTNQ -T-RFALGAK
Drosophila pseudoobscura PORA	TLHGELKD-G DSWLASLFYK LNDQIDAAVE LTK-GGGGG SDVTVGVGMI
Drosophila melanogaster	TLHGELKN-G DTWLASLFYK ASEKIDAGIE VTK-GAGGGG -DVVVNLGMI
Monodelphis domestica VDAC3	QLHTHVND-G TEFGGSIYQK VSEKIETSIN LAW-TAGSNN -T-RFGIAAK
Molgula tectiformis	QILTTVND-G TEFGGSIYQS VNKDLATGVQ LSW-TSGQAK -T-KFSVAAK
Macaca mulatta VDAC1	TL----KM-I GQFGGSIYQK VN-KLETTIN LTW-TAGNSN -T-SFGIAAK
Hydra magnipapillata	VIHSGVAD-A SKFVGSVHHQ INEQLAAAAL LRW-TSGQNA -T-SFTVAAK
Schistosoma japonicum	AFHGLITNWG KQFSANMFQR ITDRLHIAGS ITW-KRVPDE -I-IWAVGSQ
Drosophila melanogaster Porin B	EVGLKLEN-F EDLRGSIFQR IGEAWAFAIK TNL-YSSENV -K-QFAIGVQ
Drosophila pseudoobscura	EMGLKAEN-F KDLRGSLFQR IGESWAVALK MNF-YGEEQL -K-KFAIGCQ
Drosophila melanogaster Porin A	EVGLKFEN-F KALRGSIFQR IGEKWAVALK ANL-YGNVSA -K-SVSIGGQ
Fugu rubripes	QLHTNVND-G TEFGGSIYQK VNEQLETAVN LAW-TAGNSN -T-RFGIAAK
Fugu rubripes	QLHTSVND-G TEFGGSVYQK VNSNLETAVT LAW-TAGSNN -T-RFGVGAK
Xenopus tropicalis VDAC1	QLHTNVND-G SEFAGSIYQK VSDKMETAVN LAW-TSGNNS -T-RFGIAAK
Xenopus tropicalis	QLHTNVND-G SEFAGSIYQK VSDKMETAVN LAW-TSGNNS -T-RFGIAAK
Amblyomma variegatum	AVHTNVND-G QEVAGSVFQR VNPQLQTGVQ LAW-TAGTNA -T-RFGLGCV
Branchiostoma floridae	QLHTNVND-G TEFGGSIYQK VNNNLETGVN LAW-TAGSNN -T-RFGLAAK
Pratylenchus penetrans	VLHSYVND-A REFGGSFYHS VAHNLDIGAM FSW-TTGEPG -A-RFGLAMK
Meloidogyne incognita	SLHTYTND-G NEFGGSLYHK VHKNFEFGVQ YMW-KLKEVN -N-QWSLASK
Lymnaea stagnalis	SFTTFVND-G QEFVGSVHQK VSDNLEAAVN LSW-AAGTGN -T-------
Necator americanus	TLHSFVVN-S TDFGASLYHR VAHNVEIGTM LGW-KVGGNG -A-DFGLAAK
Ancylostoma ceylanicum	TLHSFVVN-S TDFGASLFHK VA-------- ---------- ----------
Nippostrongylus brasiliensis	TLHSFVVN-S TDFGAS---- ---------- ---------- ----------
Parastrongyloides trichosuri	TLHSFVLN-S QEFGASLFHK AARNVEIGAM L-----GW-- ----------
Tribolium castaneum	DSSGPLIQ-A AAVVGHQGWL AGYQTAFDTQ KSKLTKNNFA LGFSTGDFIL
Ambystoma tigrinum	DIAGPSVR-G AAVFGYEGWL AGYQMTFETT KSRVSQSNFA VGYKTDEFQL
Spermophilus lateralis	DIAGPSIR-G ALVLGYEGWL AGYQMNFETA KSRVTQSNFA VGYKTDEFQL
Ixodes scapularis	GIGGPIAH-A GAVLHYQGWL AGAQLSYDAN KSRLSKTNFA VGYQGGDFVL

				260				270				280				290			300

Species	Sequence
Ancylostoma caninum	Y S P S R D
Caenorhabditis elegans VDAC2	Y A P S R D
Caenorhabditis briggsae	Y S P S R D
Caenorhabditis elegans	Y S P S R D
Ascaris suum	Y C P A K D
Haemonchus contortus	Y S P T R D
Monodelphis domestica VDAC2	Y Q L D S T
Macaca mulatta VDAC2	Y Q L D P T
Bos taurus VDAC2	Y Q L D P T
Rattus norvegicus VDAC2	Y Q L D P T
Mus musculus VDAC2	Y Q L D P T
Homo sapiens VDAC2	Y Q L D P T
Sus scrofa VDAC2	Y Q L D P T
Homo sapiens VDAC2	Y Q L D P T
Gallus gallus VDAC2	Y K L D S T
Meleagris gallopavo VDAC2	Y K L D S T
Pan troglodytes VDAC2	Y Q L D P T
Gasterosteus aculeatus	Y Q L D K D
Fugu rubripes	Y Q L D K D
Fugu rubripes	Y Q L D K D
Tetraodon nigroviridis	Y Q L D K D
Ictalurus punctatus	Y Q L D K D
Oncorhynchus mykiss	Y Q L D K S
Oryzias latipes VDAC2	Y Q L D K D
Danio rerio	Y Q L D K D
Xenopus laevis VDAC2	Y Q L D S H
Fugu rubripes	Y Q L D S S
Fugu rubripes	Y Q L D K N
Squalus acanthias	Y Q I D S D
Danio rerio	Y Q L D K D
Salmo salar	Y Q L D K D
Monodelphis domestica VDAC1	Y Q I D P E
Gallus gallus VDAC1	Y Q I D P D
Rattus norvegicus VDAC1	Y Q V D P D
Bos taurus VDAC1	Y Q I D P D
Sus scrofa VDAC1	Y Q I D P D
Homo sapiens VDAC1	Y Q I D P D
Mus musculus VDAC1	Y Q V D P D
Rattus norvegicus VDAC1	Y Q V D P D
Xenopus laevis VDAC1	Y Q I D S D
Xenopus tropicalis	Y Q I D S D
Fundulus heteroclitus VDAC1	Y Q I D S D
Oryzias latipes VDAC1	Y Q I D P D
Danio rerio VDAC1	Y Q I D S D
Petromyzon marinus	Y Q L D S N
Tetraodon nigroviridis	Y Q L D S S
Oryctolagus cuniculus VDAC3	Y K L D C R
Sus scrofa VDAC3	Y K L D C R
Bos taurus VDAC3	Y K L D C R
Mus musculus VDAC3	Y K L D C R
Homo sapiens VDAC3	Y M L D C R
Rattus norvegicus VDAC3	Y R L D C R
Pongo pygmaeus VDAC3	Y M L D C R
Gallus gallus VDAC3	Y Q L D E K
Pimephales promelas	Y M L D S S
Argopecten irradians	Y T L D K N
Spisula solidissima	Y I L D K N
Daphnia magna	Y Q L D K D
Apis mellifera	Y N L D N D
Homalodisca coagulata	Y N L D H D
Anopheles gambiae	Y D L D K D
Drosophila melanogaster	Y Q L D D D
Drosophila pseudoobscura PORB	Y Q L G D D
Aedes aegypti	Y D L D K D
Rhipicephalus appendiculatus	Y D L D M E
Ciona intestinalis	Y N I D A D
Strongylocentrotus purpuratus	Y V L D S E
Drosophila pseudoobscura PORA	Y R L E G D
Drosophila melanogaster	Y H L E E D
Monodelphis domestica VDAC3	Y K L D H K
Molgula tectiformis	Y A M D N N
Macaca mulatta VDAC1	Y Q I D P D
Hydra magnipapillata	Y D I D K D
Schistosoma japonicum	Y I L D N Q
Drosophila melanogaster Porin B	Y D F Q N G

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                                              . . . . . . . . . 260 . . . . . . . . . 270 . . . . . . . . . 280 . . . . . . . . . 290 . . . .      300
        Drosophila pseudoobscura   ----------- ----------- ----------- ----------- -----  YQLGDG
      Drosophila melanogaster Porin A ----------- ----------- ----------- ----------- -----  YEWEPG
                     Fugu rubripes   ----------- ----------- ----------- ----------- -----  YQIDPD
                     Fugu rubripes   ----------- ----------- ----------- ----------- -----  YQLDKN
             Xenopus tropicalis VDAC1 ----------- ----------- ----------- ----------- -----  YQLDSH
                Xenopus tropicalis   ----------- ----------- ----------- ----------- -----  YQLDSH
              Amblyomma variegatum   ----------- ----------- ----------- ----------- -----  YEMDQD
               Branchiostoma floridae ----------- ----------- ----------- ----------- -----  YTIDSK
             Pratylenchus penetrans   ----------- ----------- ----------- ----------- -----  YCPTKD
              Meloidogyne incognita   ----------- ----------- ----------- ----------- -----  YQVNPD
                   Lymnaea stagnalis   ----------- ----------- ----------- ----------- -----  ------
                 Necator americanus   ----------- ----------- ----------- ----------- -----  YAPSRD
             Ancylostoma ceylanicum   ----------- ----------- ----------- ----------- -----  ------
          Nippostrongylus brasiliensis ----------- ----------- ----------- ----------- -----  ------
           Parastrongyloides trichosuri ----------- ----------- ----------- ----------- -----  ------
               Tribolium castaneum   HTNVDDGQEF GGSIYQKLSP KLETGIQLAW SAGSNNTKFG IXAKYDLDQD
                 Ambystoma tigrinum   HTNVNDGTDF GGSIYQKVND KLETAVNLAW TAGNSNTRFX IAAKYQLDSD
               Spermophilus lateralis HTNVNDGTEF GGSIYQKVNK KLETAVNLAW TAGNSNTRFG IAAKYQIDPD
                  Ixodes scapularis   HTNVNDGQEF AGSIYQRVND CLETGVQLSW TAGTNATRFG LGCVYQLDRE


                                              . . . . . . . . . 310 . . . . . . . . . 320 . . . . . . . . . 330 . . . . . . . . . 340 . . . - HK  35^
                Ancylostoma caninum   LVLRA-KLNN SSHIALAATH SLSPDLTMTL STRINLTSYD H--HKFGMVL EYEPS
          Caenorhabditis elegans VDAC2 LTVRA-KVNS SSQVAVAATH SLSPALKLTL STQFNLAAND A--HKFGLGL EFDP-
             Caenorhabditis briggsae   LALRA-KVNS SSQVAVAATH SLSPALKLTL STQFNLAAND A--HKFGLGL EFDP-
               Caenorhabditis elegans LAIRA-KVNS SSQVAVAATH SLSPALKLTL STQFNLSAND A--SQ---G- -SGL-
                       Ascaris suum   LELKA-KVDN QSKVAFAATH HLTNQLKLIL STQFGLNTLN EGGHKFGLGL VY---
                Haemonchus contortus   LTLRG-KVNN SSQVALAVTH SLSPDLKATL SSQFN----- ---------- -----
        Monodelphis domestica VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKLGLAL ELEA-
               Macaca mulatta VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKLGLAL ELEA-
                  Bos taurus VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKLGLAL ELEA-
             Rattus norvegicus VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSFN AGGHKLGLAL ELEA-
                Mus musculus VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSFN AGGHKLGLAL ELEA-
                Homo sapiens VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKVGLAL ELEA-
                  Sus scrofa VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKLGLAL ELEA-
                Homo sapiens VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKVGWSW RLNP-
                 Gallus gallus VDAC2   ASISA-KVNN SSLVGVGYTQ TLRPGVKLTL SALIDGKSIN AGGHKLGLGL ELEA-
           Meleagris gallopavo VDAC2   ASISA-KVNN SSLVGVGYTQ TLRPGVKLTL SALIDGKSIN AGGHKLGLGL ELEA-
             Pan troglodytes VDAC2   ASISA-KVNN SSLIGVGYTQ TLRPGVKLTL SALVDGKSIN AGGHKVGLAL ELEA-
               Gasterosteus aculeatus ASVSA-KVNN NSLVGVGYTQ TLRPSVKLTL SALFDGKNIN SGGHKLGLGL ELE--
                     Fugu rubripes   ASVSA-KVNN NSLVGVGYTQ TLRPGVKLTL SGLVDGKNIN AGGHKLGLGL ELEA-
                     Fugu rubripes   ASVSA-KVNN NSLVGVGYTQ TLRPGVKLTL SGLVDGKNIN AGGHKLGLGL ELEA-
              Tetraodon nigroviridis   ASVSA-KVNN SSLVGVGYTQ TLRPGVKLTL SGLVDGKNIN AGGHKLGLGL ELEA-
                Ictalurus punctatus   ASISA-KVNN TSLVGVGYTQ TLRPGVKLTL SALVDGKSIN SGGHKLGLGL ELEA-
               Oncorhynchus mykiss   ASISA-KVNN ISLVGLGYTQ TLRPGVKLTL SALVDGKSIN TGGHKLGLGL ELEA-
               Oryzias latipes VDAC2   ASISA-KVNN NSLVGVGYTQ TLRPGVKLTL AGLVDGKSIN AGGHKLGLGL ELEA-
                        Danio rerio   ASISA-KVNN TSLVGVGYTQ SLRPGVKLTL SALVDGKSIN SGGHKLGLGL ELEA-
              Xenopus laevis VDAC2   AAISA-KVNN SSLVGVGYTQ TLRPGVKLTL SALVDGKNIN AGGHKLGLGL ELEA-
                     Fugu rubripes   ASLSA-KVNN ASLVGIGYTQ TLRPGMKVTL SALVDGKNIN AGGHKLGLGL ELEA-
                     Fugu rubripes   SSLST-KVDN GCLVGLGYTQ TLRPGVKLTL SGLIDGKNVN GGGHKVGLGF ELEA-
                Squalus acanthias   AYVSA-KVNN SSLIGVGYTH TLRPGVKLTL SGLIDGKNFH AGGHKVGMGF ELEA-
                        Danio rerio   SSVSA-KVNN ASLVGVGYTQ SLRPGVKLTL SALIDAKNFN AGGHKVGMGF ELEV-
                        Salmo salar   ASLSA-KVNN ASLIGVGYTQ ALRPGVKLTL SALIDAKNFN AGGHKVGMGF ELEA-
        Monodelphis domestica VDAC1   ASFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFQA-
                 Gallus gallus VDAC1   ASFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFEA-
             Rattus norvegicus VDAC1   ACFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFQA-
                  Bos taurus VDAC1   ACFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFQA-
                  Sus scrofa VDAC1   ACFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFQA-
                Homo sapiens VDAC1   ACFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFQA-
                Mus musculus VDAC1   ACFSA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGGHKLGLGL EFQA-
             Rattus norvegicus VDAC1   ACFLA-KVNN SSLIGLGYTQ TLKPGIKLTL SALLDGKNVN AGSHKLGLGL EFQA-
              Xenopus laevis VDAC1   ASFSA-KVNN SSLIGLGYTQ TLKPGIKLTL STLVDGKNIN AGGHKLGLGL EFEA-
                Xenopus tropicalis   ASFSA-KVNN SSLIGLGYTQ TLKPGIKLTL STLLDGKNIN AGGHKLGLGL EFE--
        Fundulus heteroclitus VDAC1   ASFSA-KVNN SSLVGLGYTQ TLKPGVKLTL AALLDGKNIN SGGHKLGLGL EFQA-
               Oryzias latipes VDAC1   ASFSA-KVNN SSLVGLGYTQ TLKPGIKLTL SALLDGKNIN AGGHKLGLGL EFQA-
                   Danio rerio VDAC1   AAFSA-KVNN SSLVGLGYTQ TLKPGIKLTL SALLDGKNIN AGGHKMGLGL EFEA-
                  Petromyzon marinus TTINA-KVNN SSLVGLGYTQ TLRPGVKLSL SALLDGKNIN AGGHKLGLGL ELEA-
              Tetraodon nigroviridis   ASLSA-KVNN ASLVGIGYTQ ALRPGMKVTL SALVDGKNIN AGGHKLGLGL ELEA-
         Oryctolagus cuniculus VDAC3   TSLSA-KVNN ASLIGLGYTQ TLRPGVKLTL SALIDGKNFN AGGHKVGLGF ELEA-
                  Sus scrofa VDAC3   TSLSA-KVNN ASLIGLGYTQ TLRPGVKLTL SALIDGKNFS AGGHKVGLGF ELEA-
                  Bos taurus VDAC3   TSLSA-KVNN ASLIGLGYTQ TLRPGVKLTL SALIDGKNFN AGGHKVGLGF ELEA-
                Mus musculus VDAC3   TSLSA-KVNN ASLIGLGYTQ TLRPGVKLTL SALIDGKNFN AGGHKVGLGF ELEA-
                Homo sapiens VDAC3   TSLSA-KVNN ASLIGLGYTQ TLRPGVKLTL SALIDGKNFS AGGHKVGLGF ELEA-
             Rattus norvegicus VDAC3   TSLSA-KVNN ASLIGLGYTQ SLRPGVKLTL SALVDGKNFN AGGHKVGLGF ELEA-
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	Sequence
Lycopersion esculentum POM34	---------- -MGKGPGL YT EI GKKARDLL YKDY QSD HKF SI TTY SPTGV
Lycopersion esculentum POM34	---------- -MGKGPGL YS DI GKKTRDLL YRDY QTD HKF TI TTY SATGV
Solanum tuberosum POM34	---------- -MGKGPGL YS DI GKKTRDLL YRDY QTD HKF TI TTY SPTGV
Lotus corniculatus VDAC1.1	---------- --AKGPGL YT DI GKKARDLL FKDY HSD QKF TI TTF SPTGV
Glycine max VDAC1.1	---------- --AKGPGL YS DI GKKARDLL FKDY HSD QKF TI TTY SPTGV
Lotus corniculatus VDAC1.2	---------- --AKGPGL YT DI GKRARDLL FKDY HSD QKF TI TTY SPTGV
Medicago truncatula VDAC1.1	---------- --SKGPGL YK DI GKKARDLL YKDY NTD QKF TL TTY SPTGI
Picea glauca	---------- -MGKGPGL YS DI GKKTRDLL NKDY NFD QKF SF TSY TSTGL
Pinus pinus	---------- -MGKGPGL YS DI GKKTRDLL NKDY NFD QKF SF TSY TSTGL
Pinus pinus	---------- -MVKGPGL FS DI GKKSRDLL YKDF LVD QKF SL TTY TPTGL
Populus trichocarpa	---------- -MVKGPGL YS EI GKKARDLL YKDY QTD HKF TL NTS SPTGV
Gycine max VDAC1.3	---------- --VGKGPGL YS DI GKKARDLL FKDY QND HKF TI TTY SPTGV
Medicago truncatula VDAC1.3	---------- -MVKGPGL FS DI GKRARDLL FKDY QND HKF TI TTQ TLAGV
Arabidopsis thaliana VDAC3	---------- --VKGPGL YT EI GKKARDLL YKDH NSD QKF SI TTF SPAGV
Pisum sativum plastid porin	---------- --VKGPGL YT DI GKKARDLL YKDY HSD KKF TI STY SPTGV
Pisum sativum plastid porin	---------- --VKGPGL YT DI GKKARDLL YKDY SDKKF TI ST YSPTGV
Arabidopsis thaliana VDAC3	---------- --VKGPGL YT EI GKKARDLL YRDY QGD QKF SV TTY SSTGV
Brassica rapa	---------- --GKGPGL YT EI GKKARDLL YKDY QGD QKF SI TAY SSTGV
Solanum habrochaites VDAC1	---------- ----GPGL YT EI GKKARDLL YKDY QSD HKF SI TTY SPTGV
Citrus reticulata VDAC1	---------- ---I GPGL YS EI GKKARDLL YKDY QSD HKF TV TTY TSAGV
Gossypium raimondii VDAC3.1	---------- --MAGPAP FV DL GKKAKDLL TKDY NFD QKF TL SML STTGM
Vitis vinifera VDAC3.1	---------- --GNGPAP FS DI GKKTRDLL NKDY NFD HKF TL LMQ SPGGM
Solanum tuberosum VDAC3	---------- -MEQSPAP FS EI GRRARDLL TKDY NYD QKF TF SI PSSSGV
Lotus corniculatus VDAC3.1	---------- --ANGPAL YP DI GKKARDLL YKDY NFD QKF NL SI PSSTGL
Glycine max VDAC3.1	---------- --ANGPAP FS EI GKRAKDLL YKDY NFD HKF SL SI PNSTGL
Medicago truncatula VDAC3.1	---------- --ANGPAP FS EI GKRARDLL YKDY NFD HKF SL SI PSSTGL
Allium cepa	---------- --GSGPAP FS EI GKKARDLL TRDY NFD HKF SL STV SKDGL
Pinus pinus	---------- --GKGPGL FS DI GKKAKDLL TKDY NYD QKF TV TTF SETGL
Pinus taeda	---------- ----GPGL FS DI GKKAKDLL TKDY SYD QKF AV ATY SDDGL
Hordeum vulgare VDAC3.1	---------- --SKGPVP FP IF GKKARDIL YKDY NFD QKF SW STT SEFGL
Sorghum bicolor VDAC3.1	---------- --SKGPVP FV NI GKRAKDLL YKDY NFD QKF SL STS SSSGL
Oryza sativa POM36	---------- --SKGPAP FL NI GKRAKDLL YKDY NFD QKF SL TTT SNSGL
Arabidopsis thaliana POM36	---------- --GSSPAP FA DI GKKAKDLL NKDY IFD HKF TL TML SATGT
Pennisetum glaucum	---------- --MKGPGL FS DI GKKAKDLL TRDY TYD QKL TV STV SSSGV
Zea mays	---------- --MKGPGL FS DI GKKAKDLL SKDY TYD QKL TI STA SASGL
Triticum aestivum	---------- MAMKGPGL FT DI GKKAKDLL TRDY TYD QKL SV STV SSSGV
Vitis vinifera VDAC2.1	---------- --SKGPGL FA DI GKKAKDLL TRDY IS D QKF TV STY SDTGV
Solanum tuberosum VDAC2	---------- -MSKGPGL FA DI GKKARDLL TKDY IS D QKL SI STY SDTGV
Glycine max VDAC2.1	---------- --SKGPGL FT DI GKKSKDLL TKDY NSD QKL TI SSY SI SGV
Lotus corniculatus VDAC2.1	---------- --SKGPGL FS DI GKKPRDLL TKDY SSD QKL TV SSY STAGV
Gossypium raimondii VDAC2.1	---------- --SKGPGL FV DI GKRAKDLL TKDY TSD QKF TV STY TGAGV
Medicago truncatula VDAC2.1	---------- --SKGPGL YS DI GKKAKDLL TKDY NSD QKL TV SSY STTGV
Arabidopsis thaliana VDAC3	---------- --SKGPGL FT DI GKKAKDLL TRDY NSD QKF SI STY SASGV
Ananas comosus	---------- ---MGPGL YS EI GKKARDLL YKDY HTD QKF TI TTY TSTGV
Saccharum officinarum VDAC3.1	---------- --AKD---- -------LL YKDY NFD QKF SL STS SSSGL
Antirrhinum majus	---------- --GPGL YS DI GKRARDLL NKDY QSD QKF TL TTY SPTGV
Welwitschia mirabilis	---------- ------S D--- -I GKGARDLL YKDY NFD HKI AL TTY TSTGL
Allium cepa	---------- ---MGPGL YS EI GKKARDLL YRDY QTD QKF SI TTV TPYGA
Zantedeschia aethiopica	---------- ---MGPGL YS EI GKKARDLL YKDY QTD HKF SI TTY TANGI
Lycopersion esculentum VDAC3.1	----EQSPA P --------- FS EI GRRARDLL TKDY SYD QKF TFSI PSSSGM
Triphysaria versicolor	---------- --------- ------TL--- --TTY SPTGV
Picea sitchensis	---------- ----GPGL YS DI GKKTRDLL NKDY NFD QKF SF TSY TSTGL
Gossypium raimondii VDAC1.1	---------- --GKGPGL YT EI GKKARDLL YKDY QTD HKF TL TTS SPTGV
Saruma henryi	---------- ------Y S--- DI GKRARDLL YRDY QPD QKF TI TTL TSTGV
Brassica rapa	---------- --MGKGPGR YT DI GKKARDLL YKDH NSD QKF SI TTH SPAGV
Liriodendron tulipifera	---------- -MSKGPGL YS DI GKKARDLL CKDY QSD HKF TI TTY TSTGV
Saccharum officinarum VDAC1	---------- --MVGPGL YT EI GKKTKDLL YKDY QTD HKF TV TTY TSHGV
Panicum virgatum	---------- ---VGPGL YT EI GKKTRDLL YRDY QTD HKF TL TTY TSNGV
Lactuca sativa	---------- --GKGPGL YP DI GKKARDLL YRDY QGD HKF SL TTY TANGV
Nicotiana tabacum POM34	---------- --GKGPGL YS DI GKKARDLL YRDY QTD HKF TI TTY SPTGV
Eschscholzia californica	---------- -MGKGPGL YT DI GKRARDLL YRDY QSD QKF TV TTY TSTGV
Beta vulgaris	---------- --VNGPGL FS DI GKRAKDLL SKDY VLD QKF TI SSC SETGV
Cycas rumphii	---------- ---MGPGL FS DI GKKTRDLL YKDY LI D QKF SL TTY TSTGL
Glycine max VDAC1.2	---------- -MSKGPGL YS DI GKKARDLL FKDY HSD QKF TI TTY SPTGV
Hordeum vulgare	---------- --------- --------- ----- MAP GL -YTDI GKKTR
Lycopersion esculentum VDAC2	---------- --------- --------- ----- MGPGL -FSDI GKKAK
Citrus sinensis	---------- --------- --------- ----- GPGL -YTDI GKKAR
Gossypium raimondii VDAC1.3	---------- --------- --------- ----- GKGPGL -YTDI GKKAR
Picea glauca	---------- --------- --------- ------- L -YSDI GKKTR
Welwitschia mirabilis	---------- --------- --------- ----- GPGL -FSEI GKKTR
Acorus americanus	---------- --------- --------- --------- -
Selaginella moellendorffii	---------- --------- --------- ----- FSDI GRNTR

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                                              .........60........70........80........90.......100
Oryza sativa VDAC1                      AITATSTKKADLIFGEIQSQIKN-NITVDVKANSDSNVVTTVTV-DEL--
Triticum aestivum VDAC1                 AITATSTKKADLTVGEIQSQ-IKNNITVDVKANSASNVITTITADDLA--
Triticum aestivum                       AITATSTKKADLILGEIQSQIKNKNITVDVKANSASNVITTITADDLA--
Hordeum vulgare                         AITATSTKKADLILGEIQSQIKNKNITVDVKANSASNVITTITADDLA--
Zea mays VDAC2                          AITAASTRKDEAIFNEIQSQLKH-NVTVDVKATSESNVITTITVHELG--
Oryza sativa VDAC2                      AITVAGTKKNESIFSEIQSQVKN-NVSVDVKATSDSKLITTFTVHDLA--
Triticum aestivum VDAC2                 TITSSSTKKNEAILADLQTQVKI-NFTVDVKATSDSSVVTTITVPELY--
Horeum vulgare                         TITSSSTKKNEAILSDLQTQVKIKNFTVDVKATSDSSVVTTITIPELC--
Triticum aestivum                       TITSSSTKKNEAILADLQTQVKIKNFTVDVKATSDSSVTTITVPELY--
Asparagus officinalis VDAC1             AITASSTRKNEMIFGEIQSQLKNKNVTVDVKANSDSNLLTTVTIDELA--
Triticum aestivum VDAC3                 AITAAGTRKNESIFGELQTQLKN-NLTVDIKANSESDLLTTFTVDEFA--
Triticum aestivum                       AITAAGTRKNESIFGELQTQLKNKNLTVDIKANSESDLLTTFTVDEFA--
Zea mays plastid porin                  AITAAGTRKNESIFGELHTQIKN-KLTVDVKANSESDLLTTITVDEFG--
Zea mays plastid porin                  AITAAGTRKNESIFGELHTQIKNKKLTVDVKANSESDLLTTITVDEFG--
Sorghum bicolor plastid porin           AITAAGTRKNESIFGELHTQIKNKNLTVDIKANSESDLLTTITVDELG--
Saccharum officinarum plastid porin     AITAAGTRKNESIFGELHTQIKNKKLTVDVKANSESDLLTTITADEFG--
Oryza sativa VDAC3                      TITAAGTRKNESVFGELQTQLKNKL-TVDVKANSESDLLTTVTVDEFG--
Zea mays VDAC1a                         AVTASSTKKADLILGEIQSQI-KNNMTIDVKANSESNIITTITVDEIA--
Zea mays VDAC1b                         AVTATSTKKADLIFSEIHSQI-KNNMTVDVKANSESNIITTVTVNEIA--
Citrus paradisi VDAC1                   AITSSGVKKGELFLADVNTQLKNKNIKVDTNSN-LFTTITVDEP--
Nicotiana tabacum POM36                 AITSSGLKKGELFLADVNTQLKNKNIKVDTNSN-VYTTITVDEP--
Spinacia oleracea VDAC1                 AITSTGTKKGELFLADVSTKL-TNNITTDVKVDTNSN-LFTTITVDEP--
Mesembryanthemum crystallinum           AITSSGTKKGDLFLADVNTQL-KNNVTTDIKVDTNSN-LLTTITVDEA--
Lycopersion esculentum VDAC1.3          EITSTGVKKGEIYLADVSTKL-KNNITTDVKVDTNSN-LHTTITVDEP--
Prunus armeniaca                        AISSTGIRKGDLYLGDVSTQL-KNNITTDIKVDTDSN-LRTTITIDEP--
Solanum tuberosum VDAC1                 AITASGLKKGELFLADVSTQLKN-NITTDVKVDANSN-VYTTITVDEP--
Lycopersion esculentum POM36            AITSSGLKKGELFLADVSTQLKNKNITTDVKVDTNSN-LYTTITVDEP--
Populus trichocarpa                     AITSTGIKKGELFLADISSQLKNKNITTDVKVDTNSN-LLTTITVDEP--
Vitis vinifera VDAC1.1                  AITSSGTKKGDLFVADVNTQLKNKNIKIDTSSN-LFTVTVDEP--
Helianthus annuus POM34                 AITSSGTKKGDLFLADVNTQLKRNNVTADVKVDTGSN-LSTTIVVDEP--
Solanum tuberosum POM34                 VITSSGSKKGDLFLADVNTQL-KNNVTTDIKVDTNSN-LFTTITVDEA--
Lycopersion esculentum POM34            VITSSGSKKGDLFLADVNTQLKNKNVTTDIKVDTNSN-LFTTITVDEA--
Lycopersion esculentum POM34            AITSSGLKKGDLFLADVNTQLKNKNLTTDIKVDTNSN-LLATVTVDEA--
Solanum tuberosum POM34                 AITSSGLKKGDLFLADVNTQLKNKNITTDIKVDTNSN-LLATVTVDEA--
Lotus corniculatus VDAC1.1              AITSSGTKKGELFVADVNTQL-KNNITTDIKVDTDSN-LFTTIAVNDP--
Glycine max VDAC1.1                     AITASGTRKSELFLADVNTQLKNKNITTDIKVDTDSN-LFTTITVNEP--
Lotus corniculatus VDAC1.2              AITSSGIKKGELFVADVNTQLK-NNITTDLKVDTDSN-LFTTVTVDEP--
Medicago truncatula VDAC1.1             ALTASSTKKGELFLADINTQLKHKNVTTDIKVDTDSN-LFTTITVTEP--
Picea glauca                           AFTSTGIKKGEFFLGDLNTQLKNNNVTTDIKVDTSSN-LFATVTIDEP--
Pinus pinus                            AFTSTGIKKGEFFLGDLNTQLKNNNITTDIKVDTSSN-LFATVTIDEP--
Pinus pinus                            VFTSTGTKKGEFFLGDLTTQLKNKNVTTDIKVDTSSN-LFATVTIDEP--
Populus trichocarpa                     TITSSGTKKGDLLAADVNTQLKNKNITTDIKVDTNSN-LFTTITVDEP--
Gycine max VDAC1.3                      EITSTGVRKGEIYLADVSTKVKNKNITTDVKVDTNSN-LRTTITVDEP--
Medicago truncatula VDAC1.3             EITSAGVRKGEAFLADVSTKVKNKNVTTDIKVDTNSN-LRTTITVDET--
Arabidopsis thaliana VDAC3              AITSTGTKKGDLLLGDVAFQS-RRNITTDLKVCTDST-FLITATVDEA--
Pisum sativum plastid porin             AITSSGTKKGFLELGDVNTQL-KNNITTQIKVDTNSN-LFTTITVNEP--
Pisum sativum plastid porin             AITSSGTKKGELFLGDVNTQL-KNNITTDIKVDTNSN-LFTTITVNEP--
Arabidopsis thaliana VDAC3              AITTTGTNKGSLFLGDVATQV-KNNFTADVKVSTDSS-LLTTLTFDEP--
Brassica rapa                          AITTSGTNKGDLFLGDVVTQI-KNNFTADIKVASDSS-ILTTFTYDEA--
Solanum habrochaites VDAC1              VITSSGSKKGDLFLADVNTQLKNKNVTTDIKVDTNSN-LFTTITVDEA--
Citrus reticulata VDAC1                 AITSSGVKKGELFLADVNTQLKNKNIKVDTNSN-LFTTITVDEP--
Gossypium raimondii VDAC3.1             GLTATGLKKDQNFFGDINTVYKSGNTTVDMKVDTYSN-VSTKVTVNDV--
Vitis vinifera VDAC3.1                  GVTATGWKKDQIFFGDVSTLYKSGNTTVDLKLDTHSN-VSTKVTAHDI--
Solanum tuberosum VDAC3                 GITATGVKKDQIFIGDISTQYKLGSTVVDIKVDTYSN-VSTKVTLVDV--
Lotus corniculatus VDAC3.1              GLIATGLKKDQIFIGDINTLY-KSNLTVDVKVDTYSN-VSTKVTLNDI--
Glycine max VDAC3.1                     GLIATGLKKDKLFVGDINTLYKSGNTVVDVKVDTYSN-LYTKVTVNDI--
Medicago truncatula VDAC3.1             GLTATGLKRDKFFVGDLNTLYKSGNVTVDVKVNTDSN-VSTKVTLNDVSL
Allium cepa                            GITATGVKVDGLFFGDIGTQFKNGKTAVDVKVNTNSN-VSTTITLNEV--
Pinus pinus                            AFTSTGVKKGELFSGDLNTQYKYQNAIVDVKIDTNSN-ILTTITLDQP--
Pinus taeda                            GITSTGMNKGGLFCGDIATLYKYQKSTIDVKIDTNSN-ISTTITIDEV--
Hordeum vulgare VDAC3.1                 SVTAAGVKFDELFIGDIRVQHKSGRNTVDVTIDCDSK-VSTAVTFDEV--
Sorghum bicolor VDAC3.1                 NLTATGVKINEDFIGVIRTQHKSGRTTVDVIIDSDSK-VSTTVTVDEA--
Oryza sativa POM36                      GLTATGVKIDELFIGDIQTQHKSGKTTVDVKIDSESR-VSTTVTVDEA--
Arabidopsis thaliana POM36              EFVATGLKKDDFFFGDISTLY-KGNTIVDLKIDSHSS-VSTKVTLKNL--
Pennisetum glaucum                      GLTSTAVKKGGLYTLDVSSVYKY-NTVVDIKVDTESN-ISTTLTVLDA--
Zea mays                               GLTSTAVKKGGLYSLDVSSVYKYKNTVDVKVDTESN-ISTTLTVLDA--
Triticum aestivum                       AVTSTAVKKGGLYSLDVASAYKYKNNLVDVKVDTESN-ISTTLTVLDV--
Vitis vinifera VDAC2.1                  AITSTAVKKGGLSTGDVAVQYKYKNSVIDVKADTGSN-INTTLTFTEI--
Solanum tuberosum VDAC2                 ALTSTAVKKGGLSTGDVGAQYKYKNTLIDVKVDTASN-LSTTLTLNDI--
Glycine max VDAC2.1                     ALTSTSVKKGGLSTGDVAALYKYKNTIIDVKLDTAST-ISTTLTFNDV--
Lotus corniculatus VDAC2.1             ALTSTAVKKGGLSVGDVAALYKY-NTLIDVKLDTASI-ISTTLTFTDL--
Gossypium raimondii VDAC2.1            AITSTSLKKGGLSTGDVAALYKYKNTQFDVKVDTDSN-ISTTMTFTDI--
Medicago truncatula VDAC2.1             ALTSTALKKGGLSTGDVAGQYKYKNTLIDVKIETASI-INTTLTFTDL--
Arabidopsis thaliana VDAC3              ALTSTALKKGGVHAADVATQYKKY-NALFDVKIDTDSS-VLTTVTLTEI--
Ananas comosus                         AITASGTKKNELIFGEIHSQLKNKNVTVDVKANSDSN-LLTTVTIDELA--
Saccharum officinarum VDAC3.1           NLTATGVKINEDFIGDIRTQHKSGRTTVDVIIDSDSK-VSTTVTVDEA--
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                                          . . . . 60 . . . . . . . . 70 . . . . . . . . 80 . . . . . . . . 90 . . . . . . . . . . 100
           Antirrhinum majus   TLTSSGTKKG ELFLADVNTQ LKNKNITTDI KVDTSSN-LF TTITVDEP--
        Welwitschia mirabilis   EFSSTGIKKG DIFLGDIKTQ LKSSRVTTDL KVDTGSN-IL TTVTSDEL--
                 Allium cepa   AITASSTKKN DLIIGEIQSR LKNKNVTVDV KANSDSN-LI TTITYDEL--
       Zantedeschia aethiopica   AITSSGTKKN DLIIGEIQAQ QKSNGITTDV KVNSDSN-LF TTITVDEL--
 Lycopersion esculentum VDAC3.1   GITATGVKKD QIFIGDISTQ YKLGSTVVDI KVDTYSN-VS TKVTLVDV--
         Triphysaria versicolor   VLTSTGTKKG DLFLADVNTQ LKNKNITTDI KVDTSSN-LF TTITIDEP--
              Picea sitchensis   AFTSTGIKKG EFFLGDLNTQ LKNNNVTTDI KVDTSSN-LF ATVTIDEP--
      Gossypium raimondii VDAC1.1   AITSAGTKKG ELFLADVNTQ LKNKNVTTDI KVDTYSN-LY TTITVDQP--
                Saruma henryi   TITSTGLKKG ELFLADVNTQ LKSRNFTTDI KVDTNSN-LF TTITVDEP--
                 Brassica rapa   AITSTGTKKG DSLLGDVSLQ LKQKNITTDL KVSTDST-VL ITATVDEA--
         Liriodendron tulipifera   AITSTGTKKG ELFLADITNQ LKNKNITTDI KVDTNSR-VF TTITVNEP--
     Saccharum officinarum VDAC1   AVTASSTKKA DLIFGEIQSQ IKNKNITVDV KANSESNITT TVTVDEIA--
             Panicum virgatum   AVTATSTKKA DLIFGEIQSQ IKNKNVTIDV KANSGSNVIT TVTVDEIA--
               Lactuca sativa   SITSSGAKKG ELFLADVNTK LINKNITTDV KVDTNSKVFT TITIDE-A--
        Nicotiana tabacum POM34   AITSSGLKKG DLFLADVNTQ LKNKNVTTDI KVDTNSN-LL ATVTVDEA--
        Eschscholzia californica   AITSSGTKKG ELFLADITNQ LKNKNITTDV KVDTNSR-VF TTITVDEP--
                 Beta vulgaris   AFTSTAVKNG GLSAGDVGAL YKYKNVMADV KLHTESS-IS TTFTIADI--
               Cycas rumphii   VFTSTGTKKG ELFVGELNTQ LKNNNITTDI KVDTSSN-LF TTVTIDEP--
          Glycine max VDAC1.2   AITSSGTKKG DLFVADVNTQ LKNKNITTDI KVDTGSN-LF TTITVNEP--
              Hordeum vulgare   DLLYRDYCTH QKFTLTTCTP E-GVAITAA- ----------G TRKNESIF--
   Lycopersion esculentum VDAC2   DLLTKDY--- ---------I A-DQ------ ----------- -----KLS--
                Citrus sinensis   DLLYKDYQSD HKFTITT-YS P-TGVAITS- ----------S GTKKGELF--
      Gossypium raimondii VDAC1.3   DLLYKDYQAD HKFTLTT-YT S-NGVAITS- ----------S GTKKGELL--
                 Picea glauca   DLLNKDYNFD QKFSFTS-YT S-TGLAFTS- ----------T GIKKGEFF--
         Welwitschia mirabilis   DLLYKDYLVD QKFSVTT-YT S-SGLAFTS- ----------T GVKKGDLF--
             Acorus americanus   ---------- --------TA --NGVAITS- ----------T GTKKGDIT--
      Selaginella moellendorffii   DLLNKDYIYN HKFTVTT-TT S-SGLTFTS- ----------T GTRKGESL--


                                         . . . . . . 110 . . . . . . . . 120 . . . . . . . . 130 . . . . . . . . 140 . . . . . . . . 150
              Oryza sativa VDAC1   TPGLKSILSF AVPDQRSG-- KFELQYSHDY AGVSASIGLT ASPVVNLSSV
            Triticum aestivum VDAC1   APGLKTILSF AVPDQKSG-- KVELQYLHDY AGINASIGLT ANPVVNLSGA
                 Triticum aestivum   APGLKTILSF AVPDQKSG-- KVELQYLHDY AGINASIGLT ANPVVNLSGA
                  Hordeum vulgare   APGLKTILSF AVPDQKSG-- KVELQYLHDY AGINASIGLT ANPVVNLSAA
               Zea mays VDAC2   TPGLKAILCV PFPYQKSA-- KAELQYLHHH AGVAASVGLN ANPVVNLSGV
               Oryza sativa VDAC2   TPGLKGILSI PFPYQKSA-- KAEVQYLHPH AGLNAIVGLN ANPLVSFSGV
            Triticum aestivum VDAC2   TPGLKGVLSL PFPYQKSTPG KAELQYLHPH LGINGSVGLN SNPLVNFSGV
                   Horeum vulgare   KPGLKGVLSL PFPYQKSTPG KAELQYLHPH LGINGSVGLN PNPLVNISGV
                 Triticum aestivum   TPGLKGVLSL PFPYQKSTPG KAELQYLHPH LGINGSVGLN PNPLVNFSGV
        Asparagus officinalis VDAC1   TPGLKSIISF VVPDQKSG-- KVELQYFHEY AGVNASIGLT ANPVVNLAGV
            Triticum aestivum VDAC3   TPGLKSILSL VVPDQRSG-- KLELQYLHEF AGINASVGLN PNPMVNLSGV
                 Triticum aestivum   TPGLKSILSL VVPDQRSG-- KLELQYLHEF AGINASVGLN PNPMVNLSGV
             Zea mays plastid porin   TPGLKSIINL VVPDQRSG-- KLEFQYLHEY AGVNASVGLN SNPMVNLSGA
             Zea mays plastid porin   TPGLKSIINL VVPDQRSG-- KLEFQYLHEY AGVNASVGLN SNPMVNLSGA
          Sorghum bicolor plastid porin   TPGLKSILSL VVPDQRSG-- KFELQYLHEY AGVNASIGLN PNPMVNISGV
     Saccharum officinarum plastid porin   APGLKSILSL VVPDQRSG-- KLELQYLHKY AGVNASIGLN PNPMVNLSGV
               Oryza sativa VDAC3   TPGLKSILSL VVPDQRSG-- KLELQYLHEY AGINASVGLN SNPMVNLSGV
              Zea mays VDAC1a   TPGLKTILSF AVPDQRSG-- KVELQYLHDY AGVNASIGLT ANPVVNLSAA
              Zea mays VDAC1b   TPGLKTILSF AVPDQRSG-- KVELQYLHDY AGVNASVSLT ANPVANLSAG
            Citrus paradisi VDAC1   APGLKSIFSF IVPDQRSG-- KVELQYQHEY AGISTGIGFT ANPIVNFSGV
         Nicotiana tabacum POM36   APGLKTIVSF VLPDQRSG-- KVELQYLHEY AGISTGIGLT ASPLVNFSGV
         Spinacia oleracea VDAC1   APGLKAIFSF RVPDQRSG-- KVELQYLHEY AGISTSLGLT ANPIVNFSGV
   Mesembryanthemum crystallinum   APGLKTILSF RVPDQRSG-- KLELQYLHDY AGICTSVGLT ANPIVNFSGV
   Lycopersion esculentum VDAC1.3   APGLKTIFSF IFPDQKSG-- KVELQYQHEY AGISTSIGLT ASPIVNFSGI
               Prunus armeniaca   APGLKAIFSF IVPDQRSG-- KVELQYQHEY AGISTSIGLT ANPIVNFSGV
         Solanum tuberosum VDAC1   APGLKTIFSF VVPDQKSG-- KVELQYLHEY AGINTSIGLT ASPLVNFSGV
    Lycopersion esculentum POM36   APGLKTIFSF VVPDQKSG-- KVELQYLHEY AGINTSIGLT ASPLVNFSGV
             Populus trichocarpa   APGLKTIFSF KVPDQRSG-- KVELQYQHEY AGISTSIGLT ANPIVNFSGV
           Vitis vinifera VDAC1.1   APGLKSIFNF KVPDQRSG-- KVELQYLHDY AGISTSIGLT ANPIVNFSGT
          Helianthus annuus POM34   APGLKAIFSF KVPDQRSG-- KLELQYLHDY AGIATSIGLT ANPIVNFSGV
         Solanum tuberosum POM34   APGLKTILSF RVPDQRSG-- KLEVQYLHDY AGICTSVGLT ANPIVNFSGV
    Lycopersion esculentum POM34   APGLKTILSF RVPDQRSG-- KLEVQYLHDY AGICTSVGLT ANPIVNFSGV
    Lycopersion esculentum POM34   APGLKTIFSF RIPDQSSA-- KMELQYLHDY AGICTSVGLT ANPIVNFSGV
         Solanum tuberosum POM34   APGLKTIFSC RIPDQSSA-- KMELQYLHDY AGICTSVGLT ANPIVNFSGV
       Lotus corniculatus VDAC1.1   APGLKAIFSF RVPDQRSG-- KVELQYLHDY AGISSSIGLT ANPIVNFSGV
          Glycine max VDAC1.1   APGLKAIFSF KVPDQRSG-- KVEVQYLHDY AGISTSVGLT ANPIVNFSGV
       Lotus corniculatus VDAC1.2   APGLKSIFSF RVPDQRSG-- KVELQYLHDY AGISTSVGLT PNPIVNFSGV
      Medicago truncatula VDAC1.1   APGLKTILSF RIPEPELKSG KAEVQYLHDY AGISASIGLT ANPVANFSGV
                 Picea glauca   APGLKTILSF TVPDQRSG-- KVELQYLHDY AGVSTSIGLT PSPITHFSGV
                  Pinus pinus   APGVKAIFSF TVPDQRSG-- KVELQYLHDY AGVSASIGLT PSPITHFSGA
                  Pinus pinus   APGLKAILNF TVPDQRSG-- KVELQYLHDY AGISTSIGLT ATPVVESSVV
             Populus trichocarpa   APGLKTIFSF KVPDQRSG-- KVEIQYLHDY AAVSSSVGLT VNPTVNFSGV
          Gycine max VDAC1.3   APGLKTIFSF NFPDQRSG-- KVELQYQHEY AGINTSIGLT ANPVVNFSGV
     Medicago truncatula VDAC1.3   VPGLKTIFSF IYPEQKSG-- KVELQYLHDY AGISTSIGLT ATPTVNISAV
      Arabidopsis thaliana VDAC3   APGLRSIFSF KVPDQNSG-- KVELQYLHEY AGISTSMGLT QNPTVNFSGV
         Pisum sativum plastid porin   APGVKAILSE KVPEQRSG-- KLEFQYLHEY AGVNASVGLN SNPMVNLSGA
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Species	Sequence
Pisum sativum plastid porin	APGVKAILSF KVPEQTSG-- KVELQYLHEY AGISSSVGLK ANPIVNFSSV
Arabidopsis thaliana VDAC3	APGLKVIVQA KLPDHKSG-- KAEVQYFHDY AGISTSVGFT ATPIVNFSGV
Brassica rapa	TPGLKAIVSA KVPDQKSA-- KVELQYMPH AGICTSVGLT ANPVVNFSGV
Solanum habrochaites VDAC1	APGLKTILSF RVPDQRSG-- KLEVQYLHDY AGICTSVGLT ANPIVNFSGV
Citrus reticulata VDAC1	APGLKSIFSF IVPDQRSG-- KVELQYQHEY AGISTGIGFT ANPIVNFSGV
Gossypium raimondii VDAC3.1	WPCSKAALSF RIPDHKSG-- KLDVQYLHPH AAIDSSIGLN PTPLLELSAT
Vitis vinifera VDAC3.1	LPCTKVACSF KIPDHKSG-- KLDVQYLHPH AAIDSSIGLN PTPLLELSAA
Solanum tuberosum VDAC3	FRSTKVSLGF NIPDHKSG-- KLDVQYLHQH AAINSSIGLN PSPLLELSAA
Lotus corniculatus VDAC3.1	FRCKKVALSF NIPDHKSG-- KLDVQYLHPH AAIDSSIGLN PSPQLELSTA
Glycine max VDAC3.1	LHGTKAALSF NIPDHKSG-- KLDVQYLHPH AAVDSSIGLN PSPLLELSAA
Medicago truncatula VDAC3.1	SHSKKVALSF NIPDHKSG-- KLDVQYLHPH AAIDSSIGLN PAPKLELSAA
Allium cepa	VDGGKAILSF KLPDHKSG-- KLDLHYANGY AAVSSSTGLT PAPLLEFSAA
Pinus pinus	IPHAKTIFSF KIPDQKSG-- KVDLQYFHDH VGLSSSIGLT PTPIVEVSGA
Pinus taeda	IPGAKTVFSF KLPDDKSG-- KLEVQYLHEH VGLSSSIGLT PKPVVDFSTA
Hordeum vulgare VDAC3.1	VPGLKTSFGF KVPDQKSG-- KLDLQYLHDR FALNSTIGLT SAPVIELATT
Sorghum bicolor VDAC3.1	LTGLKTSFAF KVPDHKSG-- KLDLQYAHNR FSLNSTIGLT STPLVELAAT
Oryza sativa POM36	LTGLKTSFSF RVPDQKSG-- KLDLQYLHDH FALNSTIGLT STPLIELAAT
Arabidopsis thaliana POM36	LPSAKAVISF KIPDHKSG-- KLDVQYVPH ATLNSSIGLN PTPLLDLSAT
Pennisetum glaucum	LPSTKLVTSV KLPDYNSG-- KVELHYFHEN ASLATVVGTK PSPVVELSGT
Zea mays	LPCTKLVTSV KLPDYNSG-- KVELQYFHEN ATFATVVGTK PSPVIDLSGT
Triticum aestivum	LPSTKLVTSI KFPDYNAG-- KVEVQYFHDH ATFAAAVGMK PSPVVDFSLV
Vitis vinifera VDAC2.1	IPSTKTIASF KFPDYNSG-- KLEVQYFHDH ATLTTSVALN QSPAVDLSVT
Solanum tuberosum VDAC2	APSTKTIASL KFPDYSSG-- KLEVQYYHHH AAFSTAVGLK QSPIVDLSVT
Glycine max VDAC2.1	LPSTKTIASF KLPDYNSG-- KLEVQYFHDH ATLTTAVALK QSPVIDVSAT
Lotus corniculatus VDAC2.1	LPSTKAIASF KLPDYNSG-- KLEVQYFHDH ATLTAAAALN QSPIIDVSAT
Gossypium raimondii VDAC2.1	LPSTKTIASF KVPDYNAG-- KLEVQYFHHH ATFTTTIALY KTPVVDATAT
Medicago truncatula VDAC2.1	FPSTKTIASI KLPDYSSG-- KLEVQYFHDH ATLTSVATLK QSPIFDVSAT
Arabidopsis thaliana VDAC3	LPSTKAIASF KVPDYNSA-- KLEVQYFHDH ATVTAAAALK QNPLIDITAT
Ananas comosus	TPGLKTIFSF VVPDQRSG-- KVELQYLHDY AGVNASIGLT ANPVVNLAGV
Saccharum officinarum VDAC3.1	LTGLKTSFAF KVPDHKSG-- KLDLQYAHNR FSLNSTIGLT STPLVELAAT
Antirrhinum majus	APGLKTILSF RVPDQRSG-- KLELQYLHEY AGICSSVGLT ANPIVNFSGV
Welwitschia mirabilis	APGLKSILNF TFPDQKSG-- KVEVQYKHDY AGISSSIGLT ATPVANFSGV
Allium cepa	TPGLKSILTF VVPDQKSG-- KVELQYFHDY AGINASIGLT ASPIVNLSGI
Zantedeschia aethiopica	APGFKTILSF VVPVYQKSG-- KVELQYLHDY AGISASFGLT ANPIVNFSGV
Lycopersion esculentum VDAC3.1	FRSTKLSLGF NIPDHKSG-- KLDVQYLHQH AAINSSIGLN PSPLLELSVA
Triphysaria versicolor	TPGLKAILGF RVPDQRSG-- KLELQYLHEY AGVSSSVGLT ANPIVNFSGA
Picea sitchensis	APGLKTILSF TVPDQRSG-- KVELQYLHDY AGVSTSIGLT PSPITHFSGV
Gossypium raimondii VDAC1.1	APGLKAIFSF RVPDQRSG-- KVELQYLHEY AGISSSIGLT ANPIVNLSGV
Saruma henryi	TPGLKAIFNF VVPDQKSG-- KVELQYLHDN AGINTSVGLT SNPTINFAGV
Brassica rapa	APGLKSIFSF RAPDQNSG-- KIELQYLHEY AGISTSMGLT QNPTVNFSGV
Liriodendron tulipifera	APGLKTIVSF IVPDQKSG-- KVELQYLHDY AGIATSIGLT ANPVVNFSGV
Saccharum officinarum VDAC1	TPGLKTILSF AVPDQRSG-- KVELQYLHDY AGVNASIGLT ANPVVNLSAA
Panicum virgatum	TPGLKTILSF AVPDQRSG-- KFELQYLHDH AGVNASIGLT ANPVVNLSGS
Lactuca sativa	APGLKTIFSF VVPDQRSG-- KVELQYLHEH AGISTSIGLT ASPIVNFSGV
Nicotiana tabacum POM34	APGLKTILSF RIPDQSSA-- KLEVQYLHDY AGISTSLGLT ANPIVNFSGV
Eschscholzia californica	APGLKTIFNF VVPDQRSG-- KVELQYQHEY AGITTSIGLT ANPIVNFSGV
Beta vulgaris	LPSTKAIASF KIPNYSSG-- KLEVQYFHNH ATFTTAFALK QSPIVDVTAT
Cycas rumphii	TPGLKTIVSF TVPDQRSG-- KVEVQYLHDY AGIGTSIGLT ASPIVEASAV
Glycine max VDAC1.2	APGLKTIFSF RVPDQRSG-- KVEVQYLHDY AGISTSVGLT ANPIVNFSGV
Hordeum vulgare	GELQTQLKNK NLTVDI---- KANSESDLLT TFTVDE---- ----FATPG
Lycopersion esculentum VDAC2	IS--TYSDTG VALTST---- AVKKGGLSTG DVGAAL---- -----XNIRT
Citrus sinensis	LADVNTQLKN KNITTD---- LKVDTASNLF TTITVD---- ----EPAPG
Gossypium raimondii VDAC1.3	LADISTELKN KNITTN---- VKVDTSSKLF ATVTVD---- ----EPAPG
Picea glauca	LGDLNTQLKN NNVTTD---- IKVDTSSNLF ATVTID---- ----EPAPG
Welwitschia mirabilis	LGDLSTQLKT KNVTTD---- VKVNTDSKVF ATVTVD---- ----EPTPG
Acorus americanus	FGEIQTQLKN KNVTTD---- IKVNTDSSVF TTVTVD---- ----DAAPG
Selaginella moellendorffii	GGELCSQFKH YNITTD---- VKVDTNSNVY ATVTGN---- ----EVSQG

Species	Sequence
Oryza sativa VDAC1	FGTKALAVGA DVSLD--TAT GNLTKYNAGL SFSNDDLIAS LNLNNKGDSL
Triticum aestivum VDAC1	FGTSALAVGA DVSLD--TAT KNFAKYNAAL SYTNQDLIAS LNLNNKGDSL
Triticum aestivum	FGTSALAVGA DVSLD--TAT KNFTKYNAAL SYTNQDLIAS LNLNNKGDSL
Hordeum vulgare	FGTSALAVGG DVSLD--TAT KNFTKYNAAF SYTNQDLIAS LNLNNKGDSL
Zea mays VDAC2	FGTKAIAVGA DAAFD--TSS GDLTKYNAGL SYTTPDFVAA ATLNNKGDNI
Oryza sativa VDAC2	FGTGAFAVGT DVAFD--TAT GDFTKYNAGL SHTTPDLTAA LLLNNKGDSL
Triticum aestivum VDAC2	IGTKAFAFGV DVAFD--TAS GDFTKYNAGL SHTNQDLTAS LNLNNKVNTL
Horeum vulgare	IGTKALAFGL DVAFD--TAS GDFTKYNAGM SHTNQDLTAS LMLNNKGNTL
Triticum aestivum	IGTKAFALGI DVAFD--TAS GDFTKYNAGL SHTNQDLTAS LNLNNKANTL
Asparagus officinalis VDAC1	FGSGALAVGA DVSFD--TAT GNFTKYNAGL SLINSDLVAA LNLNNKGDSL
Triticum aestivum VDAC3	FGSKELSVGV DVSFD--TAT SNFTKYNAAL SLTYPDLIAS LHLNNHGDTL
Triticum aestivum	FGSKELSVGV DVSFD--TAT SNFTKYNAAL SLTYPDLIAS LHLNNHGDTL
Zea mays plastid porin	FGSKALSVGV DVSFD--TAT SDFTKYNAGL SLTSPDLIAS LHLNNHGDTL
Zea mays plastid porin	FGSKALSVGV DVSFD--TAT SDFTKYNAAL SLTSPDLIAS LHLNNHGDTL
Sorghum bicolor plastid porin	FGSKALSVGV DVSFD--TAT SNFTKYNAAL SLTSPDLIAS LHLNNHGDTL
Saccharum officinarum plastid porin	FGSKALSVGI DVSFD--TAT SNFTKYNAAL SLTSPDLIAS LHLNNHGDTL

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                                              .....|160|....|....|170|....|....|180|....|....|190|....|....|200
Oryza sativa VDAC3                     FGSKELSVGV DVAFD--TAT SNFTKYNAAL SLTNSDLIAS LHLNNHGDTL
Zea mays VDAC1a                        FGTKAVALGA DISLD--TAT GNLTKYNAAL RFTHEDLVAS LNLNNKGDSL
Zea mays VDAC1b                        FGTKALAVGA EISFD--SAT GNFTKYNAGL MFTHEDLIAS LNMNNKGDNL
Citrus paradisi VDAC1                  VGNNSVALGT DLSFD--TAT GNFTKCNAGL SYTHTDLIAS LTLNDKGDTL
Nicotiana tabacum POM36                AGNNTVALGT DLSFD--TAS GNFTKCNAGL SFSTSDLIAS LALNDKGDTL
Spinacia oleracea VDAC1                FGNSTLALGT DLSFD--TAS GNFTKCNAGL SFTNDDLIAS VNVNDKLDIL
Mesembryanthemum crystallinum          LGTNLLAFGT DVSFD--SKT GNFTKCNAGL SFANADLIAS LTLNDKGDTL
Lycopersion esculentum VDAC1.3         IGNNLVAVGT DLSFD--TAS GNFTKYNAGL NVTHADLIAS LTLNDKGDSL
Prunus armeniaca                       VGNNLLSLGT DLSFD--TAS GNFTKCNAGL NFTHTDLIL NDKADTV
Solanum tuberosum VDAC1                AGNNTVALGT DLSFD--TAT GNFTKCNAGL SFSSSDLIAS LALNDKGDTV
Lycopersion esculentum POM36           AGNNIVALGT DVSFD--TAT GNFTKCNAGL SFSSSDLIAS LALNDKGDTL
Populus trichocarpa                    VGSNVVALGT DLSFD--TAT GNFTSYTNSD LIAS LTVNDKGDTL
Vitis vinifera VDAC1.1                 IGTNVVALGT DLSFD--TKS GNFTKCNAGV SFCNADLIAS LTVNDKGDAV
Helianthus annuus POM34                VGTNLAAIGT DVSFD--TKT GNFTKCNAGI SFSNADLIAA LTLNDKGETL
Solanum tuberosum POM34                VGTNIIALGT DVSFD--TKT GDFTKCNAGI SFTNADLVAS LNLNNKGDNL
Lycopersion esculentum POM34           VGTNIVALGT DVSFD--TKT GDFTKCNAGL SFTNADLVAS LNLNNKGDNL
Lycopersion esculentum POM34           LGTNVLALGT DVSFD--TKA GAITKCNAGL RFTNADLITS LNLNNKGDSL
Solanum tuberosum POM34                LGTNVLALGT DVSFD--TKA GALTKCNAGL SFTNADLITS LNLNNKGDSL
Lotus corniculatus VDAC1.1             IGTNVLALGA DLTFD--TKI GELTKSNAGL SFTKDDLIAA LTLNDKGDAL
Glycine max VDAC1.1                    IGTNVLALGA DLSFD--TKI GELTKSNAGL SFTKDDLIAA LTLNDKGDAL
Lotus corniculatus VDAC1.2             VGTNALAVGT DLSFD--TKI GELTKFNAGL NFTKADLIAS LTVNDKGDSL
Medicago truncatula VDAC1.1            IGNNVVALGG DLSYD--TKT GVFTRLNAGL NFTKDDLIAS LTLNEKANAL
Picea glauca                           IGNDTFSVGG EVSFD--TAS GNFLKYDAGL SFSKPDFVSS LTLTGKGDTL
Pinus pinus                            IGNDTFAVGG EVSFD--TAS GNFLKYDAGL SFSKPDFVSS LTLTGKGDTL
Pinus pinus                            IGNESVALGG EFAFD--TAS GNFLKYNAAV NFVQPDFISS LNLTDRGDTL
Populus trichocarpa                    IGTNVASLGT DLSFD--TKT GDFIKCNAGV SLSKVDLIAS LTLNDKGDSL
Gycine max VDAC1.3                     VGNNLVAVGT DLSFD--TAS GNFTKYNAGL NITHADLIAS LTLNDKGDNL
Medicago truncatula VDAC1.3            IGNNLVSVGS DVSFE--TSS GSLNKCNFGL NVTHADLIAS LTVNDRGDSL
Arabidopsis thaliana VDAC3             IGSNVLAVGT DVSFD--TKS GNFTKINAGL SFTKEDLIAS LTVNDKGDLL
Pisum sativum plastid porin            FGSKALSVGV DVSFD--TAT SDFTKYNAV NFVKDSLIGS LTLNEKGDLL
Pisum sativum plastid porin            IGTNALAFGA DISFD--TKL GELTKSNAAV NFVKDDLIGS LTLNEKGDLL
Arabidopsis thaliana VDAC3             VGTNGLSLGT DVAYN--TES GNFKHFNAGF NFTKDDLTAS LILNDKGEKL
Brassica rapa                          IGTSVLALGT DVSFD--TES GNFKHFNTGV SFTKDDLIAS LTLNDKGEKL
Solanum habrochaites VDAC1             VGTNIVALGT DVSFD--TKT GDFTKCNAGL SFTNADLVAS LNLNNKGDNL
Citrus reticulata VDAC1                VGNNSVALGT DLSFD--TAT GNFTKCNAGL SYTHTDLIAS LTLNDKGDTL
Gossypium raimondii VDAC3.1            IGSKELALGG EIGFD--TAS ASFTEYTAGI NLNKPDFSVA LLLTDKGQAL
Vitis vinifera VDAC3.1                 IGSKDLSLGG EVGLN--TAS ASFTKYNAGI GITKPDFSVA VLLTDKGETL
Solanum tuberosum VDAC3                IGSKDLALGG EIGFN--TAS SSFTKCNAGI SFNKPDFSAA LMLTDRGQAV
Lotus corniculatus VDAC3.1             IGSKDLCMGA EVGFN--TTS ASFTKYNAGI TFNRPDFSAA LMLVDKGKSL
Glycine max VDAC3.1                    IGSKDLCLGA EVGFN--TTS ASFTKYNAGV AFNKPDFSVA LMLADKGQTL
Medicago truncatula VDAC3.1            IGSKDISMGA EVGFD--TTS ASFSTYNAGI AFNKPDFSAT LMLADKGQSL
Allium cepa                            TGTKDITLGA EIAFD--SAS ASFSKYNAGV MLTKPDFSAS LILADKGETL
Pinus pinus                            LGYDEISVGG EMAFD--TAS GTFLKYNAGL SKPEYTAA VILADKGDTV
Pinus taeda                            IGSDVFTVGG EVGFD--TSS GLFTKYNAAI GITKPFSAA VIFADKGDTI
Hordeum vulgare VDAC3.1                IGTNELAVGG EVGFD--STS ASVTKYNSGI SYTKDDFSAA LLLADKGETL
Sorghum bicolor VDAC3.1                VGTSELTIGA EVGFD--STS AAVTKYNSGI GYNKSDFSAS LLLADKGETL
Oryza sativa POM36                     IGTNELSAGA EVGFD--STS ASVTKYNSGI CYNKHDFSAA VLLADKGETL
Arabidopsis thaliana POM36             IGSQNVCLGG EVSFD--TAS SSLTKYNAGI GFNNQGVSAA LILEDKGESL
Pennisetum glaucum                     VGAQGVTFGA EAGYD--TAS GKFTKYTAAI GLTKPDYHAA FILADKGDTI
Zea mays                               VGAQGVAFGA EAGYD--TAT GKFTKYTAAI GVTKPDYHAA FILADKGDTI
Triticum aestivum                      VGPKGLAFGG EAGFD--TSS GKFTKYTGGF SVTKPDFHAS AILADKGDTI
Vitis vinifera VDAC2.1                 IGTPSIAFGA EAGYD--TTS GCFTKYTAGI SVAKPDSCAS IILGDKGDTI
Solanum tuberosum VDAC2                LGTPTFAIGA EASYE--TAE GKLAKYTAGI SVTKPDSCAA IILGDKGDTI
Glycine max VDAC2.1                    VGTPTIALGA EAGYD--TSS GGFTKYTAGI SVTRPDSASV IILGDKGDSI
Lotus corniculatus VDAC2.1             FGTPVIAVGA EAGYD--TSS GGFTKYTAGI SVTKPDSASA SIILGDKGDSI
Gossypium raimondii VDAC2.1            IGTPSIAFGA EAGYD--PTK GNFTKYTAGI SVTKPDSCAS IILGDKGDSI
Medicago truncatula VDAC2.1            VGSPTLAFGA EAGYD--TKS GSFTKYNTGI SFTKLDSSAS VIIGDKGDSI
Arabidopsis thaliana VDAC3             LGSPVKTFTGA EAGYD--TTS KFTFTKYNAGI SVTKPDACLS IILGDKGDTI
Ananas comosus                         VGTKALSVGA DVSFD--TAT GNFTKYNAGL SVTNADLVAA LTLSNKGDSL
Saccharum officinarum VDAC3.1          VGTSELTIGA EVGFD--STS ATVTKYNSGI GYNKSDFSAS LLLADKGETL
Antirrhinum majus                      LGNNKLAIGS DVSFD--TKE GALTKCNFGA SFTNSDLIAA LTLNDKGDTL
Welwitschia mirabilis                  FGNDNFALGG EVEFD--TAS GGLLKCNAGL SYSKPDFVSS FTVTNKGDVL
Allium cepa                            FGSGALSVGA DVAFD--TAT GDFTKYNAGL SYSNADLIAS LTLNNKGESL
Zantedeschia aethiopica                LGTNTLAAGV DLSFD--TSS GQFTKYNAGV NFTNADLIAS VTLNDKGDNL
Lycopersion esculentum VDAC3.1         IGNKDLALGG EIGFN--TAS SSFTKCNAGL SFNKPDFSAA LMLTDRGQAV
Triphysaria versicolor                 LGNNYLALGS DLSYD--TKE GAFTKCNFGA SFTNADLIAA LTLNDKGDTL
Picea sitchensis                       IGNDTFSVGG EVSFD--TAS GNFLKYDAGL SFSKPDFVSS LTLTGKGDTL
Gossypium raimondii VDAC1.1            IGTNVLALGT DLSFD--TKT GNFTKCNAGL SFTNADLIAS LTLNDKGDSL
Saruma henryi                          VGNSCVQAGA DVSFD--TGT GDLTKVNGGI SYSNADLIAS FTLNDKADRL
Brassica rapa                          VGTNVLALGT DVSFD--TKS GNFTKINAGV NFTKDDLIAS LTLNDKGDSV
Liriodendron tulipifera                VGSNVLAVGA DLSFD--TAS GNFTKYNAGL SYSNADLIAS LNLNDKGDNL
Saccharum officinarum VDAC1            FGAKALAVGA DVSLD--TAT GNLTKYNAGL RFTHEDLIAS LTLNDKGDRL
Panicum virgatum                       FGTKAVAVGA DISFD--TAS GSLTKYNAGL SITHEDLIAS LNLNNKGDSI
Lactuca sativa                         AGNNTVAVGT DISFD--TAS GNFTKYNAGL SFSTSDLIAS LTLNDKGDTL
Nicotiana tabacum POM34                VGTNLLSLGT DLSFD--TKV GAITKCNAGL SFTKSDLIAS WNLNDKGDAL
Eschscholzia californica               IGSNILALGT DLSFD--TAS GNLTKLHAGV NFSHADLIAS VTLNDRGDSI
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Multiple sequence alignment (residues ~155–250)

Block 1 (positions 160–200):

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                                           160        170        180        190        200
Beta vulgaris                     LGTPTIAFGV EAGYD--ANS GNFTKYTAGV NVSKPESCAS ILLGDKGDTI
Cycas rumphii                     IGNESLAFGV EVAFD--TAS GNFTKYNAGL SFVKPDFISS LILTDKGDLL
Glycine max VDAC1.2               VGTNVLALGT DVSFD--TKI GELTKFNAGL NFTKDDLVAS LTVNTKV---
Hordeum vulgare                   LKSILSLVVP DQRSGK-LEL QYLHEFASVN ASVGLNPSPM VNLSGVF-GS
Lycopersion esculentum VDAC2      PLIDVKVDTR IKFLAG-XRL NIFTITPTFT TAVALKQCPA VDLSITL-GT
Citrus sinensis                   LKTILSFKVP DQRS---GKV ELQYLHDYAG ISTSVGLTAN PIVNFSA-VI
Gossypium raimondii VDAC1.3       LKTIFSFIAP DQRSGKVELQ YQHEYAGIST SIGLTANPLV NFSGVVG-NN
Picea glauca                      LKTILSFTVP DQRSGK-VEL QYLHDYAGVS TSIGLTPSPI THFSGVI-GN
Welwitschia mirabilis             LKAILSFTIP DQKSGK-VEL QYYHDYAGVS AVIGMTASPV VETSVAV-GN
Acorus americanus                 LKTILSFVVP DQKSGK-AEI QYLHDYAGIT SSIGLTANPV VNMSGVI-GS
Selaginella moellendorffii        VKAIIGVNLP NVSTGK-VEV HYVHDNVGIT SAVGLTASPP VDVTAAF-GN
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Block 2 (positions 210–250):

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                                             210        220        230        240        250
Oryza sativa VDAC1                  TASYYHIVNH S-ATAVGAEL THSFSSNENS LTFGTQHTLD P---------
Triticum aestivum VDAC1             TASYYHIVEK S-GTAVGAEL THSFSSNENS LTFGTQHTLD P---------
Triticum aestivum                   TASYYHIVEK S-GTAVGAEL THSFSSNENS LTFGTQHTLD P---------
Hordeum vulgare                     TASYYHFVEK S-STAVGAEL THSFSSNENS LTFGTQHTLD P---------
Zea mays VDAC2                      AASYYHSVSP T--TAVGGEL SHSFSTNGNT ITFGTQHALD P---------
Oryza sativa VDAC2                 AASYYHKVSK T--SAVGAEL AHSFSSNENT LTFGTQHALD E---------
Triticum aestivum VDAC2             AASYYHQVQR T--TAVGAEI AHSFSSNENT ITIGTQHELD P---------
Horeum vulgare                     AASYYHQVQL T--TAVGAEI AHSFSSNENT ITVGTQHELD P---------
Triticum aestivum                   AASYYHQVQR T--TAVGAEI AHSFSSNENT ITVGTQHELD P---------
Asparagus officinalis VDAC1        SASYYHVVSP LTSTAVGAEM THSFSSNENT LTFGTQHALD P---------
Triticum aestivum VDAC3             TASYYHLVKY TSSTAVGAEL SHNFPRNEST LIFGSQHSLD P---------
Triticum aestivum                   TASYYHLVKS HSSTAVGAEL SHNFPRNEST LIFGSQHSLD P---------
Zea mays plastid porin              VASYYHLVKN HSGTAVGAEL SHSMSRNEST LIFGSQHSLD P---------
Zea mays plastid porin              VASYYHLVKN HSGTAVGAEL SHSMSRNEST LIFGSQHSLD P---------
Sorghum bicolor plastid porin       VGSYYHLVKH HSGTAVGAEL SHSISRNEST LIFGSQHSLD P---------
Saccharum officinarum plastid porin VASYYHLVKH HSGTAVGAEL SHSVSRNEST LIFGSQHSLD P---------
Oryza sativa VDAC3                  IASYYHLVKH HSNTAVGAEL SHSFSRNEST LIFGSQHSLD P---------
Zea mays VDAC1a                     TGAYYHKVSE LTNTAVGAEL THSFSTNENT LTFGGQHALD P---------
Zea mays VDAC1b                     TGAYYHKVSE LTNTAVGAEL THSFSTNENT LTFGGQHTLD P---------
Citrus paradisi VDAC1               NASYYHIVSP LTNTAVGAEL THSFSSNENT LTIGTQHALD P---------
Nicotiana tabacum POM36             SASYYHTVKP VTNTAVGAEL THSFSSNENT LTIGTQHLLD P---------
Spinacia oleracea VDAC1             NASYYHIVSP LTNTAVGAEL SHSFSSNENT LNIGTQHALD P---------
Mesembryanthemum crystallinum       NASYYHTISP LTSSAVGAEV AHSFSTNQNT ITLGAQHAID P---------
Lycopersion esculentum VDAC1.3      NASYYHVVSP LTNTAVGAEL SHSFSSNENI LTIGTQHALD P---------
Prunus armeniaca                    TASYYHTVSP LTNTAVGAEL SHSFSSNENS LTIGTQHALD P---------
Solanum tuberosum VDAC1             SASYYHTVKP VTNTAVGAEL THSFSSNENT LTIGTQHLLD P---------
Lycopersion esculentum POM36        SASYYHTVKP VTNTAVGAEL THSFSSNENT LTIGTQHLLD P---------
Populus trichocarpa                 SASYYHTVSP LTNTAVGAEL SHSFSSNENI LTIGTQHALD P---------
Vitis vinifera VDAC1.1              NASYFHSVNL LTNTAVGAEV THSFSTNQNT LTLGAQHKLD P---------
Helianthus annuus POM34             NASYYHIVKP LTNTSVGAEV RHNFSTNENT ITVGTQHALD P---------
Solanum tuberosum POM34             TASYYHTVSP LTSTAVGAEV NHSFSTNENI ITVGTQHRLD P---------
Lycopersion esculentum POM34        TASYYHTVSP LTSTAVGAEV NHSFSTNENI ITVGTQHRLD P---------
Lycopersion esculentum POM34        SASYYHIVSP LTSTAVGAEV THSFSTSENT ITVGTQHQLD P---------
Solanum tuberosum POM34             SASYYHIVTP LTSTAVGAEV THSFSTSENT ITVGTQHQLD P---------
Lotus corniculatus VDAC1.1          NASYYHVVSP LTNTAVGAEV THRFSTNENT LTLGTQHALD P---------
Glycine max VDAC1.1                 NAAYYHVVNP LTNTAVGAEV THRFSTNENT LTLGTQHQLD P---------
Lotus corniculatus VDAC1.2          NASYYHIVNP LTNTAFGAEV THRFSTNENT LTLGTQHALD P---------
Medicago truncatula VDAC1.1         NASYYHVVNP VTKTAVGAEV SHRFSTKENT LTLGTQHAID S---------
Picea glauca                        KASYLHTVSP LTKTAVGAEI SHSFSRSENT FTIGTQHALD P---------
Pinus pinus                         KASYLHTVSP LTKTAVGAEI SHSFSRNENT FTIGTQHALD P---------
Pinus pinus                         KASYLHIVSP LTKTAVGAEI AHSISRNENT FTMGTLHAFD P---------
Populus trichocarpa                 NASYYHIVNP LT--AVGAEV SHSFSSNENT ITVGAQHALD P---------
Gycine max VDAC1.3                  TASYYHIVNP LTNTAVGAEL SHSFSGNENI LTFGTQHALD P---------
Medicago truncatula VDAC1.3         NASYYHVVSP LTNTAVGAEL SHSFSSNENV LTIGTQHALD P---------
Arabidopsis thaliana VDAC3          NASYYHIVNP LFNTAVGAEV SHKLSSKDST ITVGTQHSLD P---------
Pisum sativum plastid porin         SASYYHAINP LSNTAVGVDI SHRFSTKEND FTLGTQHALD P---------
Pisum sativum plastid porin         SASYYHAINP LSNTAVGVDI SHRFSTKENT FTLGTQHALD P---------
Arabidopsis thaliana VDAC3          NASYYQIVSP S--TVVGAEI SHNFTTKENA ITVGTQHALD P---------
Brassica rapa                       NASYYHIVNP LKNTVVGAEV SHNLKSQVNS ITVGTQHALD P---------
Solanum habrochaites VDAC1          TASYYHTVSP LTSTAVGAEV NHSFSTNENI ITVGTQHRLD P---------
Citrus reticulata VDAC1             NASYYHIVSP LTNTAVGAEL THSFSSNENT LTIGTQHALD P---------
Gossypium raimondii VDAC3.1         KASYIHSVNP F--TCVAAEM AHRFSTYENT FTIGSSHAVD P---------
Vitis vinifera VDAC3.1              KASYIHSVNP PGGTTVAAEI THKFNTYENS FTIGSAHVVD P---------
Solanum tuberosum VDAC3             KASYVHSVDP ANGTEVGAEM IHRLSTYENS FSIGSSHKVD P---------
Lotus corniculatus VDAC3.1          KASYIHHVDR PDGFTVAAEI NHRFSSFENR FTIGSSQLID P---------
Glycine max VDAC3.1                 KASYIHYVDR PDGFTVAAEI SHRFSSLENR FTIGSSQSID P---------
Medicago truncatula VDAC3.1         KASYIHYVDR PDGLTVAAEL AHKLSSSENR FTFGTSQSID P---------
Allium cepa                         KASYIHVNP ASENAVAAEM VHRFNTFENS FTIGSSHKID T---------
Pinus pinus                         KASYTHIVNP ISKTAVAAEF THKFSTNENT YTVGSSHAID Q---------
Pinus taeda                         KASYLHELNP ITKTCFAAEI SRKFSANQNT FTVGSSYALD P---------
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                                           . . . . . . . . .210. . . . . . . . .220. . . . . . . . .230. . . . . . . . .240. . . . . . . . .250
        Hordeum vulgare VDAC3.1    KASYIHLFSPT--SAVAAEVMHRLKTKENYFTIGSSHALDP---------
        Sorghum bicolor VDAC3.1    KASYIHLFNPTNGATVAAEVTHKFKTKENYFTVGSSHALDP---------
              Oryza sativa POM36    KASYIHTFNETNGATVAAEVTHKLKTKENYFTIGSSHAIDS---------
       Arabidopsis thaliana POM36   RATYVHTVNPT--TSFGAELIRRFSNYNNSFTVGSSHSVDQ---------
              Pennisetum glaucum    KVSGVYHLDEKQKASAVAELTRRLSTNVNTLTVGGLYKIDP---------
                        Zea mays    KVSGVYHLDEKQKASAVAELTRRLSTNQNTLTVGGLYTVDP---------
                Triticum aestivum   KVSGVYHLDEKQKASAVAEFTRKLSTNENTLTVGGLYTVDP---------
           Vitis vinifera VDAC2.1   RASYVHHLDSLKKSAAVGEISRRFSTNDNTFTVGGSYAIDP---------
           Solanum tuberosum VDAC2  KASYIHHLDELKKSAAVGEITRRFSTNENTFTVGGSYAVDN---------
              Glycine max VDAC2.1   KASYLHHLDLLKKSAAVAEITRKFSTNENIFTVGGSFAVDP---------
         Lotus corniculatus VDAC2.1 KASYLHHLDQLKKSAAVAEITRKFSTNENTFTVGGSFAIDH---------
        Gossypium raimondii VDAC2.1 KASYVHYLDNLKKSAAVGEISRKFSTNENTFTVGGAYAVDH---------
        Medicago truncatula VDAC2.1 KVSYLHHLDQIKKSAAVVDITRRFSTNQNTITVGGSFAVDP---------
        Arabidopsis thaliana VDAC3  KASYLHHFDEFKRTAAVGEVYRKFSTNENTITVGGLYAIDH---------
                  Ananas comosus    GASYYHLVSPL--SAVGAELTHSFASSENTLTFGTQHQLDP---------
      Saccharum officinarum VDAC3.1 KASYIHLFNPTNGATVAAEVTHKFKTKENYFTIGSSHALDP---------
                  Antirrhinum majus SASYYHTVSPFTNTAVSAEVTHSFSSNENTITVGTQHSLDP---------
              Welwitschia mirabilis KASYLHTVSPLTKTAVGAEIQHSCSKSENTLTIGTQHALDP---------
                      Allium cepa   SASYYHLVNPLSNTAVGAEMTHSFTTNESTLTFGTQHVLDP---------
             Zantedeschia aethiopica TAAYYHLVSPLMSTAVGAELAHCFSSNENTLTFGTQHALDP---------
   Lycopersion esculentum VDAC3.1   KASYVHLVDPTNGTEVGAEMIHRLSTYENSFSIGSAHKVDS---------
             Triphysaria versicolor SASYYHVVNPLTNTAVGAEVTHSFSSNENTITVGTQHTLDP---------
                  Picea sitchensis  KASYLHTVSPLTKTAVGAEISHSFSRSENTFTIGTQHALDP---------
        Gossypium raimondii VDAC1.1 NASYYHIVNPMTNTAVGAEVTHSFSTNENTITIGTQHALDP---------
                     Saruma henryi  TASYFHIVSPLTNTAVGAELSHCFSSNDNTLTIGTQHALDP---------
                      Brassica rapa NASYYHIVNPLFNTAVGAEVNHKFSTNVNTITVGTQHSLDP---------
              Liriodendron tulipifera KASYYHTVRPLTSTAVGAELSHSFTSNENTLTIGTQHALDP---------
       Saccharum officinarum VDAC1  TGAYYHKVSELSNTAGGAELTHSFSTIENTLTF-----GG---------
                  Panicum virgatum  TAAYYHNVNLLTSTAVGAELTHSFSTNENTLTFGTQHALDP---------
                    Lactuca sativa  TASYYHTVSPTKRLWGGELTHGFSSNENSVDFWDTALVGS---------
            Nicotiana tabacum POM34 SASYYHIVSPLTYTAVSAEV----PTVS--------------
           Eschscholzia californica NASYYHTVSPL-------------TSTA--------------
                     Beta vulgaris  KASYLHHLDQLNKNAAVAEFS---RRFS--------------
                    Cycas rumphii   KASYLHTVS------------------------P--------
              Glycine max VDAC1.2   --------IP--------------------------------
                 Hordeum vulgare    KELSVGVDVSFDTATSNFTKYNAALSLAY-PDLIASLHLNNHGDTLTAS
   Lycopersion esculentum VDAC2     PTFALGAEASYETATSKLTKYTAGISVTK--PDSCAAIILGDKGDTIKAS
                    Citrus sinensis GTNVLSLGTDLSFDSKSGNFTKCNAGLSFNNADLIASLNLNNKGDSLAAS
       Gossypium raimondii VDAC1.3  SVTVGTDLSFDTASGNFTKLNAGLNFTHSDLIASLDI--VNDKGDTLNAS
                     Picea glauca   DTFSVGGEVSF--DTASGNFLKYDAGLSFSKPDFVSSLTLTGKGDTLKAS
              Welwitschia mirabilis ENVALGGEIAF--DTTSSNLTKYNAGISYIKPDFILGVNWTDKGDAVKAS
                  Acorus americanus NVLSVGTDVSF--DTATGNFLKYNAGLSFTNADLIAALNLNNKGDTLVAS
        Selaginella moellendorffii  KEYSLGGAVSL--DTAAGTVKKYEAGLGITKPDFTAALLLCDKGDTVKVS
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                                    . . . . . . . . .260. . . . . . . . .270. . . . . . . . .280. . . . . . . . .290. . . . . . . . .300
              Oryza sativa VDAC1    -------------------------------------------LTVVKARFNNSG
           Triticum aestivum VDAC1  -------------------------------------------LTLVKARINNSG
                Triticum aestivum   -------------------------------------------LTLVKARINNSG
                  Hordeum vulgare   -------------------------------------------LTLVKARINNSG
                  Zea mays VDAC2    -------------------------------------------LTTVKARFNNYG
                Oryza sativa VDAC2  -------------------------------------------LTTVKARFNNFG
            Triticum aestivum VDAC2 -------------------------------------------LTTVKGRYNNFG
                  Horeum vulgare    -------------------------------------------LTTLKGRYNNFG
                Triticum aestivum   -------------------------------------------LTTVKGRYNNFG
      Asparagus officinalis VDAC1   -------------------------------------------LTTVKARFDNYG
            Triticum aestivum VDAC3 -------------------------------------------HTSVKARFNNYG
                Triticum aestivum   -------------------------------------------HTSVKARFNNYG
              Zea mays plastid porin -------------------------------------------HTTIKTRFNNYG
              Zea mays plastid porin -------------------------------------------HTTIKTRFNNYG
         Sorghum bicolor plastid porin -------------------------------------------HTTVKARFNNYG
   Saccharum officinarum plastid porin -------------------------------------------HTTVKARFNNYG
              Oryza sativa VDAC3    -------------------------------------------HTTVKARFNNYG
                  Zea mays VDAC1a   -------------------------------------------LTVLKARINNSG
                  Zea mays VDAC1b   -------------------------------------------LTVLKARINNSG
             Citrus paradisi VDAC1  -------------------------------------------LTSVKARVNNYG
           Nicotiana tabacum POM36  -------------------------------------------LTTVKARVNSYG
             Spinacia oleracea VDAC1 -------------------------------------------LTSVKARVNNYG
      Mesembryanthemum crystallinum -------------------------------------------LTNLKGRVNNFG
   Lycopersion esculentum VDAC1.3   -------------------------------------------ITLLKARVNNYG
                  Prunus armeniaca  -------------------------------------------LTTVKGRVNNYG
           Solanum tuberosum VDAC1  -------------------------------------------LTTVKARVNSYG
     Lycopersion esculentum POM36   -------------------------------------------LTTVKARVNSYG
                Populus trichocarpa -------------------------------------------LTTVKARLNNYG
            Vitis vinifera VDAC1.1  -------------------------------------------LTTVKARVNNFG
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Multiple sequence alignment (residues ~260–300):

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                                            . . . . . . . . . . 260 . . . . . . . . . 270 . . . . . . . . . 280 . . . . . . . . . 290 . . . . . . . . . 300
Helianthus annuus POM34              ---------- ---------- ---------- -------- L T   T V K A R I D N L G
Solanum tuberosum POM34              ---------- ---------- ---------- -------- L T   S V K A R I N N F G
Lycopersion esculentum POM34         ---------- ---------- ---------- -------- L T   S V K A R I N N F G
Lycopersion esculentum POM34         ---------- ---------- ---------- -------- L T   T V K A R V N N F G
Solanum tuberosum POM34              ---------- ---------- ---------- -------- L T   V K A R V N N F G
Lotus corniculatus VDAC1.1           ---------- ---------- ---------- -------- L T   T L K A R V N N F G
Glycine max VDAC1.1                  ---------- ---------- ---------- -------- L T   T L K A R V N N F G
Lotus corniculatus VDAC1.2           ---------- ---------- ---------- -------- L T   S V K A R V N N L G
Medicago truncatula VDAC1.1          ---------- ---------- ---------- -------- L T   T A K L R F N N F G
Picea glauca                         ---------- ---------- ---------- -------- L T   T V K G R L N N Y G
Pinus pinus                          ---------- ---------- ---------- -------- L T   T V K G R L N S Y G
Pinus pinus                          ---------- ---------- ---------- -------- F T   T V K A R L N N H G
Populus trichocarpa                  ---------- ---------- ---------- -------- L T   T L K A R V N N A G
Gycine max VDAC1.3                   ---------- ---------- ---------- -------- L T   L L K S R V N N Y G
Medicago truncatula VDAC1.3          ---------- ---------- ---------- -------- V T   L L K A K V N N Y G
Arabidopsis thaliana VDAC3           ---------- ---------- ---------- -------- L T   S V K A R V N S A G
Pisum sativum plastid porin          ---------- ---------- ---------- -------- L T   T V K G R V T N S G
Pisum sativum plastid porin          ---------- ---------- ---------- -------- L T   T V K G R V T N S G
Arabidopsis thaliana VDAC3           ---------- ---------- ---------- -------- L T   T V K A R V N N A G
Brassica rapa                        ---------- ---------- ---------- -------- L T   T V K A R V N N A G
Solanum habrochaites VDAC1           ---------- ---------- ---------- -------- L T   S V K A R I N N F G
Citrus reticulata VDAC1              ---------- ---------- ---------- -------- L T   S V K A R V N N Y G
Gossypium raimondii VDAC3.1          ---------- ---------- ---------- -------- F T   V V K T R F F D N G
Vitis vinifera VDAC3.1               ---------- ---------- ---------- -------- Y T   M M K T R L S N N G
Solanum tuberosum VDAC3              ---------- ---------- ---------- -------- L T   S L K T R F S D N G
Lotus corniculatus VDAC3.1           ---------- ---------- ---------- -------- N T   V L K T R F S D D G
Glycine max VDAC3.1                  ---------- ---------- ---------- -------- K T   V L K T R F S D D G
Medicago truncatula VDAC3.1          ---------- ---------- ---------- -------- K T   V L K T R F S D D G
Allium cepa                          ---------- ---------- ---------- -------- A T   M M K T R F S S N G
Pinus pinus                          ---------- ---------- ---------- -------- L T   T M K S R L D N R G
Pinus taeda                          ---------- ---------- ---------- -------- L T   T M K T R F N N H G
Hordeum vulgare VDAC3.1              ---------- ---------- ---------- -------- S T   T V K T R F S N S G
Sorghum bicolor VDAC3.1              ---------- ---------- ---------- -------- S T   L L K T R F S N S G
Oryza sativa POM36                   ---------- ---------- ---------- -------- S T   L L K T R F S N G G
Arabidopsis thaliana POM36           ---------- ---------- ---------- -------- F T   V V K T R F S N S G
Pennisetum glaucum                   ---------- ---------- ---------- -------- Q T   A V K A R L N N T G
Zea mays                             ---------- ---------- ---------- -------- Q T   A L K A R L N N T G
Triticum aestivum                    ---------- ---------- ---------- -------- Q T   A V K A R L N N T G
Vitis vinifera VDAC2.1               ---------- ---------- ---------- -------- L T   N V K A K L N N H G
Solanum tuberosum VDAC2              ---------- ---------- ---------- -------- L T   I V K L K L N N H G
Glycine max VDAC2.1                  ---------- ---------- ---------- -------- L T   Q V K A R L N N H G
Lotus corniculatus VDAC2.1           ---------- ---------- ---------- -------- L T   Q V R A R L N N H G
Gossypium raimondii VDAC2.1          ---------- ---------- ---------- -------- L T   L I K A K L N N H G
Medicago truncatula VDAC2.1          ---------- ---------- ---------- -------- L T   Q I K A R F N N Q G
Arabidopsis thaliana VDAC3           ---------- ---------- ---------- -------- S T   A V K A K L N N H G
Ananas comosus                       ---------- ---------- ---------- -------- L T   T V K A R F N N Y G
Saccharum officinarum VDAC3.1        ---------- ---------- ---------- -------- S T   L L K T R F S N S G
Antirrhinum majus                    ---------- ---------- ---------- -------- L T   T V K A R V N N F G
Welwitschia mirabilis                ---------- ---------- ---------- -------- L T   T V R A R L D N Y G
Allium cepa                          ---------- ---------- ---------- -------- L T   M V K A R I N N Y G
Zantedeschia aethiopica              ---------- ---------- ---------- -------- L T   S V K A R V N N F G
Lycopersion esculentum VDAC3.1       ---------- ---------- ---------- -------- L T   S L K T R F S D N G
Triphysaria versicolor               ---------- ---------- ---------- -------- L T   S V K A R V N N F G
Picea sitchensis                     ---------- ---------- ---------- -------- L T   T V K G R L N N Y G
Gossypium raimondii VDAC1.1          ---------- ---------- ---------- -------- L T   M V K A R V N N A G
Saruma henryi                        ---------- ---------- ---------- -------- L T   S V K A R L N N F G
Brassica rapa                        ---------- ---------- ---------- -------- L T   T V K A R V N S - -
Liriodendron tulipifera              ---------- ---------- ---------- -------- L T   S V K A R - V - - -
Saccharum officinarum VDAC1          ---------- ---------- ---------- -------- R -   - - - - - - - - - -
Panicum virgatum                     ---------- ---------- ---------- -------- L T   - - - - - - - - - -
Lactuca sativa                       ---------- ---------- ---------- -------- V D   - - - - - - - - Y -
Nicotiana tabacum POM34              ---------- ---------- ---------- -------- - - -
Eschscholzia californica             ---------- ---------- ---------- -------- - - -
Beta vulgaris                        ---------- ---------- ---------- -------- - - -
Cycas rumphii                        ---------- ---------- ---------- -------- L T   - - - - R L Q L E -
Glycine max VDAC1.2                  ---------- ---------- ---------- -------- - - -
Hordeum vulgare                      YYHLVKSHSS TAVGAELSHN FPRNESTLIF GSQHSLDPHT S V K A R F N N Y G
Lycopersion esculentum VDAC2         YIHHMDALKT TAATGEITRR FSTNENTFTV GGSYAVDCLT I V K L K L N N H G
Citrus sinensis                      YYHFVNPLT- -AVGAEVIHS FSTTDNTITV GTQHILDPLT T L K A R V N N A G
Gossypium raimondii VDAC1.3          YYHIVSPLTN PTVGAELSHS FSSNENPFPI GTQHAFDPLT T V K A X L N N Y G
Picea glauca                         YLHTVSPLTK TAVGAEISHS FSRSENTFTI GTQHAXXXLT Y V X R L N N Y G
Welwitschia mirabilis                YLQHIDPSKN TTVGVEACHS ISXNENTFTL GAQHALDPLT M V K V R C N S H G
Acorus americanus                    YYHLVSPLTS TAVGVEMTHS FSTNENTLTI GTQHALXPLT T V K G R V N N Y G
Selaginella moellendorffii           YSHTVSPLTK ATVGAEVVHS ISKKLNTFTH RGAYNLDLLT T V K A R L N N X G
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| Species	Sequence
Oryza sativa VDAC1	KASALLQHEW RPKSVWTISA EVDT-KAIDK SSKVGIAVAL KP
Triticum aestivum VDAC1	KASALLQHEF MPKSLCTISA EVDT-KAIEK SSKVGIAIAL KP
Triticum aestivum	KASALLQHEF MPKSLCTISA EVDT-KAIEK SSKVGIAIAL KP
Hordeum vulgare	KASALLQHEF RPKSLCTISA EVDT-KAIEK SSKVGIAIAL KP
Zea mays VDAC2	MASALLQHEW RPKSLVTIST EVDT-KAIEK SSKVGLSLVL KP
Oryza sativa VDAC2	MASALLQHEF RPKSLVTIST EVDT-KAIDK SSKVGLSLVL KP
Triticum aestivum VDAC2	VASALLQHAW RPKSLITFST EVDT-KAIEK SPKFGLALSL KP
Horeum vulgare	IASALLQHAW RPKSVITFST EVDT-KAVEK SPKFGLALSL KP
Triticum aestivum	IANALLQHAW RPKSLITFST EVDT-KAIEK SPKFGLALSL KP
Asparagus officinalis VDAC1	RASALLQHQW RPKSFVTISG EVDT-KAIEK SSQVGLSLVL KP
Triticum aestivum VDAC3	MASALVQHEW RAKSLVTISG EVDT-KAIEK STKVGLSLVL KP
Triticum aestivum	MASALVQHEW RAKSLVTISG EVDT-KAIEK STKVGLSLVL KP
Zea mays plastid porin	MASALVQHEW RPKSFVTISG DVDT-KAIEK STKVGLSLVL KH
Zea mays plastid porin	MASALVQHEW RPKSFVTISG DVDT-KAIEK STKVGLSLVL KH
Sorghum bicolor plastid porin	MASALVQHEW RPKSFITISG EVDT-KAIEK STKVGLSLVL KH
Saccharum officinarum plastid porin	MASALVQHEW RPKSFITISG EIDT-KAIEK STKVGLSLVL KH
Oryza sativa VDAC3	MASALVQHEW RPKSLITISG EVDT-KAIEK STKVGLSLVL KH
Zea mays VDAC1a	KASALLQHEW RPKSLVTISA EVDT-KTIEK SSKVGIAVAL KP
Zea mays VDAC1b	KASVLLQHEW RPKSLVTISA EVDT-KTIEK SSKVGIAVAL KP
Citrus paradisi VDAC1	RASALLQHEW RPKSLFTISG EVDT-RAIEK SAKIGLALAL KP
Nicotiana tabacum POM36	KASALLQHEW RPKSLFTISG EVDT-RAIEK SAKIGLAVAL KP
Spinacia oleracea VDAC1	KASALLQHEW RPKSIVTISG EVDT-SAIEK SAKLGLALVL KP
Mesembryanthemum crystallinum	KATALLQHEW RPKSLVTFSA EVDT-KAIEK SAKVGLALAL KP
Lycopersion esculentum VDAC1.3	RASALLQHEW NPRTRVSLVG EVDT-GAIEK SAKVGLALAL KP
Prunus armeniaca	RASALLQHEW RPKSFFTISG EVDT-RAIEK SAKIGLALAL KP
Solanum tuberosum VDAC1	KASALLQHEW RPKSLFTISG EVDT-RAIEK SAKIGLAVAL KP
Lycopersion esculentum POM36	KASALLQHEW RPKSLFTISG EVDT-RAIEK SAKIGLAVAL KP
Populus trichocarpa	KVSALLQNEW RPKSLFTISG EVDT-KAIEK SAKVGLALSL KP
Vitis vinifera VDAC1.1	KASALLQHEW RPRSLFTISG EVDT-KAVDK TAKVGLALAL KP
Helianthus annuus POM34	KANALLQHEW RPKSLFTISG EVDT-KAVDK SAKFGLALAL KP
Solanum tuberosum POM34	KASALLQHEW RPKSLFTVSG EVDT-KSVDK GAKFGLALAL KP
Lycopersion esculentum POM34	KASALLQHEW RPKSLFTVSG EVDT-KSVDK GAKFGLALAL KP
Lycopersion esculentum POM34	MATALLQHEW RPKSLFTISG EVDT-KAVDK SAKFGLALAL KP
Solanum tuberosum POM34	TATALLQHEW RPKSLFTISG EVDT-KAVDK SAKFGLALAL KP
Lotus corniculatus VDAC1.1	KASALLQHEW RPKSFFTISG EVDT-KAIEK SAKVGLALAL KP
Glycine max VDAC1.1	RTSALLQHEW RPKSFFTISG EVDT-KAIEK SAKVGLGLVL KP
Lotus corniculatus VDAC1.2	KANALLQHEW RAKSFFTISG EVDT-KAIEK SAKIGLGLAL KP
Medicago truncatula VDAC1.1	LASGLLQHEW RPKSFFTISG EVDT-KAIEK SARIGIALAL K-
Picea glauca	KAAALLQHEW RPKSFVTLSG EVDT-KALHN SAKVGLSLIL KP
Pinus pinus	RAAALLQHEW RPKSFVNLSG EVDT-KALHN SAKVGLSLVL KP
Pinus pinus	KIAALVQHEW RPKSLITLSG EVDS-KALDN SSKIGLSLVL KP
Populus trichocarpa	KASALVQHQW RPKSFFTVSG EVDT-KAIEK TAKVGLALAL KP
Gycine max VDAC1.3	RASALLQHDW TPRTRFSLVG EVDT-GAIEK SAKVGLAVAL KP
Medicago truncatula VDAC1.3	KASALLQHDW SRQARFSLVG EVDT-AAIQK TAKVGLACAL KP
Arabidopsis thaliana VDAC3	IASALLQHEW KPKSFFTISG EVDT-KSIDK SAKVGLALAL KP
Pisum sativum plastid porin	KASALLQHEW RPKSLITISS EVDT-KAIEK SAKIGLSLAL K-
Pisum sativum plastid porin	KASALLQHEW RPKSLITISS EVDT-KAIEK SAKIGLSLAL KP
Arabidopsis thaliana VDAC3	VANALLQHEW RPKSFFTVSG EVDS-KAIDK SAKVGIALAL KP
Brassica rapa	IANALLQHEW RPKSFITISG EVDS-KAIEK SAKVGFALAL KP
Solanum habrochaites VDAC1	KASALLQHEW RPKSLFTISG EVDT-KSVD- ---------- --
Citrus reticulata VDAC1	RASALLQHEW RPQSLFTISG EVDT-RAIE- ---------- --
Gossypium raimondii VDAC3.1	KVAMLCQREW RPKSLVTFSA EYDS-KAINA SPKMGLALAF KP
Vitis vinifera VDAC3.1	KFVMLCQREW RPKSLITVSG EYDP-KANNG ATKLGLALAL KP
Solanum tuberosum VDAC3	KVAMLCQREW RPKSLITFSA EYDT-KANNS VPKLGLALAL KP
Lotus corniculatus VDAC3.1	KAAFLCQRAW RPKSLITLSA EYDSTKIFGS STKLGLALAL QP
Glycine max VDAC3.1	KAAFQCQRAW RPNSLITLSA EYDSTKIFGS STKFGLALAL KP
Medicago truncatula VDAC3.1	KAAFQCQRAW RPNSLITLSA EYDSKKIIGS PAKFGLALSL KP
Allium cepa	KFGVDVSMNG RPKSLATVSA EYDC-KAVGA PTKVGVALAL KW
Pinus pinus	KLGALLQHEW RPKSLVTISG EFDT-KALDK SAKIGLSLAL KP
Pinus taeda	KLGTVLQHEW KPKSLITLSS EFDT-KALDK SPKIGLALAY KP
Hordeum vulgare VDAC3.1	KAGLLCQHEW RPKSYVTLSA EYDP-KVVSS PARFGVAVAL SP
Sorghum bicolor VDAC3.1	KVGLLCQHEW RPKSLVTLSA EYDP-KVVSA PSRIGVAISL KP
Oryza sativa POM36	KVGVLCQHEW RPKSTVSISA EYDP-KVVSS PSRFGVAIAL KP
Arabidopsis thaliana POM36	KAGMVVQREW RPKSHITFSA EYDS-KAVTS SPKLGLALAL KP
Pennisetum glaucum	TLAALLQHEL KPKSLLTISG EFDT-KALDR APKFGLALAL KP
Zea mays	TLAALLQHEL KPKSLLTISG EFDT-KALDR SPKFGLALAL KP
Triticum aestivum	KLAALLQHEV KPKSLLTISG EFDT-KALDR APKFGLSLAL KP
Vitis vinifera VDAC2.1	NLGALLQHEI IPKSLLTLSG EFDT-KALDK TPKFGVALAL KP
Solanum tuberosum VDAC2	NLGALLQHEL IPKSLLTISS EFDT-KALEK TPKFGVALAL KP
Glycine max VDAC2.1	KLGALLQHEI IPKSVFTVSG EIDT-KALDK KPRFGLAIAL KP
Lotus corniculatus VDAC2.1	KLGALLQHEI IPKSVLTISS EVDT-KALDK NPRFGLAIAL KP
Gossypium raimondii VDAC2.1	RLGALLQHEV IPKSLLTVSG ELDT-KSLDK SPRLGLAFAL KP
Medicago truncatula VDAC2.1	KVGGLLQHEL FPKSVLTISG EVDT-KALEK KPKFGLSLAL KP
Arabidopsis thaliana VDAC3	TLGALLQHEV LPRSLVTVSS EIDT-KALEK HPRFGLSLAL KP
Ananas comosus	KASALLQHEW RPKSFLTIST EVDT-KAIER APR-----SA YL
Saccharum officinarum VDAC3.1	KVGLLCQHEW RPKSLVTLSA EYDP-KVVSA PSRIGVAISL KP

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	310	320	330	340
Antirrhinum majus	KANALI QHEW RPKSLITVS- ----TVLK P--------- --
Welwitschia mirabilis	KAAALVQHEW RPKSFITISG EVD------- ---------- --
Allium cepa	KASGLI QHPW RANSFVTLS- ---------- ---------- --
Zantedeschia aethiopica	KASALI PQEW RPK------- ---------- ---------- --
Lycopersion esculentum VDAC3.1	KVAMLYQREW RPKSLVTFSA EYDT-KAKSS VPKLGLALAL KP
Triphysaria versicolor	KASALI QHEW RPKSLITLSS EVDT-KSIDK SAKFGLALAL KP
Picea sitchensis	KAAALLQ--- HE-------- ---------- ---------- --
Gossypium raimondii VDAC1.1	NA---- ---- SAL------- ---------- ---------- --
Saruma henryi	KASALI Q--- ---------- ---------- ---------- --
Brassica rapa	----- ---- ---------- ---------- ---------- --
Liriodendron tulipifera	----- ---- ---------- ---------- ---------- --
Saccharum officinarum VDAC1	----- ---- ---------- ---------- ---------- --
Panicum virgatum	----- ---- ---------- ---------- ---------- --
Lactuca sativa	----- ---- ---------- ---------- ---------- --
Nicotiana tabacum POM34	----- ---- ---------- ---------- ---------- --
Eschscholzia californica	----- ---- ---------- ---------- ---------- --
Beta vulgaris	----- ---- ---------- ---------- ---------- --
Cycas rumphii	----- ---- ---------- ---------- ---------- --
Glycine max VDAC1.2	----- ---- ---------- ---------- ---------- --
Hordeum vulgare	MASALVQHEW RPKSLVTISG EVDT-KAIEK STKVGXVIGA KA
Lycopersion esculentum VDAC2	SLGAVLQHEV IPKSLLTISS EFDT-KALDK TPKFGV---- --
Citrus sinensis	IASALI QHEW RPKSLFTISG EVXH-QAIEK SAKFGLALAL KP
Gossypium raimondii VDAC1.3	RASALI QHEW RPKSLISIFG EVDP-GAIEK SAKVGVALAL KP
Picea glauca	KAAALLQHEX XPKSF----- ---------- ---------- --
Welwitschia mirabilis	KVAGLLQHEW RPKSLITIS- ---------- ---------- --
Acorus americanus	KASALI QHQW RPKS------ ---------- ---------- --
Selaginella moellendorffii	KLAALI Q--- ---------- ---------- ---------- --

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As shown in Fig. 6.1, a segment located 5-6 residues from the amino-terminus predicts an α-helix similar to previous secondary structure prediction methods (see Chapter 1, section 1.5). The primary sequences of these putative α-helices are highly conserved within the major phylogenetic groups, and based on the high level of primary sequence conservation are likely the best candidates for refining eukaryotic porin signature motifs.

In all cases, the remainder of the protein is predicted to be rich in β-strands, in agreement with existing models of porin structure (reviewed in Mannella 1997, Benz 1994, De Pinto and Palmieri 1992, Bay and Court 2002, Song *et al.* 1998, Runke *et al.* 2006, Chapter 1). Nineteen regions with β-strand propensity are predicted in the majority of porin sequences (Fig. 6.1), these are numbered 1-19 in Fig. 6.2 and for simplicity will be referred to as β1, β2 etc. for the subsequent discussion. Remarkably, each of these regions corresponds to an aligned segment in the multiple sequence analysis, although the spacing between individual putative β-strands varies between the major phylogenetic groups (Fig. 6.2).

It is unlikely that 19 TM β-strands predicted by SSPRO reflect the true porin TM topology. As discussed in Chapter 1, regions including β1-β7 are in agreement with previous prediction algorithms and models. Interestingly, in an alignment of all 244 sequences, the region including β8 is not always predicted to form a β-strand (data not shown). However, when the plant or fungal sequences are aligned alone, a 6-residue β-strand is predicted in some sequences (residues, 114-119 of *Oryza sativa* VDAC1, and 122-129 of *N. crassa* porin); in others this β-strand region is shorter (3-5 aa) (Fig. 6.1). Six residues are the minimum proposed to be sufficient for the β-strands in bacterial porins to span the thickness of a lipid bilayer (Koebnik *et al.* 2000) but a shorter strand may be possible if two short β-strands or perhaps a short β-strand/ α-helix were to occur in this region. β8 (residues 125-

Figure 6.2. Summary of the secondary structure prediction and analysis. Predicted secondary structure elements in VDAC from the crown groups of plants, animals, and fungi. Predictions were made as described in Materials and Methods. For each summary diagram, the putative N-terminal α-helix is indicated by a blue rectangle labelled "N" on the top, subsequent β-strands are indicated by black filled rectangles, and the intervening loops are shown as thin lines. Boxes in grey indicate β-strands that are weakly supported by experimental data (see text). According to the models described in Chapter 1, the N-terminal α-helix resides in the intermembrane space, and the subsequent loops and turns alternate between exposure to the cytosol and to the intermembrane space. The lower panel shows the model for *Neurospora crassa* VDAC structure derived in (Runke *et al.* 2006). Structural elements are as described for the plant, animal and fungal models, except that the hatched region indicates a C-terminal segment that does not form a TM β-strand (see text for discussion). Below the model of *N. crassa* VDAC structure, rectangles indicate segments, that when absent in porin variants, create VDAC able (white), or unable (red), to form pores in artificial membranes. The arrow indicates that the multiple alignment supports a different placement of β8 in the Runke *et al.* model to the position occupied by β9 in the current predictions. Positions of the GLK and porin signature motifs are also noted.
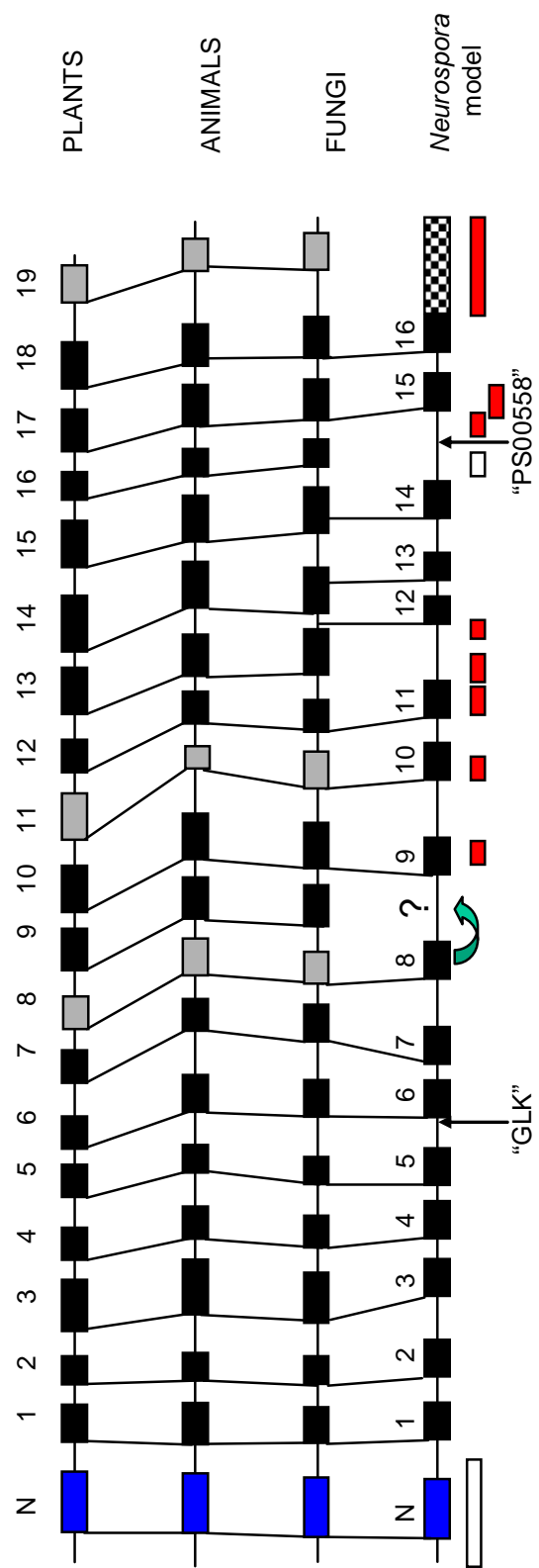
132 in *Homo sapiens* VDAC1) in the animal sequences also contains some α-helical propensity and is unlikely to satisfy this minimum TM β-strand requirement. Furthermore, this region contains a cysteine residue, which is absent from all β-strands in bacterial porins (Koebnik *et al.* 2000). Therefore, if all porins have a common structure, the region including putative β8 may not form a β-strand (Fig. 6.2).

The situation described for β8 in animals is also pertinent for β11 from all three groups, since this TM strand also has some α-helical propensity in some sequences. If these two putative β-strands are "disregarded" in this prediction, another β-strand should be omitted to maintain an even number of β-strands within the barrel (Koebnik *et al.* 2000). As discussed previously, in Chapter 1 (see also Bay and Court 2002, Runke *et al.* 2006), deletion of the β-strand predicted at the C-terminus of *N. crassa* porin resulted in pore formation (Popp *et al.* 1996) and suggests that a β-strand within this region is not critical for pore formation. Hence, β19 may also be omitted from the model (Fig. 6.2).

Based on the size of the data set and representation from all three crown groups, a common structural framework exists amongst the various porins and isoforms (Fig. 6.2). These data support a common, 16 β-strand pore structure, in which the interstrand loops and turns vary in size.

In spite of the predicted structural similarities, there is a great deal of variation in the amino acid sequences that make up the putative strands and the intervening regions. For example, pairwise sequence comparisons between the sequences of predicted β-strands reveal average sequence identity of around 47-61% for each phylogenetic crown group. To demonstrate this sequence diversity, when the regions encoding the predicted β-strands from

one representative of the animals (*Homo sapiens* VDAC1), the fungi (*Neurospora crassa*), the plants (*Oryza sativa* VDAC1) and the stramenopiles (*Phytophthora sojae*) were followed in the alignments of Fig. 6.1. Very few positions were conserved. Considering identical and chemically similar residues, there are at most three positions that are related in three β-strands among all four sequences (β7, β11, β13), and in β8, β14, β17 there are no positions consistently occupied by chemically similar residues. As observed for bacterial porins, structural similarity rather than sequence similarity is favoured in mitochondrial porins (Jap and Walian 1996, Koebnik *et al.* 2000).

6.3.2. Comparison of VDAC gene structure

Given the shared predicted structural and primary sequence elements in mitochondrial porins, it was of interest to examine representative animal, plant, and fungal VDAC genes (including paralogs) for the number of introns present, intron phasing, and the position of introns with respect to functional and structural motifs (Fig. 6.3). The comparative VDAC gene maps show that the vertebrate VDAC open reading frames usually consist of 8 exons and 7 introns, a pattern also observed within the sea urchin (*Strongylocentrolus purpuratus*) VDAC gene. The *Caenorhabditis elegans* VDAC gene has 4 introns and the *Drosophila melanogaster* paralogs contain 2 introns. Thus, the number of introns appears to decrease and the introns get shorter as one goes from the vertebrates/echinoderms/nematodes to the arthropods. The plant VDAC paralogs examined typically had 4 to 5 short introns.

Figure 6.3. **A**) Comparison of the mitochondrial porin gene structure. The exonic and intronic regions of mitochondrial porin open reading frames were mapped for a variety of organisms. The gene structure for all species was retrieved from the NCBI genome database using BLASTN or map viewer programs to analyze the ORF of each porin cDNA. Only regions of the gene containing ORFs are shown; chromosome number is indicated beside the species in Roman numerals and plus (+) or minus (-) strand reading frames are indicated beside chromosome number. Where multiple spliceoforms exist, as for human VDAC2, the form not listed as a splice variant was used. Loci of unknown location are indicated by "Un". Lengths of exons in bp are indicated inside the boxes representing the exons; the numbers of the corresponding amino acid residues are indicated below the boxes. Lines indicate introns, the sizes of which are indicated in kb above the line; the intron phase (0, 1, or 2) is indicated directly above the intron size. The positions of the coding sequence for the GLK (diamonds) and the eukaryotic porin (circles) motifs are shown above the intron/exon map. Filled and open symbols indicate matches and imperfect matches to these motifs, respectively. **B**) Intron placement with respect to coding sequences for conserved structural elements. Intron placement is indicated by vertical bars in the rows above the structural model for each crown group of organisms (see Fig. 6.2 for symbol descriptions). Patterns of intron location that are found in several VDAC genes are shown only once. The positions of the N-terminal motifs derived in this study, and the eukaryotic signature motif (PS00558) near the C-terminal of the protein are indicated by open boxes. Triangles indicate the GLK motif. Upper panel: Animal sequences. Hs, *Homo sapiens* VDAC1 (NP_003365), VDAC2 (NP_003366) and VDAC3 (NP_005653); Rn, *Rattus norvegicus* VDAC1 (NP_112643), VDAC2 (NP_112644) and VDAC3 (NP_112645); Gg, *Gallus gallus* VDAC1 (TC10222), VDAC2 (NM_204741),

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VDAC3 (TC9741): Dr, *Danio rerio* accession numbers NM_ 199585 and BC065468; Dm,

Drosophila melanogaster VDAC1 (CG6647-PA), VDAC2 (609462), and isoform B of

CG31722 (CG31722_PB); Dm-2; *D. melanogaster* isoform A (CG31722_PA); Sp,

Strongylocentrotus purpuratus (sea urchin, XM_775173); Ag, *Anopheles gambiae*

(XM_318947). Middle panel: Fungal sequences. Cn, *Cryptococcus neoformans*

(XM_569804.1); Nc, *Neurospora crassa* (XP_323644). Lower panel: Plant sequences. At,

Arabidopsis thaliana, (NP_186777, NP_201551, NP_200057, NP_197013); At-2 *A. thaliana*

NP_190561; Os, *Oryza sativa* (CAB82853_VDAC1, CAC80850_VDAC2,

CAC80851_VDAC3).

A

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S. purpuratus VDAC (284 aa) — Un +

Phase: ATG 1 0 1 ◆ 2 2 2 ● 1 TAA

Intron (kb): 0.50 0.67 0.78 1.12 0.34 1.31 1.49

Exons: 63 | 54 | 84 | 117 | 108 | 114 | 210 | 93

Residues: 1–21 | 22–40 | 41–69 | 70–109 | 110–146 | 147–185 | 186–256 | 257–284

C. elegans VDAC (283 aa) — IV –

Phase: ATG 1 2 ◆ 2 2 ○ TAG

Intron (kb): 0.07 0.16 0.16 0.04

Exons: 63 | 143 | 174 | 263 | 221

Residues: 1–21 | 22–67 | 68–124 | 125–210 | 211–283

C. neoformans var neoformans VDAC (292 aa) — III –

Phase: ATG 0 0 ◆ 0 ○ 0 TAA

Intron (kb): 0.05 0.08 0.06 0.05

Exons: 30 | 195 | 183 | 280 | 186

Residues: 1–10 | 11–75 | 76–135 | 136–231 | 232–292

N. crassa VDAC (283 aa) — V +

Phase: ATG 0 0 1 ◆ ● 0 TAG

Intron (kb): 0.31 0.14 0.36 0.07

Exons: 44 | 122 | 30 | 502 | 149

Residues: 1–15 | 16–56 | 57–66 | 67–233 | 234–283

A. thaliana VDAC1 NP_18677 (276 aa) — III +

Phase: ATG 1 0 0 ◆ 0 2 ● TGA

Intron (kb): 0.49 0.23 0.11 0.10 0.08

Exons: 48 | 67 | 110 | 86 | 208 | 304

Residues: 1–16 | 17–39 | 40–76 | 77–105 | 106–175 | 176–276

A. thaliana 34 kDa porin NP_201551 (276 aa) — V +

Phase: ATG 1 0 0 ◇ 2 ○ TGA

Intron (kb): 0.23 0.58 0.09 0.08

Exons: 48 | 67 | 110 | 208 | 304

Residues: 1–16 | 17–39 | 40–76 | 77–105 | 106–275

A. thaliana 36 kDa porin NP_200557 (274 aa) — V –

Phase: ATG 1 0 0 ◇ 0 2 ○ TAA

Intron (kb): 0.08 0.29 0.08 0.09 0.07

Exons: 48 | 67 | 110 | 86 | 208 | 302

Residues: 1–16 | 17–39 | 40–76 | 77–105 | 106–175 | 176–274

A. thaliana 34 kDa porin (274 aa) — V –

Phase: ATG 1 0 0 ◆ 0 2 ● TGA

Intron (kb): 0.45 0.18 0.10 0.10 0.09

Exons: 48 | 68 | 110 | 86 | 208 | 87

Residues: 1–16 | 17–39 | 40–76 | 77–105 | 106–175 | 176–274

O. sativa VDAC1 (274 aa) — XI +

Phase: ATG 1 0 0 ◆ 0 2 ○ TGA

Intron (kb): 0.13 0.98 0.37 0.09 0.64

Exons: 45 | 22 | 111 | 86 | 210 | 303

Residues: 1–15 | 16–23 | 23–75 | 76–104 | 105–174 | 175–274

O. sativa VDAC2 (280 aa) — V +

Phase: ATG 1 0 0 ◆ 0 2 ○ TGA

Intron (kb): 0.15 1.32 0.13 0.09 0.39

Exons: 64 | 67 | 110 | 89 | 208 | 301

Residues: 1–21 | 22–44 | 45–81 | 82–111 | 112–181 | 182–280

O. sativa VDAC3 (275 aa) — I –

Phase: ATG 1 0 0 ◆ 0 2 ● TGA

Intron (kb): 0.14 0.67 0.10 0.09 0.17

Exons: 43 | 67 | 110 | 89 | 208 | 307

Residues: 1–14 | 15–37 | 38–74 | 75–104 | 105–174 | 175–275

B

Hs, Rn,
Gg & Dr
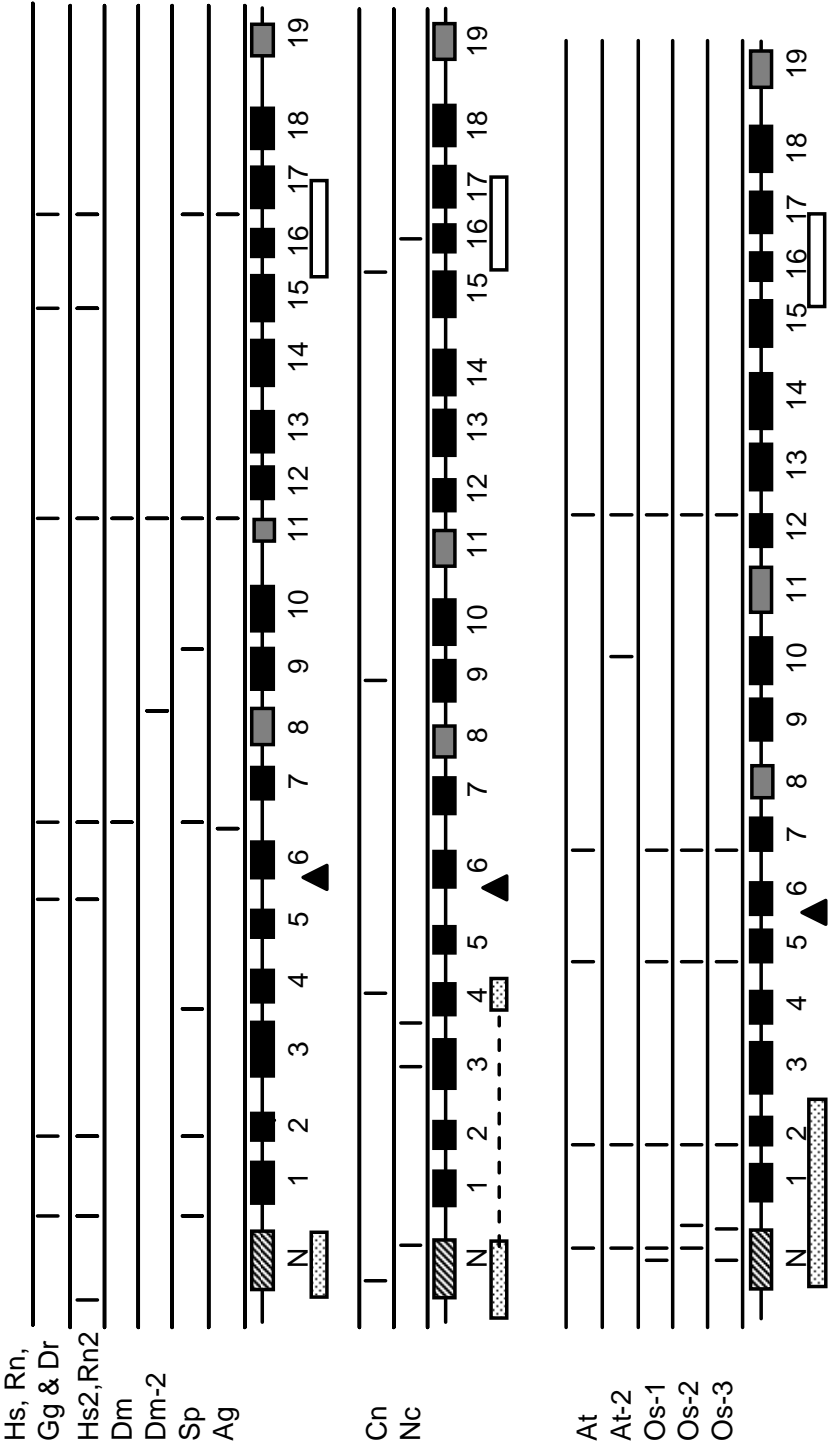Hs2,Rn2
Dm
Dm-2
Sp
Ag

Cn
Nc

At
At-2
Os-1
Os-2
Os-3

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In general, introns were longest within the mammalian sequences sampled and shorter in the invertebrate, plant and fungal taxa (Fig. 6.3A). This contrasts with the shorter exons noted within the chordate sequences, as exemplified by the human, rat, zebra fish and bird (*Gallus gallus*) VDAC genes in which exons range in size from 30 bp (human VDAC2) to 225 bps. There is a general trend among the vertebrate and echinoderm VDACs that, relative to the internal exons, the 5' terminal exons 1 and 2 are very short (30 to 75 bps), as are the 3' terminal exons 7 and 8. However, the echinoderm is an exception, with a relatively longer exon 7 (210 bp).

In the chordates, the largest exon (exon 5) appears to be quite ancient as its size is conserved from zebra fish to humans. Also, exon 5 appears to be flanked by introns that both are in phase 2, *i.e.* intron phase correlation exists, where flanking introns are in the same phase, or are found as a combination of a phase 0 and a phase 2 intron. Intron phase correlation has been viewed as evidence of non-random insertion of an intron and thus interpreted by some as the presence of an ancient intron (reviewed by Fedorova and Fedorov 2003). These observations further suggest that exon 5 and its flanking introns might have been present in the ancestor to the vertebrate lineage. Exon 6 also appears to be quite conserved both in terms of size (150 bp) and in that it is flanked downstream by a phase 0 intron.

Likewise, phase 0 introns are viewed as old by some authors (reviewed by Fedorova and Fedorov 2003). The fungal VDAC genes examined either lack introns (*S. cerevisiae VDAC1* and *VDAC2, Ustilago maydis*) or show a bias towards phase 0 introns; overall it was noted among the fungal VDAC paralogs displayed in Fig. 6.3 seven phase 0 introns and one phase 1 intron. The plant VDACs appear to have more phase 0 introns with a ratio of 21:9:8

for phase 0, 1 and 2 introns respectively. In contrast, there are 29 phase 0, 26 phase 1, and 28 phase 2 introns in vertebrate VDAC paralogs; thus there is not a strong bias towards one category of intron. In contrast to this general trend, the *C. elegans* and Arthropod porin gene sequences appear to be devoid of phase 0 introns and the ratio of phase 1 to phase 2 introns is 2 to 13.

The coding sequences for the sequence motifs discussed above are also conserved with respect to exonic location (Fig. 6.3B). In the animal sequences presented, the N-terminal motif is encoded by sequence within the first exon; the only exceptions are VDAC2 in *H. sapiens* and *Rattus norvegicus*, in which an intron is located near the middle of the coding sequence. The 42-residue plant motif begins near residue six and includes the predicted α-helix, which is encoded by a single exon, and in most cases the coding region extends through exon 2 into exon 3. The exception is the *O. sativa* VDAC1 gene, in which an additional intron is inserted past the codon for amino acid residue 15. The fungal motif also begins about six residues from the N-terminus of the protein and the N-terminal portion of the motif (see Chapter 1) spans 14-18 residues. In both fungal sequences in Fig. 6.3, this portion of the motif is encoded by exons 1 and 2. The C-terminal part of the motif is encoded by sequence including the junction between exons 2 and 3 in *Cryptococcus neoformans*, and entirely by exon 2 in *N. crassa*. In the sequences examined, the entire coding sequence for the GLK motif is in Exon 4, with the exception of VDAC2 of human and rat, in which it resides in Exon 5; VDAC2 has an additional N-terminal intron (see Fig. 6.3A). Exon 6 contains the eukaryotic porin signature motif (PS00558, Fig. 6.3A).

6.3.3 Intron location with respect to functional and structural motifs of VDAC

A more detailed examination of the position of introns with respect to the position of protein domains/or modules, such as the β-strands that are important components of the VDAC structure, showed that these modules appear to correlate with the position of introns. The introns in general do not interrupt β-strand modules or the GLK and eukaryotic porin motifs (Fig. 6.3B). This observation is most pronounced in the vertebrate, sea urchin, *Drosophila* and plant VDAC genes; for example, there appears to be an intron conserved between β6 and β7 and between β11 and β12. Other introns are frequently located between the coding sequences for the N-terminal α-helix and the first β-strand module, between β1 and β2, and between β16 and β17.

Within the plant VDAC genes examined there was a bias towards introns being located between β-strand modules (see Fig. 6.3B). Introns were located between the coding sequences for putative β1 and β2, β4 and β5, β6 and β7, and β12 and β13 strands. In addition, there is generally an intron within the coding region for the N-terminal α-helix as well. Based on the limited data for *C. elegans* and the fungal VDAC genes did not allow conclusions to be made; as more complete sequences become available a more detailed examination of these groups of organisms can be carried out.

6.4 Conclusions

Amongst members of the three groups, and isoforms within individual organisms, the lengths and positions of putative β-strands predicted by PRALINE/ SSPRO appear to be highly conserved. These data support the 16 stranded model of *N. crassa* as a candidate for

porin TM topology, assuming TM β-strand arrangements are as highly conserved within plants, animals and fungi as observed.

Analysis of the porin gene structures in the metazoans in particular has demonstrated that both the GLK and eukaryotic signature motif are located within conserved exons flanked by introns of the same phase (phase correlation). Furthermore, it appears that in general introns do not disrupt exons encoding the structural framework of porin in this case the predicted β-strands. The relevance of these observations will be come clearer as more genomic sequences and structural data become available for mitochondrial porins and other β-barrel, membrane spanning proteins.

References

Abrecht, H., E. Goormaghtigh, J. M. Ruysschaert and F. Homble (2000). "Structure and orientation of two voltage-dependent anion-selective channel isoforms. An attenuated total reflection fourier-transform infrared spectroscopy study." J Biol Chem **275**(52): 40992-9.

Abrecht, H., R. Wattiez, J. M. Ruysschaert and F. Homble (2000). "Purification and characterization of two voltage-dependent anion channel isoforms from plant seeds." Plant Physiol **124**(3): 1181-90.

Ahting, U., C. Thun, R. Hegerl, D. Typke, F. E. Nargang, W. Neupert and S. Nussberger (1999). "The TOM core complex: the general protein import pore of the outer membrane of mitochondria." J Cell Biol **147**(5): 959-68.

Aiello, R., A. Messina, B. Schiffler, R. Benz, G. Tasco, R. Casadio and V. De Pinto (2004). "Functional characterization of a second porin isoform in Drosophila melanogaster. DmPorin2 forms voltage-independent cation-selective pores." J Biol Chem **279**(24): 25364-73.

Al Bitar, F., N. Roosens, J. V. Boxtel, E. Dewaele, M. Jacobs and F. Homble (2002). "Expression of the rice vdac isoform2: histochemical localization and expression level." Biochim Biophys Acta **1579**(2-3): 133-41.

Al Bitar, F., N. Roosens, M. Smeyers, M. Vauterin, J. Van Boxtel, M. Jacobs and F. Homble (2003). "Sequence analysis, transcriptional and posttranscriptional regulation of the rice vdac family." Biochim Biophys Acta **1625**(1): 43-51.

Aljamal, J. A., G. Genchi, V. De Pinto, L. Stefanizzi, A. De Santis, R. Benz and F. Palmieri (1993). "Purification and characterization of porin from corn (*Zea mays L.*) mitochondria." Plant Physiol **102**(2): 615-621.

Anflous, K., O. Blondel, A. Bernard, M. Khrestchatisky and R. Ventura-Clapier (1998). "Characterization of rat porin isoforms: cloning of a cardiac type-3 variant encoding an additional methionine at its putative N-terminal region." Biochim Biophys Acta **1399**(1): 47-50.

Angeles, R., J. Devine, R. Barret, D. Goebel, E. Blachyl-Dyson, M. Forte and R. McCauley (1999). "Mutations in the voltage-dependent anion channel of the mitochondrial outer membrane cause a dominant nonlethal growth impairment." J Bioenerg Biomembr **31**(2): 143-51.

Arora, A., F. Abildgaard, J. H. Bushweller and L. K. Tamm (2001). "Structure of outer membrane protein A transmembrane domain by NMR spectroscopy." Nat Struct Biol **8**(4): 334-8.

Arora, A., D. Rinehart, G. Szabo and L. K. Tamm (2000). "Refolded outer membrane protein A of Escherichia coli forms ion channels with two conductance states in planar lipid bilayers." J Biol Chem **275**(3): 1594-600.

Arora, K. K., C. R. Filburn and P. L. Pedersen (1993). "Structure/function relationships in hexokinase. Site-directed mutational analyses and characterization of overexpressed fragments implicate different functions for the N- and C-terminal halves of the enzyme." J Biol Chem **268**(24): 18259-66.

Arthington-Skaggs, B. A., H. Jradi, T. Desai and C. J. Morrison (1999). "Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of Candida albicans." J Clin Microbiol **37**(10): 3332-7.

Asworth, L. A. E. and C. Green (1966). "Cholesterol is the major sterol in mammalian organisms." Science **151**: 210-211.

Bangham, A. D. (1978). "Properties and uses of lipid vesicles: an overview." Ann N Y Acad Sci **308**: 2-7.

Baker, M. A., D. J. Lane, J. D. Ly, V. De Pinto and A. Lawen (2004). "VDAC1 is a transplasma membrane NADH-ferricyanide reductase." J Biol Chem **279**(6): 4811-9.

Bathori, G., G. Csordas, C. Garcia-Perez, E. Davies and G. Hajnoczky (2006). "Ca2+-dependent control of the permeability properties of the mitochondrial outer membrane and VDAC." J Biol Chem.epub ahead of print.

Bathori, G., M. Sahin-Toth, A. Fonyo and E. Ligeti (1993). "Transport properties and inhibitor sensitivity of isolated and reconstituted porin differ from those of intact mitochondria." Biochim Biophys Acta **1145**(1): 168-76.

Bay, D. C. and D. A. Court (2002). "Origami in the outer membrane: the transmembrane arrangement of mitochondrial porins." Biochem Cell Biol **80**(5): 551-62.

Benz, R. (1994). "Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins." Biochim Biophys Acta **1197**(2): 167-96.

Beutner, G., A. Ruck, B. Riede, W. Welte and D. Brdiczka (1996). "Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore." FEBS Lett **396**(2-3): 189-95.

Blachly-Dyson, E., A. Baldini, M. Litt, E. R. McCabe and M. Forte (1994). "Human genes encoding the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane: mapping and identification of two new isoforms." <u>Genomics</u> **20**(1): 62-7.

Blachly-Dyson, E. and M. Forte (2001). "VDAC channels." <u>IUBMB Life</u> **52**(3-5): 113-8.

Blachly-Dyson, E., S. Peng, M. Colombini and M. Forte (1990). "Selectivity changes in site-directed mutants of the VDAC ion channel: structural implications." <u>Science</u> **247**(4947): 1233-6.

Blachly-Dyson, E., S. Z. Peng, M. Colombini and M. Forte (1989). "Probing the structure of the mitochondrial channel, VDAC, by site-directed mutagenesis: a progress report." <u>J Bioenerg Biomembr</u> **21**(4): 471-83.

Blachly-Dyson, E., J. Song, W. J. Wolfgang, M. Colombini and M. Forte (1997). "Multicopy suppressors of phenotypes resulting from the absence of yeast VDAC encode a VDAC-like protein." <u>Mol Cell Biol</u> **17**(10): 5727-38.

Blachly-Dyson, E., E. B. Zambronicz, W. H. Yu, V. Adams, E. R. McCabe, J. Adelman, M. Colombini and M. Forte (1993). "Cloning and functional expression in yeast of two human isoforms of the outer mitochondrial membrane channel, the voltage-dependent anion channel." <u>J Biol Chem</u> **268**(3): 1835-41.

Bousquet, J. A., C. Garbay, B. P. Roques and Y. Mely (2000). "Circular dichroic investigation of the native and non-native conformational states of the growth factor receptor-binding protein 2 N-terminal src homology domain 3: effect of binding to a proline-rich peptide from guanine nucleotide exchange factor." <u>Biochemistry</u> **39**(26): 7722-35.

Brdiczka, D. (1990). "Interaction of mitochondrial porin with cytosolic proteins." <u>Experientia</u> **46**(2): 161-7.

Brdiczka, D., K. Bucheler, M. Kottke, V. Adams and V. K. Nalam (1990). "Characterization and metabolic function of mitochondrial contact sites." <u>Biochim Biophys Acta</u> **1018**(2-3): 234-8.

Brdiczka, D., P. Kaldis and T. Wallimann (1994). "In vitro complex formation between the octamer of mitochondrial creatine kinase and porin." <u>J Biol Chem</u> **269**(44): 27640-4.

Buettner, R., G. Papoutsoglou, E. Scemes, D. C. Spray and R. Dermietzel (2000). "Evidence for secretory pathway localization of a voltage-dependent anion channel isoform." <u>Proc Natl Acad Sci U S A</u> **97**(7): 3201-6.

Burstein, E. A., E. A. Permyakov, V. A. Yashin, S. A. Burkhanov and A. Finazzi Agro (1977). "The fine structure of luminescence spectra of azurin." <u>Biochim Biophys Acta</u> **491**(1): 155-9.

Burstein, E. A., N. S. Vedenkina and M. N. Ivkova (1973). "Fluorescence and the location of tryptophan residues in protein molecules." Photochem Photobiol **18**(4): 263-79.

Casadio, R., I. Jacoboni, A. Messina and V. De Pinto (2002). "A 3D model of the voltage-dependent anion channel (VDAC)." FEBS Lett **520**(1-3): 1-7.

Cesar Mde, C. and J. E. Wilson (2004). "All three isoforms of the voltage-dependent anion channel (VDAC1, VDAC2, and VDAC3) are present in mitochondria from bovine, rabbit, and rat brain." Arch Biochem Biophys **422**(2): 191-6.

Chang, C. T., C. S. Wu and J. T. Yang (1978). "Circular dichroic analysis of protein conformation: inclusion of the beta-turns." Anal Biochem **91**(1): 13-31.

Cheng, E. H., T. V. Sheiko, J. K. Fisher, W. J. Craigen and S. J. Korsmeyer (2003). "VDAC2 inhibits BAK activation and mitochondrial apoptosis." Science **301**(5632): 513-7.

Chuang, J. Z., T. Y. Yeh, F. Bollati, C. Conde, F. Canavosio, A. Caceres and C. H. Sung (2005). "The dynein light chain Tctex-1 has a dynein-independent role in actin remodeling during neurite outgrowth." Dev Cell **9**(1): 75-86.

Colombini, M. (1980). "Structure and mode of action of a voltage dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane." Ann N Y Acad Sci **341**: 552-63.

Colombini, M., C. L. Yeung, J. Tung and T. Konig (1987). "The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion." Biochim Biophys Acta **905**(2): 279-86.

Compton, L. A. and W. C. Johnson, Jr. (1986). "Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication." Anal Biochem **155**(1): 155-67.

Conlan, S. and H. Bayley (2003). "Folding of a monomeric porin, OmpG, in detergent solution." Biochemistry **42**(31): 9453-65.

Court, D. A., R. Kleene, W. Neupert and R. Lill (1996). "Role of the N- and C-termini of porin in import into the outer membrane of *Neurospora* mitochondria." FEBS Lett **390**(1): 73-7.

Court, D. A., F. E. Nargang, H. Steiner, R. S. Hodges, W. Neupert and R. Lill (1996). "Role of the intermembrane-space domain of the preprotein receptor Tom22 in protein import into mitochondria." Mol Cell Biol **16**(8): 4035-42.

Cowan, S. W., R. M. Garavito, J. N. Jansonius, J. A. Jenkins, R. Karlsson, N. Konig, E. F. Pai, R. A. Pauptit, P. J. Rizkallah, J. P. Rosenbusch and et al. (1995). "The structure of OmpF porin in a tetragonal crystal form." Structure **3**(10): 1041-50.

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Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius and J. P. Rosenbusch (1992). "Crystal structures explain functional properties of two *E. coli* porins." Nature **358**(6389): 727-33.

Cowgill, R. W. (1966). "Fluorescence and protein structure. IX. Relationship between tyrosine fluorescence and various stages in denaturation of ribonuclease." Biochim Biophys Acta **120**(2): 196-211.

Cowgill, R. W. (1967). "Fluorescence and protein structure. X. Reappraisal of solvent and structural effects." Biochim Biophys Acta **133**(1): 6-18.

Cowgill, R. W. (1968). "Fluorescence and protein structure. XIV. Tyrosine fluorescence in helical muscle proteins." Biochim Biophys Acta **168**(3): 417-30.

Cowgill, R. W. (1968). "Fluorescence and protein structure. XV. Tryptophan fluorescence in helical muscle protein." Biochim Biophys Acta **168**(3): 431-8.

Cowgill, R. W. (1968). "Fluorescence and protein structure. XVI. Detergents bound to muscle proteins." Biochim Biophys Acta **168**(3): 439-46.

Cowgill, R. W. (1970). "Fluorescence and protein structure. XVII. On the mechanism of peptide quenching." Biochim Biophys Acta **200**(1): 18-25.

Crompton, M., E. Barksby, N. Johnson and M. Capano (2002). "Mitochondrial intermembrane junctional complexes and their involvement in cell death." Biochimie **84**(2-3): 143-52.

Crompton, M., S. Virji, V. Doyle, N. Johnson and J. M. Ward (1999). "The mitochondrial permeability transition pore." Biochem Soc Symp **66**: 167-79.

Crompton, M., S. Virji and J. M. Ward (1998). "Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore." Eur J Biochem **258**(2): 729-35.

Czub, J. and M. Baginski (2006). "Comparative molecular dynamics study of lipid membranes containing cholesterol and ergosterol." Biophys J **90**(7): 2368-82.

de Kroon, A. I. P. M., D. Dolis, A. Mayer, R. Lill and B. de Kruijff (1997). "Phospholipid composition of highly purified mitochondrial outer membranes of rat liver and *Neurospora crassa*. Is cardiolipin present in the mitochondrial outer membrane?" Biochim Biophys Acta **1325**: 108-116.

de la Maza, A. and J. L. Parra (1996). "Alterations in phospholipid bilayers caused by oxyethylenated nonylphenol surfactants." Arch Biochem Biophys **329**(1): 1-8.

de la Maza, A. and J. L. Parra (1996). "Changes in phosphatidylcholine liposomes caused by a mixture of Triton X-100 and sodium dodecyl sulfate." Biochim Biophys Acta **1300**(2): 125-34.

De Pinto, V., J. A. al Jamal and F. Palmieri (1993). "Location of the dicyclohexylcarbodiimide-reactive glutamate residue in the bovine heart mitochondrial porin." J Biol Chem **268**(17): 12977-82.

De Pinto, V., R. Benz and F. Palmieri (1989). "Interaction of non-classical detergents with the mitochondrial porin. A new purification procedure and characterization of the pore-forming unit." Eur J Biochem **183**(1): 179-87.

De Pinto, V. D. and F. Palmieri (1992). "Transmembrane arrangement of mitochondrial porin or voltage-dependent anion channel (VDAC)." J Bioenerg Biomembr **24**(1): 21-6.

Decker, W. K. and W. J. Craigen (2000). "The tissue-specific, alternatively spliced single ATG exon of the type 3 voltage-dependent anion channel gene does not create a truncated protein isoform in vivo." Mol Genet Metab **70**(1): 69-74.

Diederichs, K., J. Freigang, S. Umhau, K. Zeth and J. Breed (1998). "Prediction by a neural network of outer membrane beta-strand protein topology." Protein Sci **7**(11): 2413-20.

Dolder, M., K. Zeth, P. Tittmann, H. Gross, W. Welte and T. Wallimann (1999). "Crystallization of the human, mitochondrial voltage-dependent anion-selective channel in the presence of phospholipids." J Struct Biol **127**(1): 64-71.

Dornmair, K., H. Kiefer and F. Jahnig (1990). "Refolding of an integral membrane protein. OmpA of *Escherichia coli*." J Biol Chem **265**(31): 18907-11.

Eftink, M. R. (1994). "The use of fluorescence methods to monitor unfolding transitions in proteins." Biophys J **66**(2 Pt 1): 482-501.

Eisele, J. L. and J. P. Rosenbusch (1990). "In vitro folding and oligomerization of a membrane protein. Transition of bacterial porin from random coil to native conformation." J Biol Chem **265**(18): 10217-20.

Elkeles, A., A. Breiman and M. Zizi (1997). "Functional differences among wheat voltage-dependent anion channel (VDAC) isoforms expressed in yeast. Indication for the presence of a novel VDAC-modulating protein?" J Biol Chem **272**(10): 6252-60.

Elkeles, A., K. M. Devos, D. Graur, M. Zizi and A. Breiman (1995). "Multiple cDNAs of wheat voltage-dependent anion channels (VDAC): isolation, differential expression, mapping and evolution." Plant Mol Biol **29**(1): 109-24.

Engelman, D. M., Y. H. Chen, C. N. Chin, A. R. Curran, A. M. Dixon, A. D. Dupuy, A. S. Lee, U. Lehnert, E. E. Matthews, Y. K. Reshetnyak, A. Senes and P. J. L. (2003). "Membrane protein folding: beyond the two stage model." <u>FEBS Lett</u> **555**(1): 122-5.

Fasman, G. D. (1989). "Protein conformational prediction." <u>Trends Biochem Sci</u> **14**(7): 295-9.

Fedorova, L. and A. Fedorov (2003). "Introns in gene evolution." <u>Genetica</u> **118**(2-3): 123-31.

Ferguson, A. D., E. Hofmann, J. W. Coulton, K. Diederichs and W. Welte (1998). "Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide." <u>Science</u> **282**(5397): 2215-20.

Fernández, C., K. Adeishvili and K. Wüthrich (2001). "Transverse relaxation-optimized NMR spectroscopy with the outer membrane protein OmpX in dihexanoyl phosphatidylcholine micelles." <u>Proc Natl Acad Sci U S A</u> **98**(5): 2358-63.

Fernández, C., C. Hilty, S. Bonjour, K. Adeishvili, K. Pervushin and K. Wüthrich (2001). "Solution NMR studies of the integral membrane proteins OmpX and OmpA from *Escherichia coli*." <u>FEBS Lett</u> **504**(3): 173-8.

Fernández, C., C. Hilty, G. Wider, P. Guntert and K. Wüthrich (2004). "NMR structure of the integral membrane protein OmpX." <u>J Mol Biol</u> **336**(5): 1211-21.

Fischer, K., A. Weber, S. Brink, B. Arbinger, D. Schunemann, S. Borchert, H. W. Heldt, B. Popp, R. Benz, T. A. Link and et al. (1994). "Porins from plants. Molecular cloning and functional characterization of two new members of the porin family." <u>J Biol Chem</u> **269**(41): 25754-60.

Forst, D., W. Welte, T. Wacker and K. Diederichs (1998). "Structure of the sucrose-specific porin ScrY from *Salmonella typhimurium* and its complex with sucrose." <u>Nat Struct Biol</u> **5**(1): 37-46.

Forte, M., D. Adelsberger-Mangan and M. Colombini (1987). "Purification and characterization of the voltage-dependent anion channel from the outer mitochondrial membrane of yeast." <u>J Membr Biol</u> **99**(1): 65-72.

Freitag, H., G. Genchi, R. Benz, F. Palmieri and W. Neupert (1982). "Isolation of mitochondrial porin from Neurospora crassa." <u>FEBS Lett</u> **145**(1): 72-6.

Freitag, H., M. Janes and W. Neupert (1982). "Biosynthesis of mitochondrial porin and insertion into the outer mitochondrial membrane of Neurospora crassa." <u>Eur J Biochem</u> **126**(1): 197-202.

Freitag, H., W. Neupert and R. Benz (1982). "Purification and characterisation of a pore protein of the outer mitochondrial membrane from Neurospora crassa." Eur J Biochem **123**(3): 629-36.

Freudl, R., S. MacIntyre, M. Degen and U. Henning (1988). "Alterations to the signal peptide of an outer membrane protein (OmpA) of Escherichia coli K-12 can promote either the cotranslational or the posttranslational mode of processing." J Biol Chem **263**(1): 344-9.

Garavito, R. M. and S. Ferguson-Miller (2001). "Detergents as tools in membrane biochemistry." J Biol Chem **278**: 32403-32406.

Garcia-Borron, J. C., J. Escribano, M. Jimenez and J. L. Iborra (1982). "Quantitative determination of tryptophanyl and tyrosyl residues of proteins by second-derivative fluorescence spectroscopy." Anal Biochem **125**(2): 277-85.

Gentle, I., K. Gabriel, P. Beech, R. Waller and T. Lithgow (2004). "The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria." J Cell Biol **164**(1): 19-24.

Gilbert, R. J. (2002). "Pore-forming toxins." Cell Mol Life Sci **59**(5): 832-44.

Gincel, D., H. Zaid and V. Shoshan-Barmatz (2001). "Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function." Biochem J **358**(Pt 1): 147-55.

Gonzalez-Gronow, M., T. Kalfa, C. E. Johnson, G. Gawdi and S. V. Pizzo (2003). "The voltage-dependent anion channel is a receptor for plasminogen kringle 5 on human endothelial cells." J Biol Chem **278**(29): 27312-8.

Graham, B. H. and W. J. Craigen (2005). "Mitochondrial Voltage-dependent anion channel gene family in Drosophila melanogaster: Complex patterns of evolution, genomic organization, and developmental expression." Mol Genet Metab.

Guo, X. W. and C. A. Mannella (1993). "Conformational change in the mitochondrial channel, VDAC, detected by electron cryo-microscopy." Biophys J **64**(2): 545-9.

Guo, X. W., P. R. Smith, B. Cognon, D. D'Arcangelis, E. Dolginova and C. A. Mannella (1995). "Molecular design of the voltage-dependent, anion-selective channel in the mitochondrial outer membrane." J Struct Biol **114**(1): 41-59.

Guzow, K., A. Rzeska, J. Mrozek, J. Karolczak, R. Majewski, M. Szabelski, T. Ossowski and W. Wiczk (2005). "Photophysical properties of tyrosine and its simple derivatives in organic solvents studied by time-resolved fluorescence spectroscopy and global analysis." Photochem Photobiol **81**(3): 697-704.

Ha, H., P. Hajek, D. M. Bedwell and P. D. Burrows (1993). "A mitochondrial porin cDNA predicts the existence of multiple human porins." J Biol Chem **268**(16): 12143-9.

Harkness, T. A., F. E. Nargang, I. van der Klei, W. Neupert and R. Lill (1994). "A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria." J Cell Biol **124**(5): 637-48.

Harms, N., G. Koningstein, W. Dontje, M. Muller, B. Oudega, J. Luirink and H. de Cock (2001). "The early interaction of the outer membrane protein phoe with the periplasmic chaperone Skp occurs at the cytoplasmic membrane." J Biol Chem **276**(22): 18804-11.

Havel, H. A. (1996). Derivative near-ultraviolet absorption techniques for investigating protein structure. Spectroscopic methods for determining protein structure in solution. H. A. Havel. New York, VCH Publishers, Inc.**:** 62-68.

Havel, H. A., E. W. Kauffman, S. M. Plaisted and D. N. Brems (1986). "Reversible self-association of bovine growth hormone during equilibrium unfolding." Biochemistry **25**(21): 6533-8.

Heins, L., H. Mentzel, A. Schmid, R. Benz and U. K. Schmitz (1994). "Biochemical, molecular, and functional characterization of porin isoforms from potato mitochondria." J Biol Chem **269**(42): 26402-10.

Heringa, J. (2000). "Computational methods for protein secondary structure prediction using multiple sequence alignments." Curr Protein Pept Sci **1**(3): 273-301.

Heringa, J. (2002). "Local weighting schemes for protein multiple sequence alignment." Comput Chem **26**(5): 459-77.

Hoppins, S. C. and F. E. Nargang (2004). "The Tim8-Tim13 complex of Neurospora crassa functions in the assembly of proteins into both mitochondrial membranes." J Biol Chem **279**(13): 12396-405.

Jacotot, E., K. F. Ferri, C. El Hamel, C. Brenner, S. Druillennec, J. Hoebeke, P. Rustin, D. Metivier, C. Lenoir, M. Geuskens, H. L. Vieira, M. Loeffler, A. S. Belzacq, J. P. Briand, N. Zamzami, L. Edelman, Z. H. Xie, J. C. Reed, B. P. Roques and G. Kroemer (2001). "Control of mitochondrial membrane permeabilization by adenine nucleotide translocator interacting with HIV-1 viral protein rR and Bcl-2." J Exp Med **193**(4): 509-19.

Jacotot, E., L. Ravagnan, M. Loeffler, K. F. Ferri, H. L. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J. P. Briand, T. Irinopoulou, E. Daugas, S. A. Susin, D. Cointe, Z. H. Xie, J. C. Reed, B. P. Roques and G. Kroemer (2000). "The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore." J Exp Med **191**(1): 33-46.

Jansen, C., M. Heutink, J. Tommassen and H. de Cock (2000). "The assembly pathway of outer membrane protein PhoE of Escherichia coli." Eur J Biochem **267**(12): 3792-800.

Jap, B. K. and P. J. Walian (1996). "Structure and functional mechanism of porins." Physiol Rev **76**(4): 1073-88.

Johnson, W. C., Jr. (1990). "Protein secondary structure and circular dichroism: a practical guide." Proteins **7**(3): 205-14.

Kakorin, S., U. Brinkmann and E. Neumann (2005). "Cholesterol reduces membrane electroporation and electric deformation of small bilayer vesicles." Biophys Chem **117**(2): 155-71.

Kawahara, K. and C. Tanford (1966). "Viscosity and density of aqueous solutions of urea and guanidine hydrochloride." J Biol Chem **241**(13): 3228-32.

Kayser, H., H. D. Kratzin, F. P. Thinnes, H. Gotz, W. E. Schmidt, K. Eckart and N. Hilschmann (1989). "[Identification of human porins. II. Characterization and primary structure of a 31-lDa porin from human B lymphocytes (Porin 31HL).]." Biol Chem Hoppe Seyler **370**(12): 1265-78.

Kim, R., M. Emi, K. Tanabe, S. Murakami, Y. Uchida and K. Arihiro (2006). "Regulation and interplay of apoptotic and non-apoptotic cell death." J Pathol **208**(3): 319-26.

Kleene, R., N. Pfanner, R. Pfaller, T. A. Link, W. Sebald, W. Neupert and M. Tropschug (1987). "Mitochondrial porin of *Neurospora crassa*: cDNA cloning, in vitro expression and import into mitochondria." EMBO J **6**(9): 2627-33.

Kleinschmidt, J. H. (2003). "Membrane protein folding on the example of outer membrane protein A of Escherichia coli." Cell Mol Life Sci **60**(8): 1547-58.

Kleinschmidt, J. H. (2006). "Folding kinetics of the outer membrane proteins OmpA and FomA into phospholipid bilayers." Chem Phys Lipids **141**(1-2): 30-47.

Kleinschmidt, J. H., T. den Blaauwen, A. J. Driessen and L. K. Tamm (1999). "Outer membrane protein A of *Escherichia coli* inserts and folds into lipid bilayers by a concerted mechanism." Biochemistry **38**(16): 5006-16.

Kleinschmidt, J. H. and L. K. Tamm (1996). "Folding intermediates of a beta-barrel membrane protein. Kinetic evidence for a multi-step membrane insertion mechanism." Biochemistry **35**(40): 12993-3000.

Kleinschmidt, J. H. and L. K. Tamm (1999). "Time-resolved distance determination by tryptophan fluorescence quenching: probing intermediates in membrane protein folding." Biochemistry **38**(16): 4996-5005.

Kleinschmidt, J. H. and L. K. Tamm (2002). "Secondary and tertiary structure formation of the beta-barrel membrane protein OmpA is synchronized and depends on membrane thickness." J Mol Biol **324**(2): 319-30.

Kleinschmidt, J. H., M. C. Wiener and L. K. Tamm (1999). "Outer membrane protein A of *E. coli* folds into detergent micelles, but not in the presence of monomeric detergent." Protein Sci **8**(10): 2065-71.

Koebnik, R. (1995). "Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell-surface proteins." Mol Microbiol **16**(6): 1269-70.

Koebnik, R., K. P. Locher and P. Van Gelder (2000). "Structure and function of bacterial outer membrane proteins: barrels in a nutshell." Mol Microbiol **37**(2): 239-53.

Komarov, A. G., B. H. Graham, W. J. Craigen and M. Colombini (2004). "The physiological properties of a novel family of VDAC-like proteins from Drosophila melanogaster." Biophys J **86**(1 Pt 1): 152-62.

Konstantinova, S. A., C. A. Mannella, V. P. Skulachev and D. B. Zorov (1995). "Immunoelectron microscopic study of the distribution of porin on outer membranes of rat heart mitochondria." J Bioenerg Biomembr **27**(1): 93-9.

Konstantinova, S. A., K. A. Mannella, V. P. Skulachev and D. B. Zorov (1994). "[Detection of porin in inter-mitochondrial contacts]." Biokhimiia **59**(8): 1109-21.

Koppel, D. A., K. W. Kinnally, P. Masters, M. Forte, E. Blachly-Dyson and C. A. Mannella (1998). "Bacterial expression and characterization of the mitochondrial outer membrane channel. Effects of n-terminal modifications." J Biol Chem **273**(22): 13794-800.

Krimmer, T., D. Rapaport, M. T. Ryan, C. Meisinger, C. K. Kassenbrock, E. Blachly-Dyson, M. Forte, M. G. Douglas, W. Neupert, F. E. Nargang and N. Pfanner (2001). "Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex." J Cell Biol **152**(2): 289-300.

Kumar, T. K., G. Jayaraman, C. S. Lee, T. Sivaraman, W. Y. Lin and C. Yu (1995). "Identification of 'molten globule'-like state in all beta-sheet protein." Biochem Biophys Res Commun **207**(2): 536-43.

Künkele, K. P., P. Juin, C. Pompa, F. E. Nargang, J. P. Henry, W. Neupert, R. Lill and M. Thieffry (1998). "The isolated complex of the translocase of the outer membrane of mitochondria. Characterization of the cation-selective and voltage-gated preprotein-conducting pore." J Biol Chem **273**(47): 31032-9.

Kyte, J. and R. F. Doolittle (1982). "A simple method for displaying the hydropathic character of a protein." J Mol Biol **157**(1): 105-32.

Ladokhin, A. S. (2000). Fluorescence spectroscopy in peptide and protein analysis. Encyclopedia of analytical chemistry. R. A. Meyers. Chichester, John Wiley and Sons Ltd.**:** 5762-5779.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-5.

Lau, F. W. and J. U. Bowie (1997). "A method for assessing the stability of a membrane protein." Biochemistry **36**(19): 5884-92.

Lazar, S. W. and R. Kolter (1996). "SurA assists the folding of Escherichia coli outer membrane proteins." J Bacteriol **178**(6): 1770-3.

Lee, J. and R. Ross (1998). "Absorption and fluorescence of tyrosine hydrogen-bonded to amide-like ligands." J Phys Chem B **102**: 4612-18.

Lemasters, J. J. and E. Holmuhamedov (2006). "Voltage-dependent anion channel (VDAC) as mitochondrial governator--thinking outside the box." Biochim Biophys Acta **1762**(2): 181-90.

Lemeshko, S. V. and V. V. Lemeshko (2000). "Metabolically derived potential on the outer membrane of mitochondria: a computational model." Biophys J **79**(6): 2785-800.

Lemeshko, S. V. and V. V. Lemeshko (2004). "Energy flux modulation on the outer membrane of mitochondria by metabolically-derived potential." Mol Cell Biochem **256-257**(1-2): 127-39.

Lemeshko, V. V. and S. V. Lemeshko (2004). "The voltage-dependent anion channel as a biological transistor: theoretical considerations." Eur Biophys J **33**(4): 352-9.

Li, X. X. and M. Colombini (2002). "Catalyzed insertion of proteins into phospholipid membranes: specificity of the process." Biophys J **83**(5): 2550-9.

Lin, K., V. A. Simossis, W. R. Taylor and J. Heringa (2005). "A simple and fast secondary structure prediction method using hidden neural networks." Bioinformatics **21**(2): 152-9.

Linke, D., J. Frank, J. F. Holzwarth, J. Soll, C. Boettcher and P. Fromme (2000). "In vitro reconstitution and biophysical characterization of OEP16, an outer envelope pore protein of pea chloroplasts." Biochemistry **39**(36): 11050-6.

Liu, M. Y. and M. Colombini (1991). "Voltage gating of the mitochondrial outer membrane channel VDAC is regulated by a very conserved protein." Am J Physiol **260**(2 Pt 1): C371-4.

Liu, M. Y. and M. Colombini (1992). "Regulation of mitochondrial respiration by controlling the permeability of the outer membrane through the mitochondrial channel, VDAC." Biochim Biophys Acta **1098**(2): 255-60.

Liu, M. Y. and M. Colombini (1992). "A soluble mitochondrial protein increases the voltage dependence of the mitochondrial channel, VDAC." J Bioenerg Biomembr **24**(1): 41-6.

Liu, M. Y., A. Torgrimson and M. Colombini (1994). "Characterization and partial purification of the VDAC-channel-modulating protein from calf liver mitochondria." Biochim Biophys Acta **1185**(2): 203-12.

Lopez-Llano, J., S. Maldonado, S. Jain, A. Lostao, R. Godoy-Ruiz, J. M. Sanchez-Ruiz, M. Cortijo, J. Fernandez-Recio and J. Sancho (2004). "The long and short flavodoxins: II. The role of the differentiating loop in apoflavodoxin stability and folding mechanism." J Biol Chem **279**(45): 47184-91.

Madesh, M. and G. Hajnoczky (2001). "VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release." J Cell Biol **155**(6): 1003-15.

Manavalan, P. and W. C. Johnson, Jr. (1987). "Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra." Anal Biochem **167**(1): 76-85.

Manley, D. and J. D. O'Neil (2003). "Preparation of glycerol facilitator for protein structure and folding studies in solution." Methods Mol Biol **228**: 89-101.

Mannella, C. A. (1989). "Fusion of the mitochondrial outer membrane: use in forming large, two-dimensional crystals of the voltage-dependent, anion-selective channel protein." Biochim Biophys Acta **981**(1): 15-20.

Mannella, C. A. (1997). "Minireview: on the structure and gating mechanism of the mitochondrial channel, VDAC." J Bioenerg Biomembr **29**(6): 525-31.

Mannella, C. A. (1998). "Conformational changes in the mitochondrial channel protein, VDAC, and their functional implications." J Struct Biol **121**(2): 207-18.

Mannella, C. A., M. Forte and M. Colombini (1992). "Toward the molecular structure of the mitochondrial channel, VDAC." J Bioenerg Biomembr **24**(1): 7-19.

Mannella, C. A. and J. Frank (1984). "Negative staining characteristics of arrays of mitochondrial pore protein: use of correspondence analysis to classify different staining patterns." Ultramicroscopy **13**(1-2): 93-102.

Mannella, C. A., X. W. Guo and J. Dias (1992). "Binding of a synthetic targeting peptide to a mitochondrial channel protein." J Bioenerg Biomembr **24**(1): 55-61.

Mannella, C. A., A. F. Neuwald and C. E. Lawrence (1996). "Detection of likely transmembrane beta strand regions in sequences of mitochondrial pore proteins using the Gibbs sampler." J Bioenerg Biomembr **28**(2): 163-9.

Manting, E. H. and A. J. Driessen (2000). "Escherichia coli translocase: the unravelling of a molecular machine." Mol Microbiol **37**(2): 226-38.

Mao, D. and B. A. Wallace (1984). "Differential light scattering and absorption flattening optical effects are minimal in the circular dichroism spectra of small unilamellar vesicles." Biochemistry **23**(12): 2667-73.

Marsh, D., B. Shanmugavadivu and J. H. Kleinschmidt (2006). "Membrane elastic fluctuations and the insertion and tilt of beta-barrel proteins." Biophys J **91**(1): 227-32.

Martin, N. L., E. G. Rawling, R. S. Wong, M. Rosok and R. E. Hancock (1993). "Conservation of surface epitopes in Pseudomonas aeruginosa outer membrane porin protein OprF." FEMS Microbiol Lett **113**(3): 261-6.

Masui, R., T. Mikawa and S. Kuramitsu (1997). "Local folding of the N-terminal domain of *Escherichia coli* RecA controls protein-protein interaction." J Biol Chem **272**(44): 27707-15.

Matsuura, J. E., A. E. Morris, R. R. Ketchem, E. H. Braswell, R. Klinke, W. R. Gombotz and R. L. Remmele, Jr. (2001). "Biophysical characterization of a soluble CD40 ligand (CD154) coiled-coil trimer: evidence of a reversible acid-denatured molten globule." Arch Biochem Biophys **392**(2): 208-18.

Mayer, A., R. Lill and W. Neupert (1993). "Translocation and insertion of precursor proteins into isolated outer membranes of mitochondria." J Cell Biol **121**(6): 1233-43.

McEnery, M. W. (1992). "The mitochondrial benzodiazepine receptor: evidence for association with the voltage-dependent anion channel (VDAC)." J Bioenerg Biomembr **24**(1): 63-9.

McGuire, R. and I. Feldman (1973). "The quenching of tyrosine and tryptophan fluorescence by H20 and D20." <u>Photochem Photobiol</u> **18**(2): 119-24.

Messina, A., M. Oliva, C. Rosato, M. Huizing, W. Ruitenbeek, L. P. van den Heuvel, M. Forte, M. Rocchi and V. De Pinto (1999). "Mapping of the human Voltage-Dependent Anion Channel isoforms 1 and 2 reconsidered." <u>Biochem Biophys Res Commun</u> **255**(3): 707-10.

Mihara, K., G. Blobel and R. Sato (1982). "In vitro synthesis and integration into mitochondria of porin, a major protein of the outer mitochondrial membrane of Saccharomyces cerevisiae." <u>Proc Natl Acad Sci U S A</u> **79**(23): 7102-6.

Mihara, K. and R. Sato (1985). "Molecular cloning and sequencing of cDNA for yeast porin, an outer mitochondrial membrane protein: a search for targeting signal in the primary structure." <u>EMBO J</u> **4**(3): 769-74.

Minetti, C. A., M. S. Blake and D. P. Remeta (1998). "Characterization of the structure, function, and conformational stability of PorB class 3 protein from *Neisseria meningitidis*. A porin with unusual physicochemical properties." <u>J Biol Chem</u> **273**(39): 25329-38.

Minetti, C. A., J. Y. Tai, M. S. Blake, J. K. Pullen, S. M. Liang and D. P. Remeta (1997). "Structural and functional characterization of a recombinant PorB class 2 protein from *Neisseria meningitidis*. Conformational stability and porin activity." <u>J Biol Chem</u> **272**(16): 10710-20.

Muller, A., D. Gunther, V. Brinkmann, R. Hurwitz, T. F. Meyer and T. Rudel (2000). "Targeting of the pro-apoptotic VDAC-like porin (PorB) of Neisseria gonorrhoeae to mitochondria of infected cells." <u>Embo J</u> **19**(20): 5332-43.

Muller, A., J. Rassow, J. Grimm, N. Machuy, T. F. Meyer and T. Rudel (2002). "VDAC and the bacterial porin PorB of Neisseria gonorrhoeae share mitochondrial import pathways." <u>Embo J</u> **21**(8): 1916-29.

Murzin, A. G., A. M. Lesk and C. Chothia (1994). "Principles determining the structure of beta-sheet barrels in proteins. I. A theoretical analysis." <u>J Mol Biol</u> **236**(5): 1369-81.

Narhi, L. O., J. S. Philo, T. Li, M. Zhang, B. Samal and T. Arakawa (1996). "Induction of alpha-helix in the beta-sheet protein tumor necrosis factor-alpha: thermal- and trifluoroethanol-induced denaturation at neutral pH." <u>Biochemistry</u> **35**(35): 11447-53.

Nayar, S., A. Brahma, C. Mukherjee and D. Bhattacharyya (2002). "Second derivative fluorescence spectra of indole compounds." <u>J Biochem (Tokyo)</u> **131**(3): 427-35.

Ninomiya, Y., K. Suzuki, C. Ishii and H. Inoue (2004). "Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining." Proc Natl Acad Sci U S A **101**(33): 12248-53.

Noronha, M., J. Lima, P. Lamosa, H. Santos, C. Maycock, R. Ventura and A. Macanita (2004). "Intramolecular fluorescence quenching of tyrosine by the peptide α-carbonyl group revisited." J Phys Chem A **108**: 2155-66.

Ohnishi, S. and K. Kameyama (2001). "*Escherichia coli* OmpA retains a folded structure in the presence of sodium dodecyl sulfate due to a high kinetic barrier to unfolding." Biochim Biophys Acta **1515**(2): 159-66.

Ohnishi, S., K. Kameyama and T. Takagi (1998). "Characterization of a heat modifiable protein, Escherichia coli outer membrane protein OmpA in binary surfactant system of sodium dodecyl sulfate and octylglucoside." Biochim Biophys Acta **1375**(1-2): 101-9.

Otzen, D. E. (2003). "Folding of DsbB in mixed micelles: a kinetic analysis of the stability of a bacterial membrane protein." J Mol Biol **330**(4): 641-9.

Park, K., A. Perczel and G. D. Fasman (1992). "Differentiation between transmembrane helices and peripheral helices by the deconvolution of circular dichroism spectra of membrane proteins." Protein Sci **1**(8): 1032-49.

Paschen, S. A. and W. Neupert (2001). "Protein import into mitochondria." IUBMB Life **52**(3-5): 101-12.

Paschen, S. A., W. Neupert and D. Rapaport (2005). "Biogenesis of beta-barrel membrane proteins of mitochondria." Trends Biochem Sci **30**(10): 575-82.

Paschen, S. A., T. Waizenegger, T. Stan, M. Preuss, M. Cyrklaff, K. Hell, D. Rapaport and W. Neupert (2003). "Evolutionary conservation of biogenesis of beta-barrel membrane proteins." Nature **426**(6968): 862-6.

Pautsch, A. and G. E. Schulz (1998). "Structure of the outer membrane protein A transmembrane domain." Nat Struct Biol **5**(11): 1013-7.

Pavlica, R. J., C. B. Hesler, L. Lipfert, I. N. Hirshfield and D. Haldar (1990). "Two-dimensional gel electrophoretic resolution of the polypeptides of rat liver mitochondria and the outer membrane." Biochim Biophys Acta **1022**(1): 115-25.

Peng, S., E. Blachly-Dyson, M. Forte and M. Colombini (1992). "Large scale rearrangement of protein domains is associated with voltage gating of the VDAC channel." Biophys J **62**(1): 123-31; discussion 131-5.

Perczel, A., K. Park and G. D. Fasman (1992). "Analysis of the circular dichroism spectrum of proteins using the convex constraint algorithm: a practical guide." Anal Biochem **203**(1): 83-93.

Pfaller, R., H. Freitag, M. A. Harmey, R. Benz and W. Neupert (1985). "A water-soluble form of porin from the mitochondrial outer membrane of Neurospora crassa. Properties and relationship to the biosynthetic precursor form." J Biol Chem **260**(13): 8188-93.

Pocanschi, C. L., H. J. Apell, P. Puntervoll, B. Hogh, H. B. Jensen, W. Welte and J. H. Kleinschmidt (2006). "The major outer membrane protein of Fusobacterium nucleatum (FomA) folds and inserts into lipid bilayers via parallel folding pathways." J Mol Biol **355**(3): 548-61.

Pocanschi, C. L., G. J. Patel, D. Marsh and J. H. Kleinschmidt (2006). "Curvature elasticity and refolding of OmpA in large unilamellar vesicles." Biophys J.

Pollastri, G., D. Przybylski, B. Rost and P. Baldi (2002). "Improving the prediction of protein secondary structure in three and eight classes using recurrent neural networks and profiles." Proteins **47**(2): 228-35.

Popot, J. L. and D. M. Engelman (1990). "Membrane protein folding and oligomerization: the two-stage model." Biochemistry **29**(17): 4031-7.

Popot, J. L. and D. M. Engelman (2000). "Helical membrane protein folding, stability, and evolution." Annu Rev Biochem **69**: 881-922.

Popp, B., D. A. Court, R. Benz, W. Neupert and R. Lill (1996). "The role of the N and C termini of recombinant *Neurospora* mitochondrial porin in channel formation and voltage-dependent gating." J Biol Chem **271**(23): 13593-9.

Popp, B., S. Gebauer, K. Fischer, U. I. Flügge and R. Benz (1997). "Study of structure and function of recombinant pea root plastid porin by biophysical methods." Biochemistry **36**(10): 2844-52.

Popp, B., A. Schmid and R. Benz (1995). "Role of sterols in the functional reconstitution of water-soluble mitochondrial porins from different organisms." Biochemistry **34**(10): 3352-61.

Privalov, P. L. and S. A. Potekhin (1986). "Scanning microcalorimetry in studying temperature-induced changes in proteins." Methods Enzymol **131**: 4-51.

Proulx, P., H. Aubry, I. Brglez and D. G. Williamson (1984). "Studies on the mechanism of cholesterol uptake and on the effects of bile salts on this uptake by brush-border membranes isolated from rabbit small intestine." Biochim Biophys Acta **778**(3): 586-93.

Provencher, S. W. and J. Glockner (1981). "Estimation of globular protein secondary structure from circular dichroism." Biochemistry **20**(1): 33-7.

Przybylski, M., M. O. Glocker, U. Nestel, V. Schnaible, M. Bluggel, K. Diederichs, J. Weckesser, M. Schad, A. Schmid, W. Welte and R. Benz (1996). "X-ray crystallographic and mass spectrometric structure determination and functional characterization of succinylated porin from Rhodobacter capsulatus: implications for ion selectivity and single-channel conductance." Protein Sci **5**(8): 1477-89.

Ragone, G., G. Colonna, L. Servillo and G. Irace (1985). "Resolution of overlapping bands in the near-UV absorption spectrum of indole derivatives." Photochem. Photobiol. **42**: 505-8.

Ragone, R., G. Colonna, C. Balestrieri, L. Servillo and G. Irace (1984). "Determination of tyrosine exposure in proteins by second-derivative spectroscopy." Biochemistry **23**(8): 1871-5.

Rahmani, Z., C. Maunoury and A. Siddiqui (1998). "Isolation of a novel human voltage-dependent anion channel gene." Eur J Hum Genet **6**(4): 337-40.

Rapaport, D. (2005). "How does the TOM complex mediate insertion of precursor proteins into the mitochondrial outer membrane?" J Cell Biol **171**(3): 419-23.

Rauch, G. and O. Moran (1994). "On the structure of mitochondrial porins and its homologies with bacterial porins." Biochem Biophys Res Commun **200**(2): 908-15.

Rawling, E. G., N. L. Martin and R. E. Hancock (1995). "Epitope mapping of the Pseudomonas aeruginosa major outer membrane porin protein OprF." Infect Immun **63**(1): 38-42.

Reader, J. S., N. A. Van Nuland, G. S. Thompson, S. J. Ferguson, C. M. Dobson and S. E. Radford (2001). "A partially folded intermediate species of the beta-sheet protein apo-pseudoazurin is trapped during proline-limited folding." Protein Sci **10**(6): 1216-24.

Reshetnyak, Y. K., Y. Koshevnik and E. A. Burstein (2001). "Decomposition of protein tryptophan fluorescence spectra into log-normal components. III. Correlation between fluorescence and microenvironment parameters of individual tryptophan residues." Biophys J **81**(3): 1735-58.

Roman, I., J. Figys, G. Steurs and M. Zizi (2006). "Hunting interactomes of a membrane protein: obtaining the largest set of VDAC-interacting protein epitopes." Mol Cell Proteomics.

Roos, N., R. Benz and D. Brdiczka (1982). "Identification and characterization of the pore-forming protein in the outer membrane of rat liver mitochondria." <u>Biochim Biophys Acta</u> **686**(2): 204-14.

Rostovtseva, T. and M. Colombini (1996). "ATP flux is controlled by a voltage-gated channel from the mitochondrial outer membrane." <u>J Biol Chem</u> **271**(45): 28006-8.

Rostovtseva, T. and M. Colombini (1997). "VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function." <u>Biophys J</u> **72**(5): 1954-62.

Rostovtseva, T. K., B. Antonsson, M. Suzuki, R. J. Youle, M. Colombini and S. M. Bezrukov (2004). "Bid, but not Bax, regulates VDAC channels." <u>J Biol Chem</u> **279**(14): 13575-83.

Runke, G., E. Maier, J. D. O'Neil, R. Benz and D. A. Court (2000). "Functional characterization of the conserved "GLK" motif in mitochondrial porin from *Neurospora crassa*." <u>J Bioenerg Biomembr</u> **32**(6): 563-70.

Runke, G., E. Maier, W. A. Summers, D. C. Bay, R. Benz and D. A. Court (2006). "Deletion variants of Neurospora mitochondrial porin: electrophysiological and spectroscopic analysis." <u>Biophys J</u> **90**(9): 3155-64.

Sampson, M. J., W. K. Decker, A. L. Beaudet, W. Ruitenbeek, D. Armstrong, M. J. Hicks and W. J. Craigen (2001). "Immotile sperm and infertility in mice lacking mitochondrial voltage-dependent anion channel type 3." <u>J Biol Chem</u> **276**(42): 39206-12.

Sampson, M. J., R. S. Lovell and W. J. Craigen (1996). "Isolation, characterization, and mapping of two mouse mitochondrial voltage-dependent anion channel isoforms." <u>Genomics</u> **33**(2): 283-8.

Sampson, M. J., R. S. Lovell and W. J. Craigen (1997). "The murine voltage-dependent anion channel gene family. Conserved structure and function." <u>J Biol Chem</u> **272**(30): 18966-73.

Sampson, M. J., L. Ross, W. K. Decker and W. J. Craigen (1998). "A novel isoform of the mitochondrial outer membrane protein VDAC3 via alternative splicing of a 3-base exon. Functional characteristics and subcellular localization." <u>J Biol Chem</u> **273**(46): 30482-6.

Schein, S. J., M. Colombini and A. Finkelstein (1976). "Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from paramecium mitochondria." <u>J Membr Biol</u> **30**(2): 99-120.

Schleiff, E., G. C. Shore and I. S. Goping (1997). "Human mitochondrial import receptor, Tom20p. Use of glutathione to reveal specific interactions between Tom20-glutathione S-transferase and mitochondrial precursor proteins." <u>FEBS Lett</u> **404**(2-3): 314-8.

Schleiff, E., J. R. Silvius and G. C. Shore (1999). "Direct membrane insertion of voltage-dependent anion-selective channel protein catalyzed by mitochondrial Tom20." <u>J Cell Biol</u> **145**(5): 973-8.

Schmidt, S., A. Strub, K. Rottgers, N. Zufall and W. Voos (2001). "The two mitochondrial heat shock proteins 70, Ssc1 and Ssq1, compete for the cochaperone Mge1." <u>J Mol Biol</u> **313**(1): 13-26.

Schulz, G. E. (2000). "beta-Barrel membrane proteins." <u>Curr Opin Struct Biol</u> **10**(4): 443-7.

Schwarzer, C., S. Barnikol-Watanabe, F. P. Thinnes and N. Hilschmann (2002). "Voltage-dependent anion-selective channel (VDAC) interacts with the dynein light chain Tctex1 and the heat-shock protein PBP74." <u>Int J Biochem Cell Biol</u> **34**(9): 1059-70.

Schweizer, M., I. Hindennach, W. Garten and U. Henning (1978). "Major proteins of the Escherichia coli outer cell envelope membrane. Interaction of protein II with lipopolysaccharide." <u>Eur J Biochem</u> **82**(1): 211-7.

Scotto, A. W. and D. Zakim (1988). "Reconstitution of membrane proteins. Spontaneous incorporation of integral membrane proteins into preformed bilayers of pure phospholipid." <u>J Biol Chem</u> **263**(34): 18500-6.

Shao, L., K. W. Kinnally and C. A. Mannella (1996). "Circular dichroism studies of the mitochondrial channel, VDAC, from *Neurospora crassa*." <u>Biophys J</u> **71**(2): 778-86.

Sherman, E. L., N. E. Go and F. E. Nargang (2005). "Functions of the small proteins in the TOM complex of Neurospora crasssa." <u>Mol Biol Cell</u> **16**(9): 4172-82

Sherman, E. L., R. D. Taylor, N. E. Go and F. E. Nargang (2006). "Effect of mutations in Tom40 on stability of the translocase of the outer mitochondrial membrane (TOM) complex, assembly of Tom40, and import of mitochondrial preproteins." <u>J Biol Chem</u> **281**(32): 22554-65.

Shimizu, S., T. Ide, T. Yanagida and Y. Tsujimoto (2000). "Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c." <u>J Biol Chem</u> **275**(16): 12321-5.

Shimizu, S., A. Konishi, T. Kodama and Y. Tsujimoto (2000). "BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death." <u>Proc Natl Acad Sci U S A</u> **97**(7): 3100-5.

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Shimizu, S., M. Narita and Y. Tsujimoto (1999). "Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC." Nature **399**(6735): 483-7.

Shimizu, S. and Y. Tsujimoto (2000). "Proapoptotic BH3-only Bcl-2 family members induce cytochrome c release, but not mitochondrial membrane potential loss, and do not directly modulate voltage-dependent anion channel activity." Proc Natl Acad Sci U S A **97**(2): 577-82.

Shoshan-Barmatz, V., A. Israelson, D. Brdiczka and S. S. Sheu (2006). "The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death." Curr Pharm Des **12**(18): 2249-70.

Simons, K. and E. Ikonen (2000). "How cells handle cholesterol." Science **290**(5497): 1721-6.

Simossis, V. A. and J. Heringa (2003). "The PRALINE online server: optimising progressive multiple alignment on the web." Comput Biol Chem **27**(4-5): 511-9.

Simossis, V. A. and J. Heringa (2005). "PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information." Nucleic Acids Res **33**(Web Server issue): W289-94.

Singh, S. P., Y. U. Williams, S. Miller and H. Nikaido (2003). "The C-terminal domain of Salmonella enterica serovar typhimurium OmpA is an immunodominant antigen in mice but appears to be only partially exposed on the bacterial cell surface." Infect Immun **71**(7): 3937-46.

Sivaraman, T., T. K. Kumar, G. Jayaraman, C. C. Han and C. Yu (1997). "Characterization of a partially structured state in an all-beta-sheet protein." Biochem J **321 (Pt 2)**: 457-64.

Smith, M. D., M. Petrak, P. D. Boucher, K. N. Barton, L. Carter, G. Reddy, E. Blachly-Dyson, M. Forte, J. Price, K. Verner and et al. (1995). "Lysine residues at positions 234 and 236 in yeast porin are involved in its assembly into the mitochondrial outer membrane." J Biol Chem **270**(47): 28331-6.

Song, J., C. Midson, E. Blachly-Dyson, M. Forte and M. Colombini (1998). "The sensor regions of VDAC are translocated from within the membrane to the surface during the gating processes." Biophys J **74**(6): 2926-44.

Song, J., C. Midson, E. Blachly-Dyson, M. Forte and M. Colombini (1998). "The topology of VDAC as probed by biotin modification." J Biol Chem **273**(38): 24406-13.

Spyroacopoulos, L. and J. D. O'Neil (1994). "Effect of a hydrophobic environment on the hydrogen exchange kinetics of model amides determined by ^{1}H-NMR spectroscopy." J Am Chem Soc **116**: 1395-1402.

Sreerama, N., S. Y. Venyaminov and R. W. Woody (2000). "Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis." Anal Biochem **287**(2): 243-51.

Stanley, S., J. A. Dias, D. D'Arcangelis and C. A. Mannella (1995). "Peptide-specific antibodies as probes of the topography of the voltage-gated channel in the mitochondrial outer membrane of *Neurospora* crassa." J Biol Chem **270**(28): 16694-700.

Sugawara, E. and H. Nikaido (1992). "Pore-forming activity of OmpA protein of Escherichia coli." J Biol Chem **267**(4): 2507-11.

Sugawara, E. and H. Nikaido (1994). "OmpA protein of Escherichia coli outer membrane occurs in open and closed channel forms." J Biol Chem **269**(27): 17981-7.

Surrey, T. and F. Jahnig (1992). "Refolding and oriented insertion of a membrane protein into a lipid bilayer." Proc Natl Acad Sci U S A **89**(16): 7457-61.

Surrey, T. and F. Jahnig (1995). "Kinetics of folding and membrane insertion of a beta-barrel membrane protein." J Biol Chem **270**(47): 28199-203.

Surrey, T., A. Schmid and F. Jahnig (1996). "Folding and membrane insertion of the trimeric beta-barrel protein OmpF." Biochemistry **35**(7): 2283-8.

Tamm, L. K., F. Abildgaard, A. Arora, H. Blad and J. H. Bushweller (2003). "Structure, dynamics and function of the outer membrane protein A (OmpA) and influenza hemagglutinin fusion domain in detergent micelles by solution NMR." FEBS Lett **555**(1): 139-43.

Tamm, L. K., H. Hong and B. Liang (2004). "Folding and assembly of beta-barrel membrane proteins." Biochim Biophys Acta **1666**(1-2): 250-63.

Thomas, L., E. Blachly-Dyson, M. Colombini and M. Forte (1993). "Mapping of residues forming the voltage sensor of the voltage-dependent anion-selective channel." Proc Natl Acad Sci U S A **90**(12): 5446-9.

Thomas, L., E. Kocsis, M. Colombini, E. Erbe, B. L. Trus and A. C. Steven (1991). "Surface topography and molecular stoichiometry of the mitochondrial channel, VDAC, in crystalline arrays." J Struct Biol **106**(2): 161-71.

Thundimadathil, J., R. W. Roeske and L. Guo (2005). "A synthetic peptide forms voltage-gated porin-like ion channels in lipid bilayer membranes." Biochem Biophys Res Commun **330**(2): 585-90.

Thundimadathil, J., R. W. Roeske and L. Guo (2006). "Conversion of a porin-like peptide channel into a gramicidin-like channel by glycine to D-alanine substitutions." Biophys J **90**(3): 947-55.

Thundimadathil, J., R. W. Roeske and L. Guo (2006). "Effect of membrane mimicking environment on the conformation of a pore-forming (xSxG)6 peptide." Biopolymers **84**(3): 317-28.

Thundimadathil, J., R. W. Roeske, H. Y. Jiang and L. Guo (2005). "Aggregation and porin-like channel activity of a beta sheet peptide." Biochemistry **44**(30): 10259-70.

Traikia, M., D. B. Langlais, G. M. Cannarozzi and P. F. Devaux (1997). "High-resolution spectra of liposomes using MAS NMR. The case of intermediate-size vesicles." J Magn Reson **125**(1): 140-4.

Traikia, M., D. E. Warschawski, M. Recouvreur, J. Cartaud and P. F. Devaux (2000). "Formation of unilamellar vesicles by repetitive freeze-thaw cycles: characterization by electron microscopy and 31P-nuclear magnetic resonance." Eur Biophys J **29**(3): 184-95.

Traurig, M. and R. Misra (1999). "Identification of bacteriophage K20 binding regions of OmpF and lipopolysaccharide in *Escherichia coli* K-12." FEMS Microbiol Lett **181**(1): 101-8.

Troll, H., D. Malchow, A. Muller-Taubenberger, B. Humbel, F. Lottspeich, M. Ecke, G. Gerisch, A. Schmid and R. Benz (1992). "Purification, functional characterization, and cDNA sequencing of mitochondrial porin from *Dictyostelium discoideum*." J Biol Chem **267**(29): 21072-9.

Tsujimoto, Y. and S. Shimizu (2002). "The voltage-dependent anion channel: an essential player in apoptosis." Biochimie **84**(2-3): 187-93.

van Stokkum, I. H., H. J. Spoelder, M. Bloemendal, R. van Grondelle and F. C. Groen (1990). "Estimation of protein secondary structure and error analysis from circular dichroism spectra." Anal Biochem **191**(1): 110-8.

Vander Heiden, M. G., X. X. Li, E. Gottleib, R. B. Hill, C. B. Thompson and M. Colombini (2001). "Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane." J Biol Chem **276**(22): 19414-9.

Verschoor, A., W. F. Tivol and C. A. Mannella (2001). "Single-particle approaches in the analysis of small 2D crystals of the mitochondrial channel VDAC." J Struct Biol **133**(2-3): 254-65.

Visser, L. and E. R. Blout (1971). "Elastase. II. Optical properties and the effects of sodium dodecyl sulfate." Biochemistry **10**(5): 743-52.

Visudtiphole, V., M. B. Thomas, D. A. Chalton and J. H. Lakey (2005). "Refolding of Escherichia coli outer membrane protein F in detergent creates LPS-free trimers and asymmetric dimers." Biochem J **392**(Pt 2): 375-81.

Vogel, H. and F. Jahnig (1986). "Models for the structure of outer-membrane proteins of Escherichia coli derived from raman spectroscopy and prediction methods." J Mol Biol **190**(2): 191-9.

Voos, W. and K. Rottgers (2002). "Molecular chaperones as essential mediators of mitochondrial biogenesis." Biochim Biophys Acta **1592**(1): 51-62.

Voulhoux, R., M. P. Bos, J. Geurtsen, M. Mols and J. Tommassen (2003). "Role of a highly conserved bacterial protein in outer membrane protein assembly." Science **299**(5604): 262-5.

Waldispuhl, J., B. Berger, P. Clote and J. M. Steyaert (2006). "Predicting transmembrane beta-barrels and interstrand residue interactions from sequence." Proteins **65**(1): 61-74.

Waldispuhl, J., B. Berger, P. Clote and J. M. Steyaert (2006). "transFold: a web server for predicting the structure and residue contacts of transmembrane beta-barrels." Nucleic Acids Res **34**(Web Server issue): W189-93.

Wallace, B. A. and D. Mao (1984). "Circular dichroism analyses of membrane proteins: an examination of differential light scattering and absorption flattening effects in large membrane vesicles and membrane sheets." Anal Biochem **142**(2): 317-28.

Wandrey, M., B. Trevaskis, N. Brewin and M. K. Udvardi (2004). "Molecular and cell biology of a family of voltage-dependent anion channel porins in Lotus japonicus." Plant Physiol **134**(1): 182-93.

Webb, D. C. and A. W. Cripps (1999). "A method for the purification and refolding of a recombinant form of the nontypeable Haemophilus influenzae P5 outer membrane protein fused to polyhistidine." Protein Expr Purif **15**(1): 1-7.

Weiss, M. S., U. Abele, J. Weckesser, W. Welte, E. Schiltz and G. E. Schulz (1991). "Molecular architecture and electrostatic properties of a bacterial porin." Science **254**(5038): 1627-30.

Whitmore, L. and B. A. Wallace (2004). "DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data." <u>Nucleic Acids Res</u> **32**(Web Server issue): W668-73.

Whittington, S. J., B. W. Chellgren, V. M. Hermann and T. P. Creamer (2005). "Urea promotes polyproline II helix formation: implications for protein denatured states." <u>Biochemistry</u> **44**(16): 6269-75.

Woodfield, K., A. Ruck, D. Brdiczka and A. P. Halestrap (1998). "Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition." <u>Biochem J</u> **336 (Pt 2)**: 287-90.

Xu, X. and M. Colombini (1996). "Self-catalyzed insertion of proteins into phospholipid membranes." <u>J Biol Chem</u> **271**(39): 23675-82.

Xu, X. and M. Colombini (1997). "Autodirected insertion: preinserted VDAC channels greatly shorten the delay to the insertion of new channels." <u>Biophys J</u> **72**(5): 2129-36.

Xu, X., W. Decker, M. J. Sampson, W. J. Craigen and M. Colombini (1999). "Mouse VDAC isoforms expressed in yeast: channel properties and their roles in mitochondrial outer membrane permeability." <u>J Membr Biol</u> **170**(2): 89-102.

Yang, J. T., C. S. Wu and H. M. Martinez (1986). "Calculation of protein conformation from circular dichroism." <u>Methods Enzymol</u> **130**: 208-69.

Yeh, T. Y., D. Peretti, J. Z. Chuang, E. Rodriguez-Boulan and C. H. Sung (2006). "Regulatory Dissociation of Tctex-1 Light Chain from Dynein Complex Is Essential for the Apical Delivery of Rhodopsin." <u>Traffic</u>.

Zaid, H., S. Abu-Hamad, A. Israelson, I. Nathan and V. Shoshan-Barmatz (2005). "The voltage-dependent anion channel-1 modulates apoptotic cell death." <u>Cell Death Differ</u> **12**(7): 751-60.

Zakharian, E. and R. N. Reusch (2003). "Outer membrane protein A of Escherichia coli forms temperature-sensitive channels in planar lipid bilayers." <u>FEBS Lett</u> **555**(2): 229-35.

Zakharian, E. and R. N. Reusch (2005). "Kinetics of folding of Escherichia coli OmpA from narrow to large pore conformation in a planar bilayer." <u>Biochemistry</u> **44**(17): 6701-7.

Zalk, R., A. Israelson, E. S. Garty, H. Azoulay-Zohar and V. Shoshan-Barmatz (2005). "Oligomeric states of the voltage-dependent anion channel and cytochrome c release from mitochondria." <u>Biochem J</u> **386**(Pt 1): 73-83.

Zizi, M., M. Forte, E. Blachly-Dyson and M. Colombini (1994). "NADH regulates the gating of VDAC, the mitochondrial outer membrane channel." J Biol Chem **269**(3): 1614-6.

Zizi, M., L. Thomas, E. Blachly-Dyson, M. Forte and M. Colombini (1995). "Oriented channel insertion reveals the motion of a transmembrane beta strand during voltage gating of VDAC." J Membr Biol **144**(2): 121-9.