Oligomycin-Resistant ATPase and Cyclic AMP-Dependent

Protein Kinase Activities in Bovine Heart Mitochondria

BY

JAMES W. BURGESS

A thesis
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES   PAGE

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
OR-ATPase	Oligomycin-resistant ATPase
OS-ATPase	Oligomycin-sensitive ATPase
C	Catalytic subunit of protein kinase
CB1, CB2, CB3	From the top, the first, second and third bands in the Comte and Gautheron sucrose density gradients
C-int	A fraction enriched in soluble mitochondrial material in fractionation procedure 1
Cyclic AMP	Cyclic adenosine 3':5' monophosphate
Cyclic GMP	Cyclic guanosine 3':5' monophosphate
EDTA	Ethylenediamine-tetra-acetate
EGTA	Ethylene glycol bis (B-aminoethyl -ether) N,N'-tetraacetic acid
NADH	Reduced form of nicotinamide-adenine dinucleotide
pCMB	p-Chloromercuribenzoic acid
R.S.A.	Relative specific activity
SB1, SB2, SB3	From the top, the first, second and third bands in the Scholte sucrose density gradients
SDS	Sodium dodecyl sulfate

ABSTRACT

This work was prompted by the suggestion of others that mitochondria of some tissues, including heart, contain an oligomycin-resistant ATPase (OR-ATPase) activity that may be involved in mitochondrial calcium efflux. Cyclic AMP has also been implicated in the efflux of mitochondrial calcium. Accordingly, beef heart mitochondria were fractionated by two different methods and the component parts examined for OR-ATPase and cyclic AMP-dependent protein kinase activities. Fractions enriched in the outer membrane marker, monoamine oxidase, were also enriched in OR-ATPase when combined light and heavy mitochondria were used. These fractions were, however, also enriched in acid phosphatase. Thus, this resistant ATPase was probably lysosomal in origin; it was not present in outer membranes of heavy mitochondria. About 12% of the ATPase activity of enriched inner membrane preparations was resistant to oligomycin. However, oligomycin and azide in combination were found to inhibit as much as 97% of the inner membrane ATPase activity. This effect, and results obtained from electrophoresis and gel staining for ATPase activity, suggest that most of the resistant ATPase activity of the inner membrane results from incomplete inhibition of the mitochondrial energy-transducing ATPase.

Mitochondrial soluble fractions, although not enriched in resistant ATPase activity, did yield two cyclic AMP-dependent protein kinases, designated Type I-m and Type II-m. Protein kinase activity was found to exist in mitochondrial soluble fractions and enrichment of mitochondrial protein kinase activity in membrane fractions suggests that at least a portion of the activity exists in a membrane-bound state. Sonication or treatment of mitochondrial preparations with Triton X-100 resulted in a 2-fold activation of mitochondrial protein kinase activity. Type I-m eluted from DEAE-cellulose at concentrations of less than 0.1 M NaCl and was a minor component of mitochondrial soluble fractions. Type II-m eluted at salt concentrations greater than 0.1 M NaCl and was further purified 74-fold from mitochondria. Both protein kinase preparations exhibit cyclic AMP-stimulated ATPase activity. Kinase and ATPase activities of both enzymes exhibit similar pH and Mg2+ optima suggesting that observed ATPase activities result from reactions involving the catalytic subunit of cyclic AMP-dependent protein kinase. Mitochondrial oligomycin-resistant ATPase, however, does not always co-localize with cyclic AMP-dependent protein kinase activity in mitochondrial fractionation schemes. It is unlikely that ATPase activity of the free catalytic subunit of cyclic AMP-dependent protein kinase can account solely, if at all, for oligomycin-resistant ATPase activity of mitochondria. The possibility exists that cyclic AMP-

dependent phosphorylation of mitochondrial proteins regulates the transport of calcium across heart mitochondrial membranes.

INTRODUCTION

I   Mitochondrial Calcium and Heart Function

Calcium and ATP are the two principal substances involved in active contraction-relaxation cycles of the heart. The normally functioning heart derives essentially all of its energy (ATP) from mitochondrial oxidative phosphorylation. Calcium is known to play an important role in the regulation of oxidative phosphorylation (1, 2, 3) and of key intramitochondrial enzymes (4). In the ischemic myocardium, calcium overloading of the mitochondria results in an uncoupling of oxidative phosphorylation with a resulting decrease of cytoplasmic ATP levels and loss of cellular viability (5, 6). The study of mitochondrial calcium influx and efflux may further understanding of the mechanisms causing heart failure in the ischemic myocardium.

II   Mitochondrial Calcium Transport

Mitochondria will take up calcium when supplied with a suitable oxidation substrate or ATP (7). The uptake is mediated by the electrophoretic calcium uniporter (8, 9) and is powered by the proton gradient generated by substrate oxidation or ATP hydrolysis. The process of mitochondrial calcium efflux is poorly understood at present. In heart the major mechanism is the Na+/Ca2+ antiport system (10) which couples the entry of sodium to the efflux of calcium. It has been proposed that phosphate ions are important in the efflux of calcium from mitochondria (11). The role of phosphate in this process is uncertain as, under diverse experimental conditions, phosphate has been reported to

stimulate (12), inhibit (13) or efflux in parallel with calcium efflux (11). Cyclic AMP has been reported to stimulate the efflux of calcium from kidney (14), liver (14, 15, 16), adrenal cortex (16) and heart mitochondria (14). These early results, however, were not readily duplicated by other investigators (17, 18). More recent work with liver mitochondria has suggested that the cyclic AMP-mediated release of calcium is reproducible but is a very labile process (15). The effect is not observed when aged preparations of mitochondria are used. It has been suggested that mitochondrial oligomycin-resistant ATPase (OR-ATPase) activity may be involved in mitochondrial calcium efflux (19).

III Mitochondrial OR-ATPase Activity

OR-ATPase activity has been localized to the outer membrane of rat kidney cortex (19) and spleen (20,21) mitochondria. This activity has also been reported in rabbit bone marrow (22) and rat heart mitochondria (20); however, no precise intramitochondrial location or function was suggested. The importance of calcium in myocardial homeostasis and a potential role for OR-ATPase activity in mitochondrial calcium efflux suggested that this enzyme should be studied in heart tissue.

IV   Problems Associated With The Study of Mitochondrial

   OR-ATPase Activity

   a) Contamination With Other Membrane Systems

   Early reports of the outer membrane OR-ATPase in

mitochondria were complete in ruling out the possibility

that the activity resulted from plasmalemmal or endoplasmic

reticular contamination (19, 20, 21). The activity was also

insensitve to azide, an inhibitor of the free form of the

mitochondrial energy-transducing ATPase (21). Unfortunately

these early studies were performed before the finding that

lysosomes exhibit ATPase activity (23-26) that is

insensitive to inhibition by oligomycin and azide (25).

Consequently, the possibility that the outer membrane

OR-ATPase activity may result from lysosomal contamination

was ignored. Lysosomes are a common contaminant of

mitochondrial preparations but can be effectively separated

from mitochondria by a number of different techniques

(23, 25, 26, 27). Bovine heart mitochondria, which are used

in the present study, exhibit heavy and light layers of

mitochondria. Lysosomal contamination can be effectively

reduced by removal of the light mitochondrial layer (27).

Following this simple manipulation, OR-ATPase activities and

distributions amongst the various mitochondrial components

can be compared for both heavy and light mitochondria. This

form of analysis should differentiate lysosomal from

mitochondrial OR-ATPase activity.

b)   The Mitochondrial Energy-Transducing ATPase

Studies of OR-ATPase activity in the outer or inner membranes of mitochondria would be complicated by the presence of the energy-transducing ATPase. This enzyme also known as oligomycin-sensitive ATPase (28), Fo-Fl complex (29) or the H+-translocating ATPase (30) is a multimeric enzyme complex, capable of utilizing the transmembrane proton gradient to synthesize ATP. The complex consists of an extrinsic sector Fl that hydrolyses ATP and a membrane-bound sector designated Fo forming the proton channel. The membrane-bound form of the enzyme is sensitive to inhibition by oligomycin or azide; however inhibition of ATPase activity by either inhibitor alone is not complete (for review see 31 and 32). In beef heart mitochondria, the residual ATPase activity seen in the presence of oligomycin is susceptible to inhibition by azide (33), suggesting that more complete inhibition of the energy-transducing ATPase can be achieved by using a combination of these inhibitors. The extrinsic sector of the energy-transducing ATPase can also exist in a soluble form that is resistant to oligomycin, but is sensitive to azide (32). Therefore, used in combination, these inhibitors may provide a superior measure to assess mitochondrial ATPase activities that are distinct from the energy-transducing ATPase.

c) Mitochondrial Membranes and Fractionation Methods

The existence of a putative outer membrane mediator of calcium efflux poses a problem when the properties of the outer mitochondrial membrane are considered. It is generally accepted that this membrane system is freely permeable to calcium, most metabolites, and quite high molecular weight compounds (34, 35). In contrast, the inner membrane is a highly selective permeability barrier suggesting that this would be a logical emplacement for calcium efflux effectors. Accordingly, mitochondrial OR-ATPase activity should be examined in both the outer and inner membrane.

Unlike in other tissues, the outer and inner mitochondrial membranes of heart are joined together by proteinaceous cross-bridges (36). For this reason, fractionation procedures designed to separate out the various components of heart mitochondria are rather severe. During these procedures, inner membrane rupture often occurs with a resulting contamination of mitochondrial components with matrix material (37-40). Outer and inner membrane preparations are usually heavily contaminated with each other (36-38); probably the result of cross-bridging between these components. Some of these fractionation methods involve the use of proteinases, for example trypsin, to aid in breaking the cross-links between the outer and inner membrane system and improving yields and purity of these two components (36, 38). However, proteolytic alterations of

the enzymes under investigation may also occur. These
problems have often resulted in the erroneous association of
an enzymatic activity with a specific mitochondrial
compartment that could not be duplicated when a different
fractionation procedure was used. Therefore localization
data derived from one method should always be verified by a
second fractionation scheme.

V   Mitochondrial Protein Kinase Activity

Two casein kinases, designated MPK1 and MPK2, have been
identified and purified from bovine heart mitochondria (41).
Similar enzymes have been noted in yeast mitochondria and
localized to the inner mitochondrial membrane (42, 43).
Cyclic nucleotide-independent protein kinase with a
preference for mixed histones as substrate has also been
localized to the inner membranes of mouse heart and liver
mitochondria (44). Several reports in the literature
describe cyclic AMP-dependent protein kinase activity in rat
liver (45, 46) and ovarian tissue mitochondria (47-50). _In_
vivo phosphorylation of mitochondrial membrane was observed
in rat liver and this activity was stimulated by glucagon
(51).

VI   Cyclic AMP-Dependent Protein Kinases

Cyclic AMP has been implicated in the release of
calcium from mitochondria (14-16). Cyclic AMP-dependent
protein kinase activity has been demonstrated in
mitochondria from various tissues (45-50) but not in heart
(44). It is possible that cyclic AMP provokes the release

of mitochondrial calcium through the cyclic AMP-dependent
protein phosphorylation of mitochondrial proteins. The
description of cyclic AMP-dependent protein kinase activity
in heart mitochondria may suggest a mechanism for the cyclic
AMP-mediated regulation of mitochondrial calcium transport
in this tissue.

Studies of cytosolic fractions of cells have revealed
that two forms, designated Types I and II, of cyclic AMP-
dependent protein kinases exist. The inactive holoenzyme
form of these enzymes is a tetrameric structure, composed of
two catalytic subunits (C) and two cyclic AMP-binding
regulatory subunits (R). Binding of cyclic AMP to the R
subunits results in the dissociation of C and R and a
resulting activation of the phosphorylating activity of C.
It is generally accepted that the C subunit of Types I and
II cyclic AMP-dependent protein kinases is identical; the
differences between the isozymes residing in the R subunit
(for review see ref 52 and 53).

VI   Problems Associated With the Study of Mitochondrial

Protein Kinases

a)   Cytosolic Protein Kinases

The major problem associated with research in this
field resides in the fact that all the protein kinases so
far described in mitochondria also exist in the cytoplasm of
the cell. Compounding this problem are findings that
protein kinases may exist in a membrane-bound state in the
cell. For example, in bovine heart as much as 30% of the

cellular cyclic AMP-dependent protein kinase activity is membrane bound (54). Therefore protein kinase activity may appear to be mitochondrial when it is actually cytosolic in origin but bound to the outer surface of the mitochondria. Usually the only criteria available to label a protein kinase activity "intramitochondrial" is the ability of membrane disruptive agents, such as sonication or detergent treatment, to activate that activity (41, 44, 45, 47). Such techniques act by eliminating membrane barriers that separate exogenously applied phosphorylation substrates from the enzymatic activity within mitochondria.

b) Mitochondrial ATPase activity

Heart mitochondria exhibit substantial hydrolysis of ATP even in the presence of oligomycin (33). This is likely to create problems in assays designed to measure protein phosphorylation in mitochondrial fractions. Maximum inhibition of the mitochondrial energy-transducing ATPase with a combination of oligomycin and azide (39) should overcome this problem.

c) Localization of Mitochondrial Protein Kinase Activity

Localization of the cyclic AMP-dependent protein kinase activity to a specific mitochondrial compartment is likely to prove difficult. Problems may result from the use of trypsin and soybean trypsin inhibitor in some mitochondrial fractionation schemes. Trypsin has no effect on enzymatic activity; however, it can result in the release of the

holoenzyme from membranes (54). The end result would be the appearance of cyclic AMP-dependent protein kinase activity in soluble mitochondrial fractions. Although the mechanism is currently unknown, soybean trypsin inhibitor has an inhibitory effect on protein phosphorylation catalyzed by the C subunit of protein kinase (55, 56). Therefore at least one fractionation technique that does not use trypsin and soybean trypsin inhibitor should be used in the localization of cyclic AMP-dependent protein kinases in mitochondria.

VIII Research Aims

The ability of mitochondria to take up calcium far exceeds calcium release through the Na+/Ca2+ antiport system (57). This has led to the proposal that other calcium efflux mechanisms may exist in mitochondria. In heart, elucidation of other effectors of mitochondrial calcium efflux may further understanding of mitochondrial dysfunction in the ischemic myocardium. The suggestion that mitochondrial OR-ATPase is involved in calcium efflux (19) prompted us to study this enzymatic activity in bovine heart mitochondria. Emphasis placed in the current study has been on examining the possibility that lysosomal ATPase or incomplete inhibition of the energy-transducing ATPase by oligomycin are factors contributing to mitochondrial OR-ATPase activity. It has been demonstrated that ATPase activity (defined as the phosphoryl transfer to water) is an integral part of the catalytic mechanism of cyclic AMP-

dependent protein kinases (58-61). ATPase activities of protein kinases could influence results for mitochondrial OR-ATPase activity. It was the intent to unravel the complex origins of mitochondrial OR-ATPase activity first so that implications with regard to mitochondrial calcium efflux could be studied later.

Phosphorylation of proteins catalyzed by protein kinases has been recognized as a major regulatory mechanism of biological processes (52, 53). Mitochondrial protein kinase activity is poorly characterized at present and cyclic AMP, possibly acting through cyclic AMP-dependent protein kinases, has been implicated in mitochondrial calcium efflux (14-16). Previous studies (44) have been unable to demonstrate cyclic AMP-dependent protein kinase activity in heart mitochondria, suggesting a study of this enzyme was warranted.

MATERIALS

Cyclic AMP, cyclic GMP, casein, phosvitin, trypsin, protamine, phosphatidylserine, diolein, oligomycin, azide, calmodulin (P2277), vanadate, ouabain, EGTA, NADH and whole calf thymus histones (H2-A No. 92-50) were purchased from Sigma. Soybean trypsin inhibitor was obtained from Calbiochem, and [γ-32P] ATP from New England Nuclear. Histones H2A and H2B from trout testis were gifts from Dr. J.R. Davie of this department. Whatman 3 MM filter disks (1.9 cm) were obtained from McLeester Research Equipment.

EXPERIMENTAL METHODS

I   Preparation of Bovine Heart Mitochondria

Freshly excised bovine hearts were obtained from East-West Packers of Winnipeg and stored on ice during transport. Mitochondria were prepared by the method of Azzone, Colonna and Ziche (62) with the exception that the number of washing steps (including the removal of the light mitochondrial layer) was increased to four.

In some experiments, light mitochondria (washed twice) were further purified by density gradient centrifugation on Percoll gradients. Mitochondria were suspended in 30% Percoll and 0.25 M sucrose and centrifuged at 78,500 g for 1 hour (Beckman 60 Ti rotor) at 4 $^\circ$C. Gradient bands were aspirated with a J-shaped hypodermic syringe and centrifuged at 105,000 g for 1 hour (Beckman 60 Ti rotor). Gentle swirling of the centrifuge tubes was sufficient to separate the overlying mitochondrial material from the Percoll pellets. Mitochondrial material was then collected by simply decanting off the supernatant.

II   Mitochondrial Marker Enzyme Assays

Monoamine oxidase, an established outer membrane marker, was assayed according to Schnaitmann and Greenawalt (63) after a modification of the procedure of Tabor et al (64). Cytochrome c oxidase, an inner membrane marker, was assayed by the method of Yonetani (65). Adenylate kinase activity, assayed by the method of Schnaitmann and Greenawalt (63), was used as a marker for the mitochondrial intermembrane space. Malate dehydrogenase, a marker for the

mitochondrial matrix, was assayed by the method of Ochoa (66). Acid phosphatase activity, assessed according to Lindhart and Walters (67) was used to determine the extent of lysosomal contamination. 5'-Nucleotidase, a marker for sarcolemmal contamination was assayed by the method of Arkesteijn (68). All marker enzyme assays were carried out under conditions in which the reaction velocities were constant with respect to time and proportional to enzyme concentration.

III   Other Enzyme Assays

ATPase activity was measured by three methods.

Method 1

The sodium dodecyl sulfate termination method of Monk and Kellerman (69) as modified by Yamada, Schiffman and Huzel (1) was employed. The reaction mixture, containing 50 mM Tris/sulfate buffer (pH 7.7), 0.36 M mannitol as well as 1.67 µg of antimycin, 2 mM phosphoenolpyruvate, 8.5 units of pyruvate kinase, 15 units of lactate dehydrogenase, 2 mM MgATP, and 0.205 µmol of NADH, in a total volume of 1 ml, was equilibrated for 10 min at 30 °C. The reaction was started by the addition of enzyme. Incubation was for 10 min at 30 °C. The reaction was terminated by sodium dodecyl sulfate to a final concentration of 0.33% (w/v). Control tubes lacked enzyme which was added after the addition of sodium dodecyl sulfate. Reaction blanks were prepared containing the complete reaction mixture but with NADH omitted. Samples were read against the blank at

340 nm.   Oligomycin-resistant ATPase activity was
determined in the presence of 10 µg oligomycin/mg protein.
Oligomycin was preincubated with enzyme for 5 min at 30 °C
before the reaction was initiated as described before (3).
All other inhibitors were added directly to the assay.

Method 2

In some experiments it was convenient to follow the
ATPase reaction in a continuous fashion under conditions
identical to those used in method 1. In this case the
assays were performed at 30 °C in thermostatted
spectrophometer cuvettes, and the reaction was started by
the addition of enzyme after a 10 minute preincubation of
the assay mixture with ATP. Reaction blanks lacked ATP, and
control rates (in the absence of enzyme) were used to assess
the non-enzymatic breakdown of ATP during the course of the
reaction.

Method 3

The ATPase activity of partially purified preparations
of protein kinase (measured in the absence of phosphoryl-
ation substrate) was determined at 30 °C by the release of
[32P] inorganic phosphate from [γ-32P]ATP. Reaction
conditions were identical to those used to measure protein
phosphorylation in partially purified mitochondrial protein
kinase fractions. The reaction was terminated by the
addition of 175 µl of ice-cold 30% trichloroacetic acid.
[32P]Inorganic phosphate was quantitated by the method of
Racker (70).

Protein Phosphorylation Assay

Protein kinase activity of mitochondrial fractions was assessed by a modification of the method of Vardanis (44); the reaction mixture included 5 mM sodium azide, 20 µg oligomycin/mg of enzyme protein, 10 mM 2-mercaptoethanol, 20 mM NaF, 5 mM MgSO4, 10 mM Tris-HCL buffer, pH 8.0, 250 µg of histone substrate, 0.25 mM [δ-32P]ATP (8,000-20,000 cpm/nmol) and enzyme in a total volume of 75 µl. Assays were carried out at 30 °C in the presence and absence of 0.5 µM cyclic AMP. Protein substrates were heated for 5 min at 80 °C prior to use. The reaction was initiated by the addition of ATP.

Conditions used to assay partially purified preparations of mitochondrial protein kinases were slightly modified. Type I-m preparations were assayed with 4 mM MgSO4 and 10 mM imidazole-HCl buffer, pH 7.0. Type II-m kinase preparations were assayed at 2.5 mM MgSO4 and 10 mM Tris-HCl buffer, pH 7.5. All other reaction conditions were identical to those described for the assay of mitochondrial fractions. Reactions were allowed to proceed for 20 min at 30 °C and were terminated by spotting a 40 µl aliquot onto Whatman 3 MM filter disks and immediately immersing in ice-cold 10% trichloroacetic acid-20 mM sodium pyrophosphate. Disks were further washed in the trichloracetic acid-sodium pyrophosphate solution according to Vardanis (44). Heat-inactivated enzyme (100 °C for 4 min) was added to control tubes.

Units for protein kinase activity (both protein phosphorylation or ATPase as measured by method 3) are defined as the quantity that resulted in the incorporation or release of one pmol of phosphate per min. Enzyme units for all other enzymes are defined as the quantity that resulted in the formation of one μmol of product per minute. Protein was determined by the method of Hess and Lewin (71) with the exception that fractions solubilized with Triton X-100 were assayed by the method of Wang and Smith (72).

IV   Fractionation of Mitochondria.

In agreement with the results of Scholte (36), when digitonin was used to prepare outer membranes from heart mitochondria very poor recoveries were found. Several other methods (Smoly et al., 38), (Whereat et al., 73), (Hayashi and Capaldi, 74) proved to be less satisfactory on the basis of purity and yields of membranes than those described below.

a)   Procedure 1

This was an abbreviated version of the method of Comte and Gautheron (40); no attempts were made to fractionate the mitoplast fraction (inner membrane and matrix). In brief, mitochondria were allowed to swell in 10 mM potassium phosphate buffer, pH 7.4 at 1 mg protein/ml, for 20 min on ice. After light homogenization, the suspension was centrifuged at 105,000 g for one hour in a Beckman 60 Ti rotor. The supernatant was saved and designated C-int fraction. The pellet was suspended in 0.25 M sucrose, 10 mM

Tris-HCl buffer, pH 7.4 and mitoplasts (C-mitoplast fraction) sedimented at 11,500 g for 15 minutes. The resulting supernatant fraction was centrifuged at 105,000 g for one hour to pellet the crude outer membrane fraction. This pellet was suspended in the sucrose-Tris buffer and layered in 2 ml aliquots onto a discontinuous sucrose density gradient consisting of 3 ml each of 51.7%, 31.7% and 25.2% sucrose (w/v). Centrifugation was at 165,000 g for 45 minutes in a Beckman SW 40 Ti rotor at 4 °C. The three resulting bands, designated CB1, CB2 and CB3 (from the top) were aspirated, diluted with buffer, and pelleted at 105,000 g for one hour at 4 °C. The pellets were suspended in 0.25 M sucrose and stored at -70 °C.

b)   Procedure 2

To prepare outer and inner membrane fractions the method of Scholte (36) was modified to process larger amounts of mitochondrial protein. Mitochondria were suspended in 10 mM Tris-phosphate buffer, pH 7.5 (7.32 ml/ 50 mg protein) to which was added 1.7 mg of trypsin. The suspension was mixed in a centrifuge tube with a loose-fitting pestle. After 5 seconds, one-third volume of 1.8 M sucrose, 2 mM $MgSO_4$ and 2 mM ATP was added to the suspension with continuous mixing. One minute after the addition of trypsin, the suspension was sonicated on ice in 10 ml aliquots with four 5 second bursts at a setting of 6.5 (Heat-Systems Ultrasonic sonifier, model W 185). Each burst of sonication was followed by a 15 second cooling period.

Six minutes after the addition of trypsin, soybean trypsin
inhibitor in 0.45 M sucrose was added to give a ratio of
soybean trypsin inhibitor to trypsin of 1.5 µg to 1.0 µg.
The suspension was then layered in 5 ml aliquots onto
sucrose density gradients composed of 2 ml each of 1.2 M,
1.0 M and 0.6 M sucrose, and centrifuged at 249,000 g for
one hour in a Beckman SW 40 Ti rotor at 4 °C. Band 1 (top
of gradient), designated SB1, included the supernatant and
material located at the 0.45 M/0.60 M sucrose interface.
SB2 was material found at the 0.6 M/1.0 M sucrose interface;
SB3 was material at 1.0 M/1.2 M sucrose interface and
S-mitoplast fraction was the pellet. The gradient bands
were aspirated with a J-shaped hypodermic syringe and
S-mitoplast pellets were removed with a glass stirring rod
and lightly homogenized to resuspend this material. All
fractions were diluted with 0.25 M sucrose and pelleted at
105,000 g for one hour in a Beckman 70 Ti rotor at 4 °C.
The pellets were then suspended in 0.25 M sucrose and stored
at -70 °C.

c)   Analysis of the Fractionation Data

Oligomycin-resistant ATPase activity is defined as that
activity observed in the presence of 10 µg of oligomycin/mg
protein. Oligomycin-sensitive ATPase activity is the
difference between activities derived in the absence of
oligomycin and those observed in the presence of oligomycin.
Cyclic nucleotide-independent histone kinase activity has
been described in bovine heart mitochondria (44).

Consequently, protein kinase activities derived from localization studies have been broken down into basal activities (derived in the absence of cyclic AMP) and cyclic AMP-dependent activities (derived in the presence of cyclic AMP minus basal activities).

V   Preparation and Analysis of Histone Phosphorylation

Substrate

Whole calf thymus histones (Sigma H2-A, No. 92-50) were resolved into four peaks by chromatography on Bio-Gel P-30 columns as described by Davie (75). The third histone peak eluting from the column was routinely used as the phosphorylation substrate. Histone preparations were analyzed by electrophoresis by the acetic acid/urea/Triton X-100 mini slab gel system of Davie (75).

VI   Electrophoresis and Staining for ATPase Activity

Electrophoresis in polyacrylamide tube gels was carried out as described by Apps and Glover (76) with the exception that Triton X-100 (0.1% v/v) was present in the gels and electrophoresis buffers. The gels were subjected to pre-electrophoresis for 20 min prior to addition of sample as suggested by Dulaney and Touster (77). Samples for electrophoresis were solubilized in 2% Triton X-100 by the method of Barbero et al (78) and then preincubated at 25 $^{\circ}$C in 50 mM 2-mercaptoethanol for 2 hr. Samples were then diluted with an equal volume of two times concentrated electrophoresis buffer and aliquots containing 70 µg of protein were loaded on gels. ATPase activity (in the

presence and absence of 5 mM azide) was stained in gels by the methods of Apps and Glover (76).

VII Partial Purification of Mitochondrial Protein Kinases

a) Sonication and Ultracentrifugation

The following procedure is based on the enzyme purification from 9.1 g of heavy mitochondrial protein. All procedures were performed at 0-4 °C. Mitochondria were suspended to 20 mg protein/ml in 5 mM Tris-HCl, 1 mM EDTA and 5 mM 2-mercaptoethanol, pH 7.5 (purification buffer) and sonicated on ice in 10 ml aliquots at maximum amperage (Bronwill sonifier, model 1510) with three 1 min bursts interspersed with 30 second cooling periods. The sonicated suspension was centrifuged at 105,000 g for 1 hr (Beckman 70 Ti rotor). The pelleted membranes were washed again in half the volume of buffer and the two supernatant fractions were pooled.

b) DEAE-Cellulose Chromatography

Mitochondrial supernatant fractions (2.1 g in 750 ml) were processed in three batches. A 250 ml sample of the supernatant fraction was dialyzed against 20 volumes of purification buffer overnight and loaded onto a 1.6 cm x 32 cm column of DEAE-cellulose (Whatman DE-52), previously equilibrated with purification buffer. The column was washed at a flow rate of 2.5 ml/min until no further protein was detected in the eluate. Protein kinase was then eluted with a linear salt gradient running from 0 to 0.4 M NaCl in a total volume of 1000 ml. Fractions of 15 ml were

collected, and kinase activity was determined by adding 10 μl aliquots of each fraction to the phosphorylation assay. Individual peak fractions, containing Type I-m and Type II-m protein kinases were collected and concentrated by precipitation with 70% ammonium sulfate, redissolved and dialyzed overnight against purification buffer.

c) Sephacryl S-200 Column Chromatography

Concentrated preparations of Type I-m (205 mg in 12 ml) and Type II-m (170 mg in 11.5 ml) from the previous step were loaded on a column of Sephacryl S-200 (2.5 cm X 77 cm) previously equilibrated with purification buffer. Each kinase was processed by two separate runs through the column. The column was eluted with the same buffer at a flow rate of 1.8 ml/min and 3.6 ml fractions were saved and analyzed for the presence of protein kinase activity. Peak fractions were collected and concentrated by disposable Minicon 10 concentrator units (Amicon).

d) Sephacryl S-300 Column Chromatography

Samples from the previous step were loaded onto a column of Sephacryl S-300 (1.6 cm x 60 cm) previously equilibrated with purification buffer and run at a flow rate of 0.75 ml/min. Protein loads in the column never exceeded 12 mg. Fractions of 1.5 ml were collected, analyzed for protein kinase activity and the peak fractions concentrated on Minicon 10 units.

e)   Hydroxylapatite Column Chromatography

Samples from the Sephacryl S-300 step were dialyzed overnight against 100 volumes of 50 mM potassium phosphate, 5 mM 2-mercaptoethanol buffer, pH 7.0, then loaded onto a column (2.6 cm x 14 cm) of hydroxylapatite (Biogel HTP). The column was washed in step-wise fashion with 50 mM, 0.10 M, 0.15 M and 0.25 M potassium phosphate solutions, pH 7.0, containing 5 mM 2-mercaptoethanol. Each concentration of potassium phosphate was run through the column until no further protein was detected in the eluate. Types I-m and II-m eluted with the 0.15 M potassium phosphate step, were concentrated on Minicon 10 units and dialyzed against purification buffer.

Steps for purification of protein kinase Type II-m were performed in the order presented. For the purification of Type I-m the order of the last two steps was reversed: hydroxylapatite column chromatography was performed first, followed by application to the Sephacryl S-300 column.

VIII SDS-gel Electrophoresis of Protein Kinase Fractions

Samples were boiled for 90 seconds just prior to electrophoresis in 0.063 M Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol. Electrophoresis was performed in a mini slab gel electrophoresis unit (Ideal Scientific) by the method of Laemmli (79). The stacking gel had a total monomer content of 4.0% and the proportion of N',N'-methylene bisacrylamide was 2.7%. Corresponding values for the separating gel were 10.0% and 2.7%

respectively. Both gels contained 1.0% SDS and the gel
buffers were 0.125 M Tris-HCl, pH 6.8, and 0.375 M Tris-HCl,
pH 8.8, respectively for the stacking and separating gels.
Electrophoresis was carried out at a constant voltage of
200 mV/gel until the visible phenol red front reached the
bottom (usually 60 min). Gels were stained overnight in
0.125% Coomassie blue (R-250), 50% methanol and 10% acetic
acid. They were destained for 30 min in 50% methanol and
10% acetic acid, and then overnight in 5% methanol and 7%
acetic acid.

EXPERIMENTAL RESULTS

I   OR-ATPase Activity of Bovine Heart Mitochondria

Fig. 1 shows the effect of varying concentrations of oligomycin on the ATPase activity of heavy mitochondria. It is apparent that the concentration of oligomycin must be raised to 8-10 µg/mg of protein in order that a plateau of inhibition be reached. Consequently, a concentration of 10 µg of oligomycin/mg of protein was used routinely to determine the activity of OR-ATPase. The rates of the ATPase reaction (method 1) in the absence of oligomycin were constant for the 10 minute time interval used and were directly proportional to enzyme concentration up to 15 µg of mitochondrial protein. Fractions with higher specific ATPase activities (SB3 of Table 11) were linear for the same time interval with 5 µg of protein (Fig. 2). In the presence of oligomycin, rates were constant for the 10 minute time interval used and were directly proportional to enzyme concentration up to 20 µg of enzyme protein (Fig. 3).

Mitochondrial pellets derived from bovine heart are characteristically made up of two distinct layers; the upper, light brown layer contains light mitochondria, and the darker brown layer heavy mitochondria. Table 1 shows the effect of oligomycin on different mitochondrial preparations. In four separate experiments, inhibition of light mitochondrial ATPase activity by oligomycin ranged from 74% to 82% (average of 79%). These light mitochondrial preparations also exhibited the highest OR-ATPase activities. Heavy mitochondrial preparations were found to

Figure 1   <u>Inhibition of heavy mitochondrial ATPase</u>

<u>activity by oligomycin</u>

Oligomycin was preincubated for 5 min at 30 °C

with 100 µg of mitochondrial protein in 6.6 mM

Tris-sulfate buffer, pH 7.5, in a volume of

0.76 ml. 76 µl of the preincubated enzyme

(10 µg) was then added to the standard ATPase

assay mixture (method 1) to start the reaction.

Controls were preincubated without oligomycin

which was added after termination of the ATPase

reaction. Each value is the mean of two

experiments.
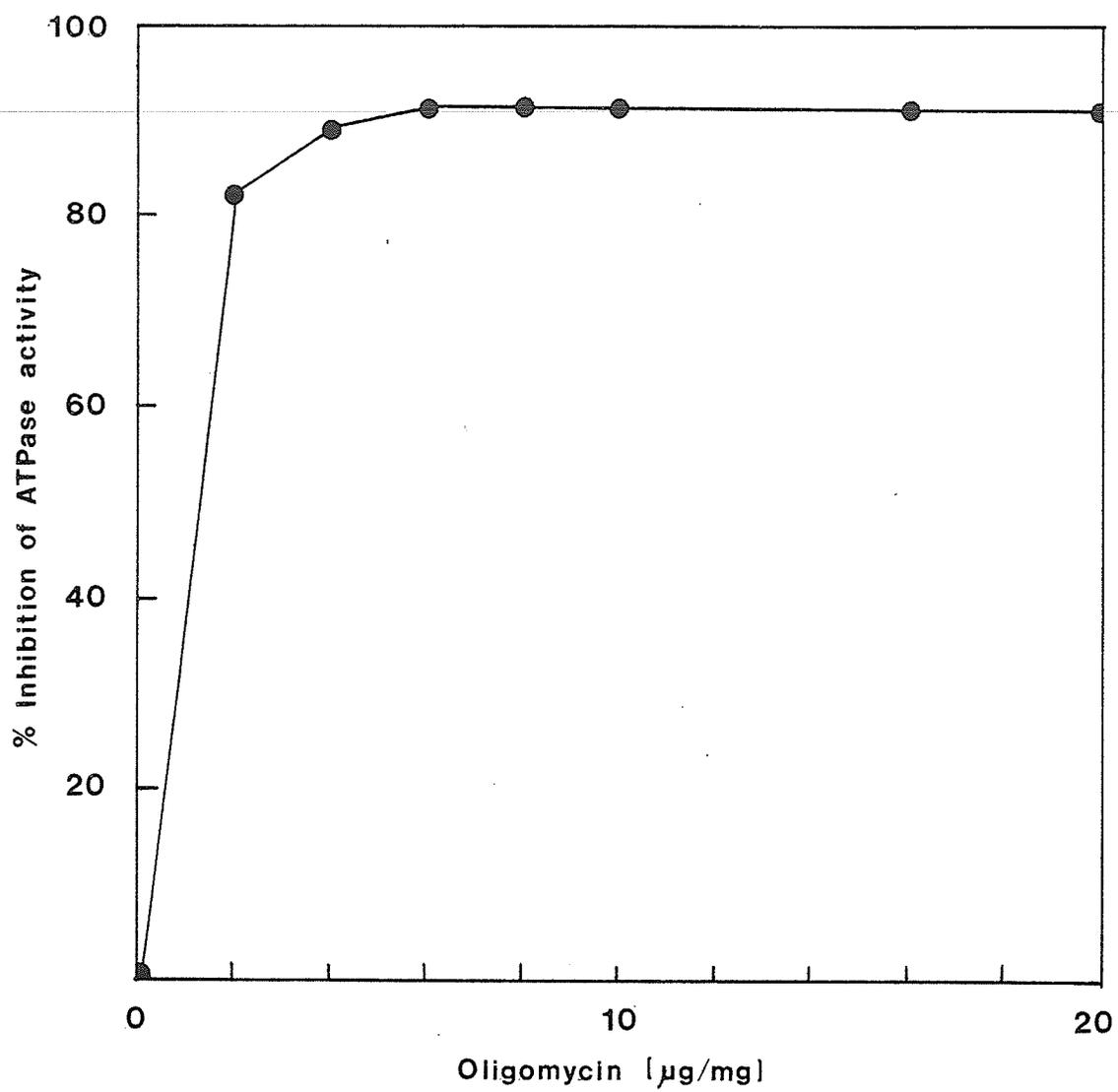
Figure 2   <u>Response of mitochondrial ATPase activity to</u>

<u>incubation time and enzyme concentration</u>

ATPase assay method 1 was used and all values

were determined in the absence of oligomycin.

Heavy mitochondrial protein (●——●), SB3 derived

from fractionation procedure 2 (■——■).   Time

points were obtained with 10 μg of mitochondrial

protein or 2.5 μg of SB3.   Activities at various

enzyme concentrations were determined over an

assay interval of 10 minutes.   Each point

represents the mean of two experiments.
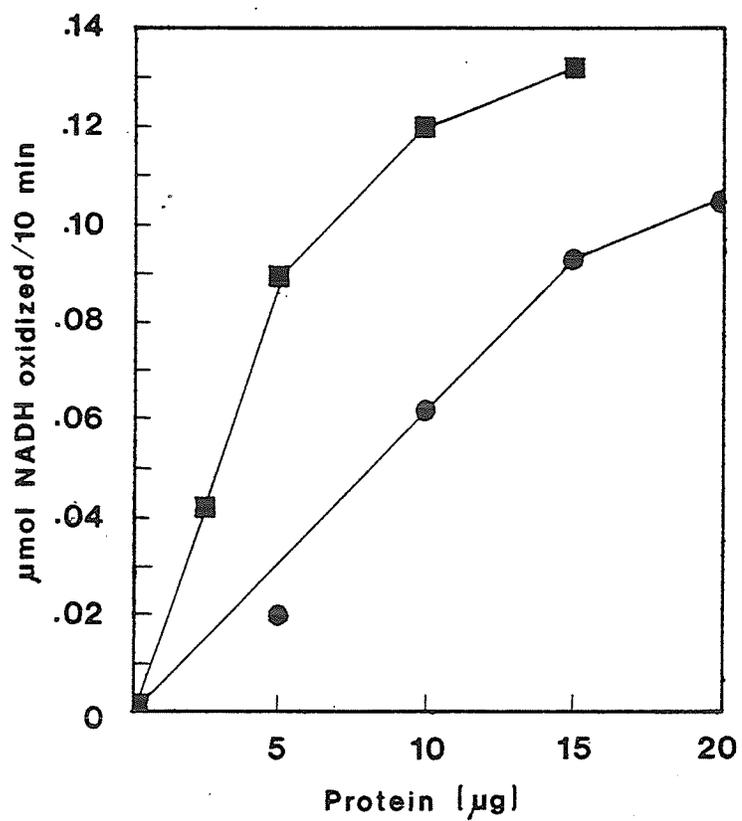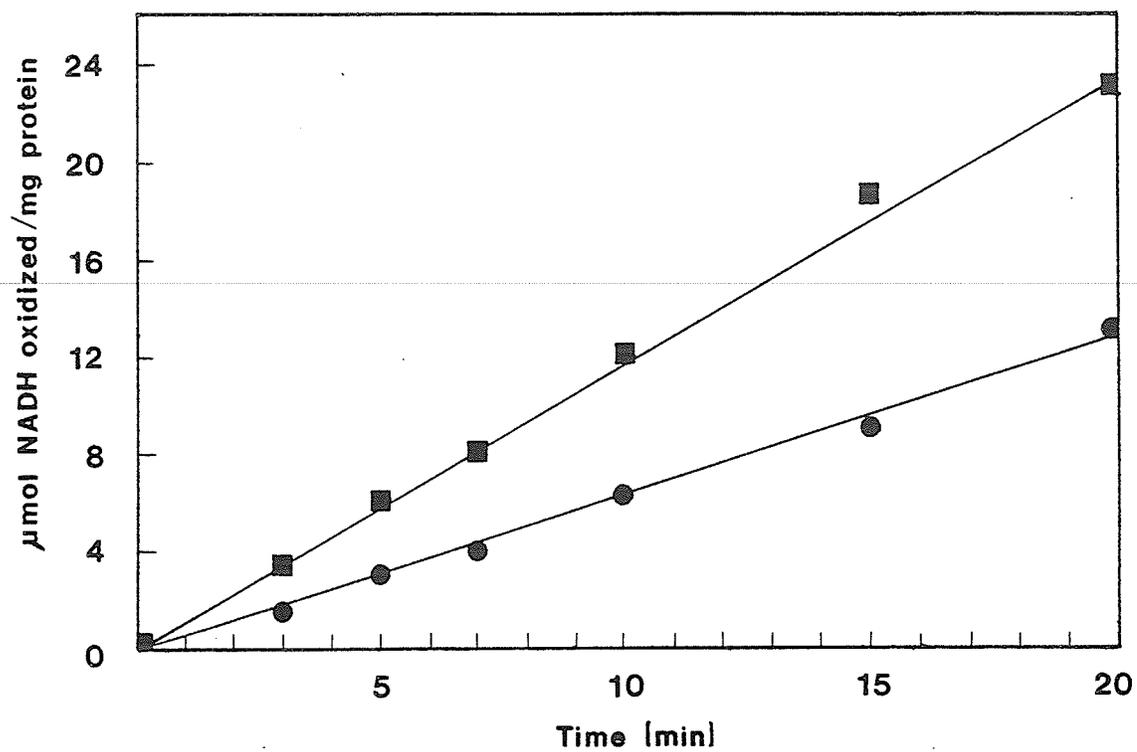
Figure 3   Response of mitochondrial OR-ATPase activity

to incubation time and enzyme concentration

ATPase assay method 1 was used and all values

were determined in the presence of 10 µg

oligomycin / mg of protein. Heavy mitochondrial

protein (●——●), SB3 derived from fractionation

procedure 2 (■——■). Time points were obtained

with 20 µg of enzyme protein and activities

at each enzyme concentration were determined over

an assay interval of 10 minutes. Each point is

the mean of two experiments.
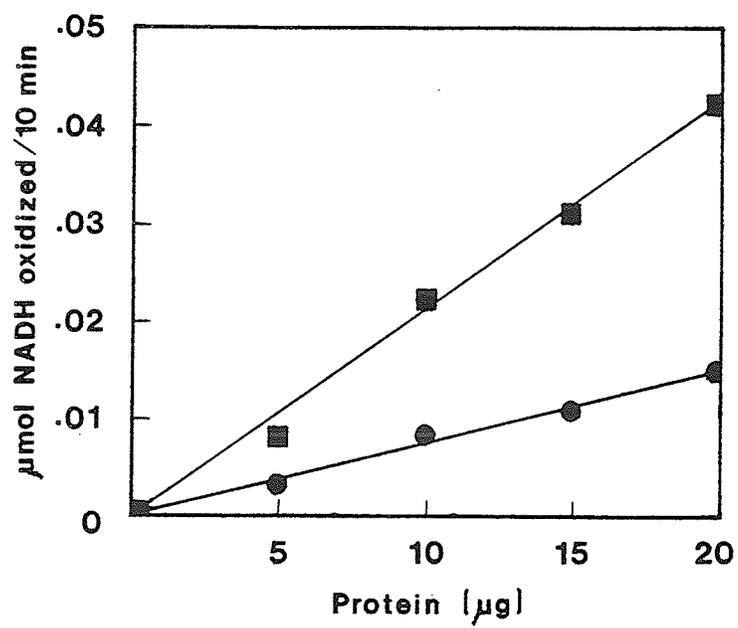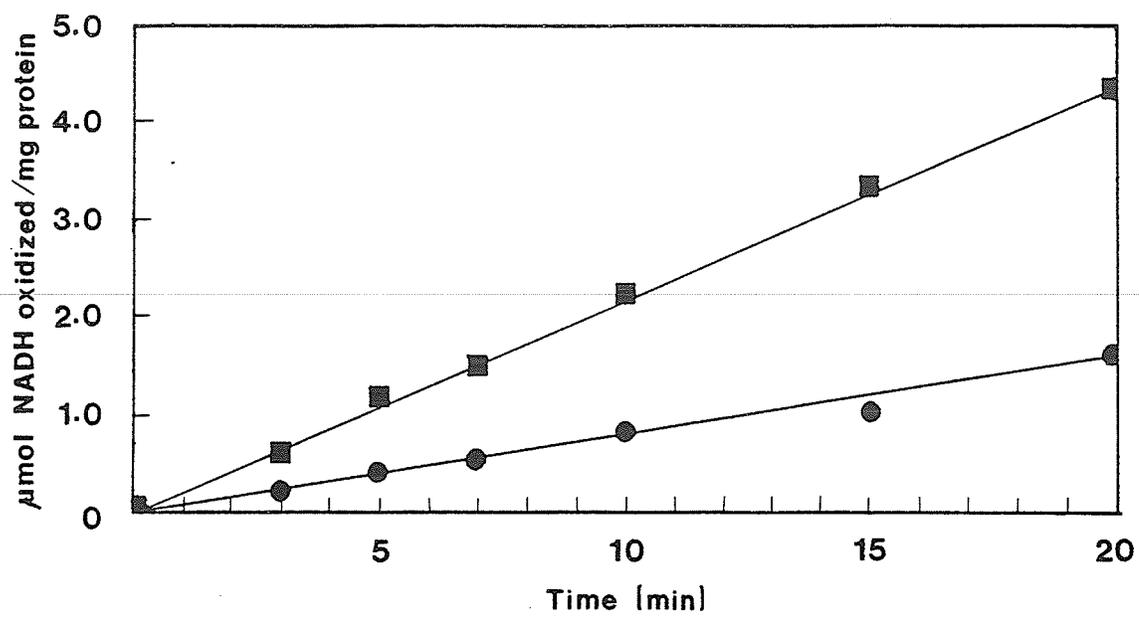
TABLE 1

Effect of Oligomycin on Different Mitochondrial Populations

Heavy and light mitochondria were separated by the method of Azzone et al (62). 10 μg oligomycin/mg protein was used in each experiment and ATPase assays were done by method 1. The number of experiments is indicated in brackets.

Mitochondrial type	CR-ATPase units/mg ± S.E.M.	% inhibition of mitochondrial ATPase activity by oligomycin
Heavy	0.08 ± 0.01 (6)	90.5
Light	0.29 ± 0.09 (4)	78.7
Mixed	0.15 ± 0.04 (5)	87.2

be more sensitive to oligomycin, with inhibitions ranging from 88% to 92% (average of 90.5%) over six experiments. Heavy mitochondrial preparations were associated with much lower ATPase activities in the presence of oligomycin. Results for mixed populations of heavy and light mitochondria are intermediate between the two separate populations in terms of specific activities for OR-ATPase.

Percoll gradients were used to examine the nature of the OR-ATPase activity in light mitochondrial preparations. Light mitochondrial material resolved into four bands on these gradients and the distributions of lysosomal acid phosphatase, mitochondrial cytochrome c oxidase and OR-ATPase were examined by De Duve's (80) plots (Fig. 4). The results of two experiments demonstrate that OR-ATPase activity was most enriched in gradient band 2 (specific activity of 0.46 $\pm$ 0.10 μmol/min/mg). Acid phosphatase was also most enriched in gradient band 2 (specific activity of 0.027 $\pm$ 0.006 μmol/min/mg) and to a lesser extent in gradient band 1. In contrast, cytochrome c oxidase activity was not enriched in gradient band 2 and the distribution of this mitochondrial enzyme in the gradient fractions varied noticeably from that of the other enzymes. These results suggested that the OR-ATPase activity of light mitochondrial preparations was largely of lysosomal origin. Further work attempted to differentiate between lysosomal and mitochond-rial OR-ATPases in heavy and mixed mitochondrial samples.

Figure 4   The partition of cytochrome oxidase, OR-ATPase
             and acid phosphatase of light mitochondria in
             Percoll gradients

             1, 2, 3 and 4 refer to bands in the Percoll
             gradient from the top. Relative specific
             activity (R.S.A.) is defined as the % recovered
             activity in a fraction / % recovered protein in
             that fraction. All data are the means of two
             experiments.
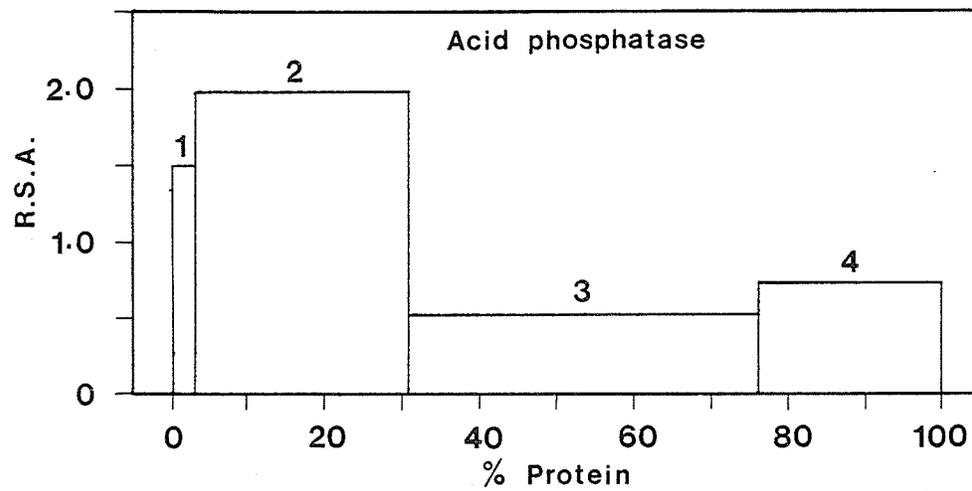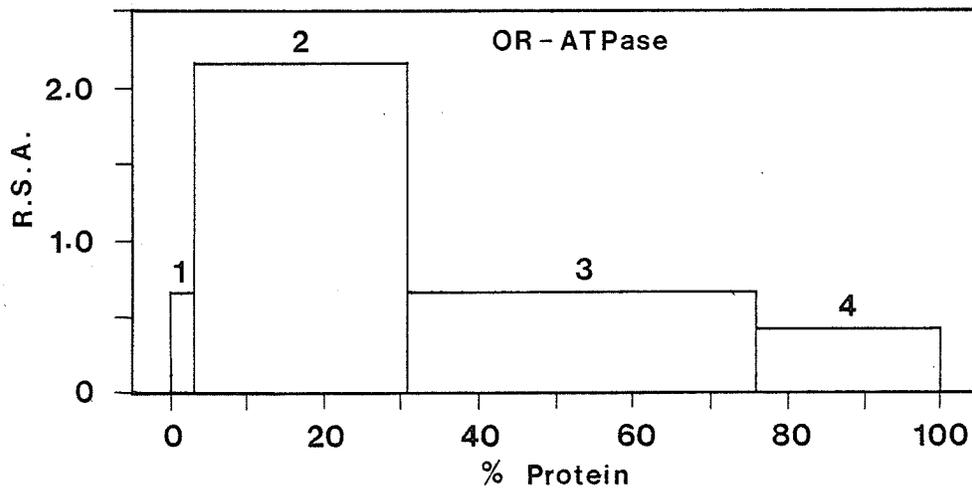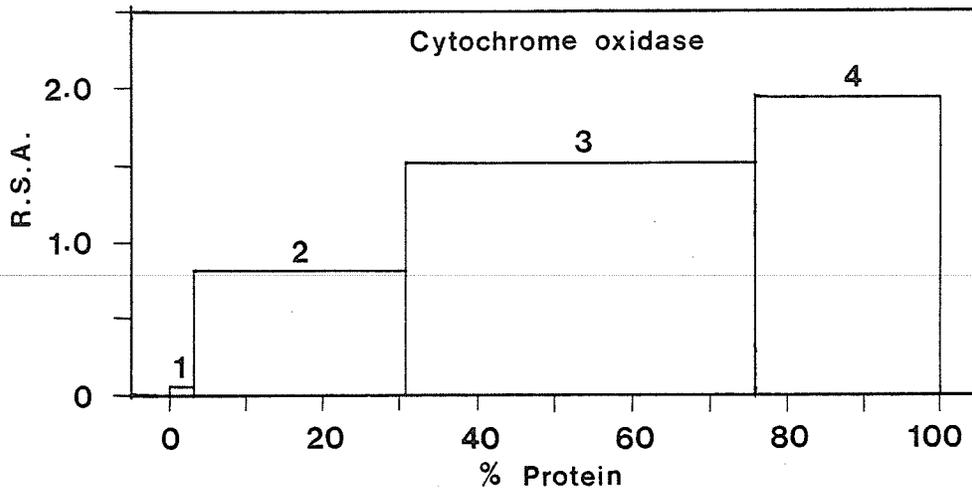
II   Mitochondrial Protein Kinase Activity

In this section a series of preliminary experiments is described which demonstrate that cyclic nucleotide-dependent protein kinase occurs in mitochondria and which establish suitable experimental conditions for the study of the enzyme.

a)   Phosphorylation Substrate and Assay Conditions

Protein kinase activity of both heavy mitochondrial particulate and soluble fractions was found to phosphorylate protamine, casein and phosvitin poorly. Table 2 demonstrates that activity was greater with crude calf thymus histone preparations (Histone IIA, Sigma). Fractions of trout testis histones comprised mainly of H2B were found to be very good substrates (data not shown). Fractionation of the crude calf thymus histone preparations by Bio-Gel P-30 column chromatography resolved histones into four peaks; the third peak eluting from the column was found to be a superior substrate (Table 2). Gel electrophoresis of the histone column fractions and densitometric tracing of the stained gels (see Fig. 5) demonstrated that H2B was the predominant species in Peak III. This histone fraction was used in all subsequent assays as the phosphorylation substrate.

Initially intact mitochondria and membranous subfractions were found to exhibit little protein kinase activity (specific activity < 2 pmol/min/mg protein) in assays lacking 2-mercaptoethanol and containing only

TABLE 2

Substrate Specificity of the Protein Kinase From Bovine Heart Mitochondria

C-int fraction (Table 9) was used. The histone fractions are
designated as described in the legend for Figure 5. Activity was assayed
in the absence of cAMP. Values are the mean of two experiments.

Substrate	Protein kinase activity (units/mg of protein)
Protamine	3.1
Casein	1.9
Phosvitin	5.2
Histone IIA (Sigma)	27.3
Histone H1 (Peak I)	12.3
Histones H2A + H3 (Peak II)	22.4
Histone H2B (Peak III)	243.9
Histone H4 (Peak IV)	7.6

Figure 5   Electrophoresis of histone fractions

Whole calf thymus histones were separated into
four peaks by gel filtration on Bio-Gel P-30.
Aliquots of each were applied to acetic acid/
urea/Triton X-100 mini slab gels (75); electro-
phoresis was in one dimension. The lanes are
numbered from left to right. Lane 1, 12 μg of
unfractionated histones; Lane 2, 6 μg of peak I;
Lane 3, 6 μg of peak II; Lane 4, 6 μg of peak III
Lane 5, 7.4 μg of peak IV. Densitometer tracings
of the stained gels gave the following: peak I,
10.1, 48.2, 29.4 and 12.4% respectively of H2A,
H1, H3 and H4; peak II, 29.7% of H2A and 70.2%
of H3; peak III, 53% H2B with the remainder
distributed among H2A, H1 and H3; peak IV, 89%
H4. The variants of each histone type are
included in the values.

H2A —
H3.1 —

H2A.Z —

H3.2 —
H3.3 =
H1 =
H2B —

H4 =

1 2 3 4 5
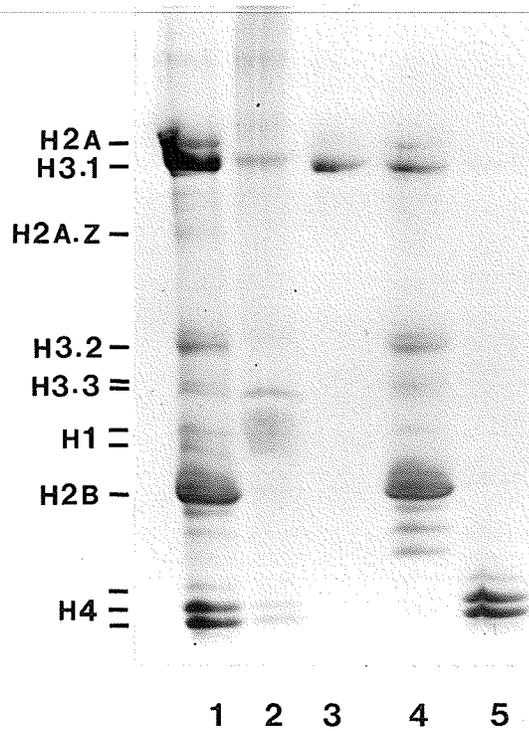
oligomycin (20 µg/mg protein) as the inhibitor of mitochondrial ATPase activity. With this assay system the units of protein kinase activity recovered in soluble mitochondrial fractions far exceeded the total units in mitochondrial starting material. Inclusion of 5 mM azide, in addition to oligomycin, was found to greatly stimulate protein kinase activity in mitochondrial membranous fractions, and to a lesser extent in mitochondrial soluble fractions (see Table 3). Protein kinase activity was reduced approximately 60% when 2-mercaptoethanol was omitted from the complete reaction mixture; however preincubation of enzyme mixtures with this reducing agent (as described in the legend to Fig. 10) had no additional effect over simple inclusion in the assay.

Protein kinase activity in non-disrupted mitochondrial preparations exhibited a 1.9-fold activation following disruption by sonic vibration, or treatment with 0.1% Triton X-100 (Table 4).

b)   Effect of Modulators on Protein Kinase Activity

Mitochondrial protein kinase activity was activated approximately 2-fold by the inclusion of 0.5 µM cyclic AMP (Table 5). Concentrations of cyclic AMP exceeding this value had no additional stimulating effect (Fig. 6). Cyclic GMP (0.5 µM) had little effect on mitochondrial protein kinase activity (Table 5). Calmodulin (Sigma P2277) or phosphatidylserine-diolein had negligible effect in the

TABLE 3

Effect of Azide and 2-mercaptoethanol on

Mitochondrial Protein Kinase Activity

Fractions to be assayed for protein kinase activity were preincubated with 0.1% Triton X-100 for 20 min on ice prior to addition to the assay. Mitochondrial soluble fractions were C-int (Table 6). Assays were done in the absence of cyclic AMP. The number of experiments is indicated in brackets.

Assay system: (see Experimental Methods)	Mitochondrial sonicates pmol/min/mg	Mitochondrial soluble fraction pmol/min/mg
with azide (5 mM) and 2-mercaptoethanol (10 mM)	77.8 (6)	243.0 (3)
without azide	15.0 (1)	23.0 (1)
without 2-mercaptoethanol	35.0 (1)	114.8 (1)
without azide and 2-mercaptoethanol	2.0 (3)	11.8 (2)

TABLE 4

Activation of Mitochondrial Protein Kinase Activity

By Membrane Disruptive Agents

Mitochondria	Protein kinase activity pmol/min/mg $\pm$ S.E.M.
Intact	39.9 ± 5.1 (5)
Sonicated	77.8 ± 21.2 (6)
+ 0.1% Triton X-100	71.0 ± 9.7 (4)

Sonication was performed as described under section VII a) in 'Experimental Methods'. Triton X-100 was preincubated with the enzyme preparation for 20 min on ice prior to addition to the assay. The number of experiments is indicated in brackets. Assays were performed in the absence of cyclic AMP.

TABLE 5

Effect of Modulators on Histone Kinase Activity

Of Bovine Heart Mitochondria

C-int fraction (Table 9) was used with histone H2B (peak III) as
substrate. Phosphatidylserine and diolein were sonicated in a ratio
of 10 mg/0.2 mg respectively, before use. Values are the mean of
two experiments.

Additions to reaction medium	Concentration	Activity (pmol/min/mg)
None	--	243.9
Cyclic AMP	0.5 μM	435.6
Cyclic GMP	0.5 μM	297.1
Calmodulin	20 μg/ml	224.3
Phosphatidylserine-diolein	0.04 mg/ml	276.5
Ca^{2+}	0.5 mM	118.1
+ calmodulin	20 μg/ml	132.1
+ phosphatidylserine-diolein	0.04 mg/ml	162.7

Figure 6   <u>Response of mitochondrial protein kinase</u>

<u>activity to cyclic AMP</u>

The enzyme was 10 µg of the C-int fraction

derived from heavy bovine heart mitochondria by

fractionation procedure 1 (Table 9).   The assay

was performed as described for mitochondrial

subfractions under `Experimental Methods'.   Each

point is the mean of two experiments.
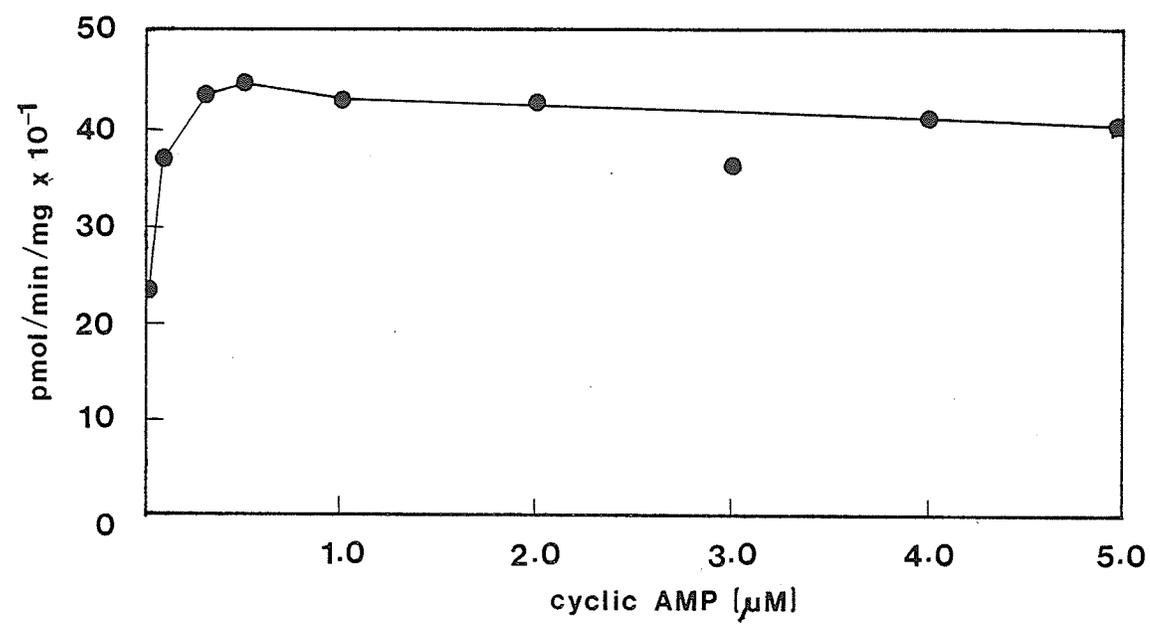
presence or absence of 0.5 mM Ca2+. Calcium itself was found to be inhibitory at concentrations exceeding 10^{-5} M.

The pH optimum for histone phosphorylation was estimated to be 8.0 in the presence or absence of cyclic AMP with optimal activity at a magnesium concentration of 5 mM (Fig. 7). At this pH and magnesium concentration, rates were constant for 30 minutes and were directly proportional to enzyme concentration up to 10 µg of soluble mitochondrial protein (Fig. 8). Cyclic AMP-dependent histone phosphorylation was maximal with 250 µg of phosphorylation substrate (Fig. 9).

III Localization of OR-ATPase and Cyclic AMP-Dependent

   Protein Kinase by Fractionation Procedure 1

a)   OR-ATPase

Table 6 demonstrates the specific activities and recoveries of mitochondrial marker enzymes and oligomycin-resistant and sensitive ATPases derived from heavy bovine heart mitochondria by fractionation method 1. The inner membrane enzymes (cytochrome c oxidase and oligomycin-sensitive ATPase) were largely associated with the C-mitoplast fraction. This fraction accounted for 91.3% of the starting mitochondrial protein and approximately 87% of the monoamine oxidase activity. C-int fractions, accounting for 7.5% of the mitochondrial protein, were enriched in the soluble mitochondrial enzymes adenylate kinase and malate dehydrogenase (3.7 and 5.2-fold respectively from heavy mitochondria) and lacked detectable amounts of outer and

Figure 7   <u>Response of mitochondrial protein kinase</u>

<u>to pH and magnesium</u>

The enzyme was 10 μg of the C-int fraction

derived from heavy mitochondria by fractionation

procedure 1 (Table 9). Protein kinase activity

in the presence (●——●) and in the absence (O——O)

of cyclic AMP. Protein kinase activity was

determined as described in 'Experimental Methods'

with the exception that the buffers used were

imidazole-HCl for pH 6.0 - 7.0, Tris-HCl for pH

7.5 - 8.5, and glycine-KOH for pH 9.0 - 10.0.

Each point is the mean of two experiments.
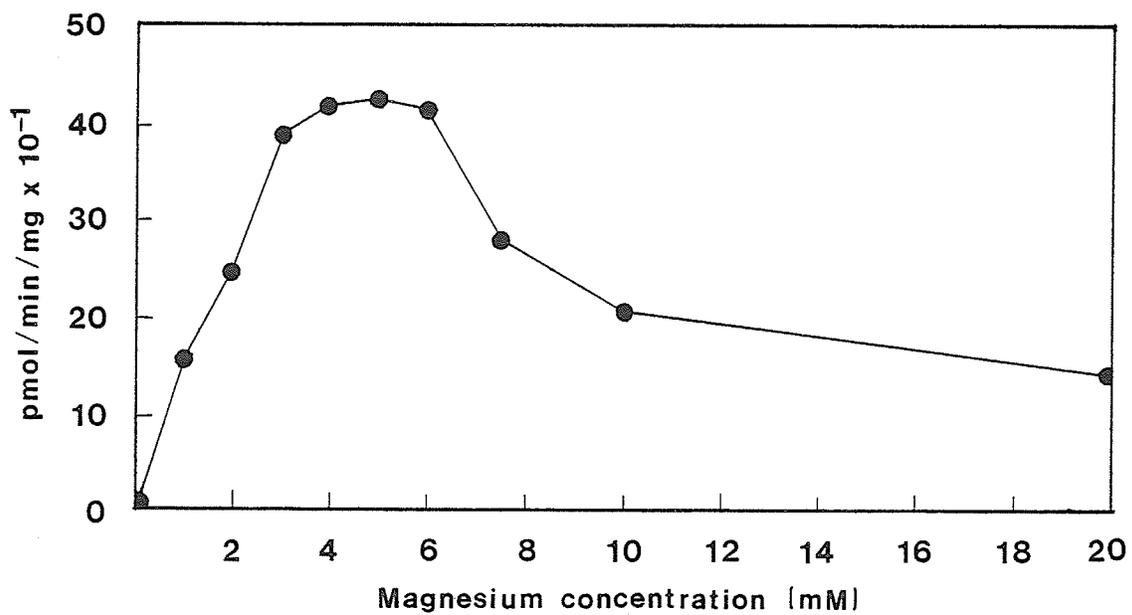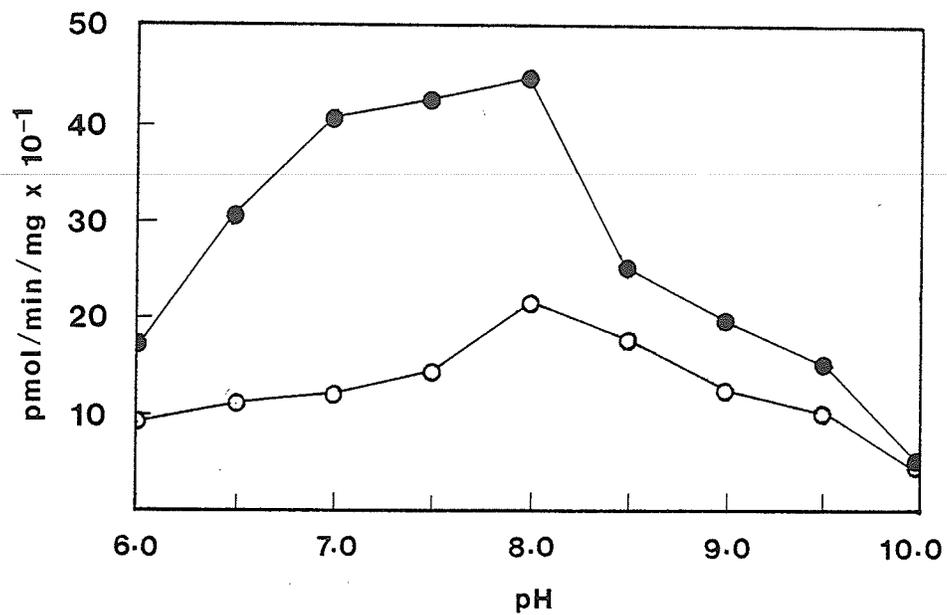
Figure 8   <u>Response of mitochondrial protein kinase</u>

<u>activity to incubation time and enzyme</u>

<u>concentration</u>

The enzyme used was the C-int fraction of

fractionation procedure 1 (Table 9). The assay

was performed as described in 'Experimental

Methods' in the presence of 0.5 μM of cyclic AMP.

Each point is the mean of two experiments.
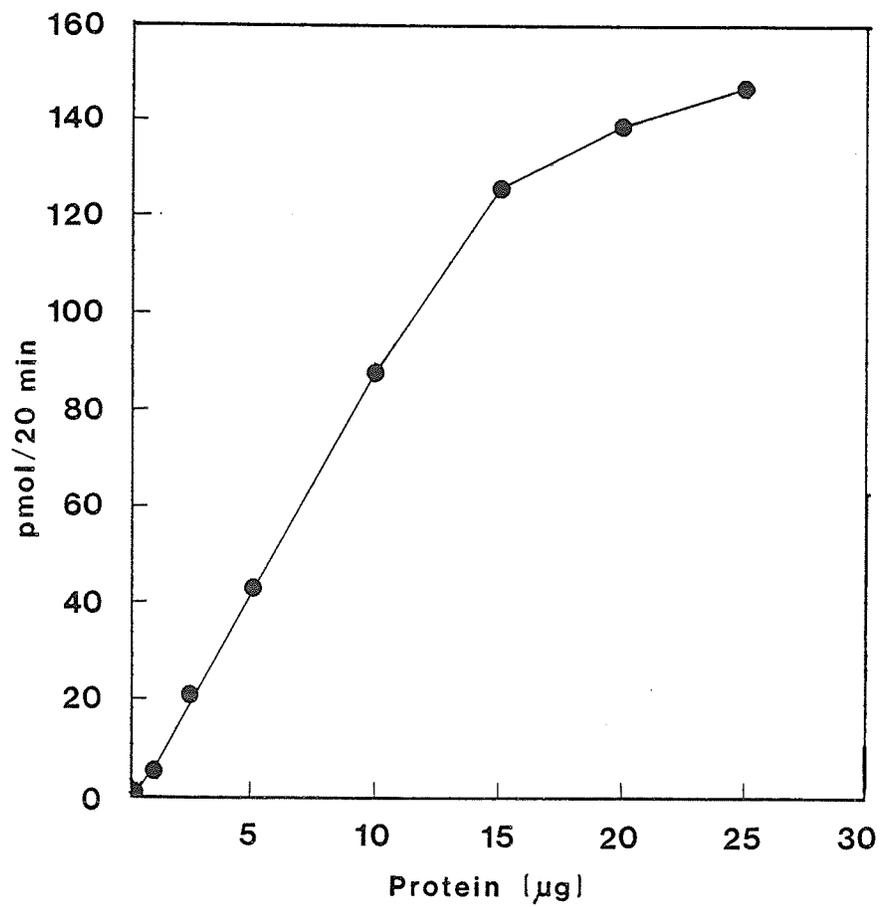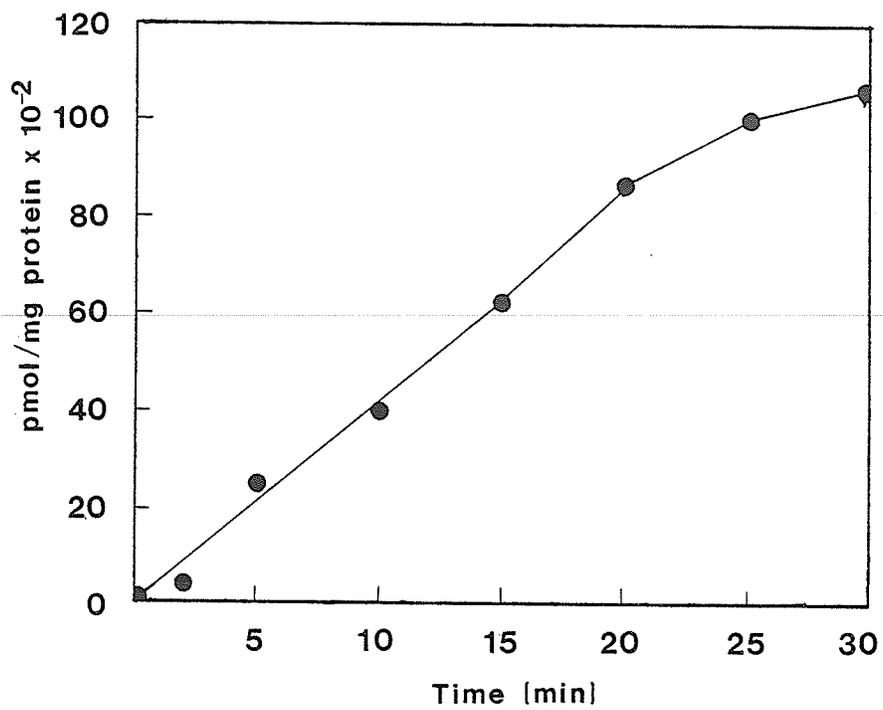
Figure 9   <u>Response of mitochondrial protein kinase to</u>

<u>substrate concentration</u>

The enzyme was 10 μg of C-int fraction prepared

from heavy mitochondria by fractionation

procedure 1.   The substrate was peak III from a

Bio-Gel P-30 column (see Figure 5 and Table 2).

Each point is the mean of two experiments done

in the presence of 0.5 μM cyclic AMP.
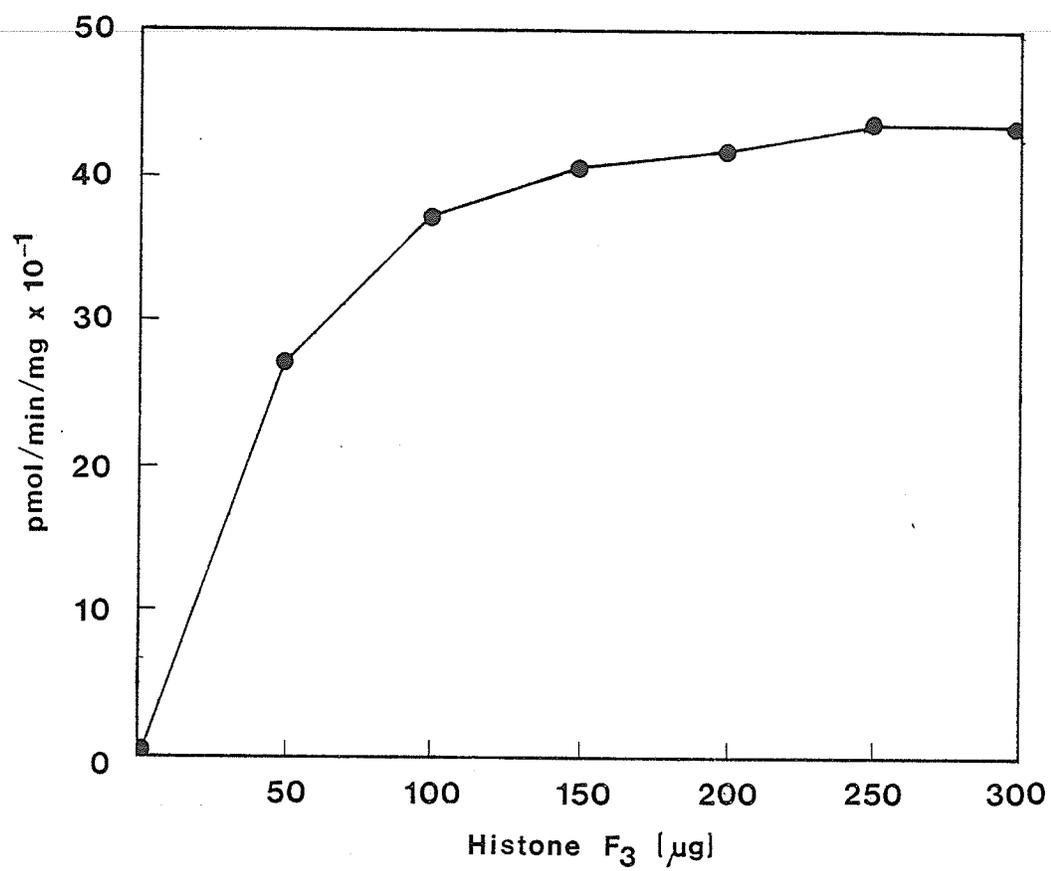
TABLE 6

Specific Activities of Enzymes in Fractions Prepared

From Heavy Mitochondria of Bovine Heart by Procedure 1

Enzyme	Specific Activities (μmol/min/mg protein ± S.E.M.)						Recovery
	Mitochondria	C-mitoplast	C-int	CB_1	CB_2	CB_3	%
Monoamine oxidase	2.0 ± 0.7	1.9 ± 0.8	0.0	29.6 ± 7.0	12.7 ± 2.5	6.3 ± 1.5	94.0
Cytochrome oxidase	3.1 ± 0.8	4.4 ± 1.0	0.0	0.8 ± 0.2	6.8 ± 1.4	4.8 ± 1.0	127.0
Malate dehydrogenase	2.2 ± 0.2	1.0 ± 0.2	11.4 ± 1.8	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	81.0
Adenylate kinase	0.3 ± 0.1	0.19 ± 0.10	1.1 ± 0.3	0.05 ± 0.02	0.13 ± 0.07	0.13 ± 0.04	86.0
OR-ATPase	0.07 ± 0.02	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.19 ± 0.05	0.35 ± 0.10	100.6
OS-ATPase	0.55 ± 0.15	0.69 ± 0.20	0.02 ± 0.00	0.19 ± 0.09	0.82 ± 0.25	1.82 ± 0.60	117.0
Acid phosphatase	0.005 ± 0.001	0.005 ± 0.001	0.0	0.004 ± 0.001	0.005 ± 0.002	0.004 ± 0.001	87.8
5' nucleotidase	0.018 ± 0.005	0.015 ± 0.005	0.0	0.015 ± 0.006	0.017 ± 0.006	0.017 ± 0.009	77.2

Note: All data are the means for three preparations. The mean protein (%) in the fractions was: mitochondria (100), C-mitoplast (91.3), C-int (7.5), CB_1 (0.2), CB_2 (0.4), and CB_3 (0.5).

inner membrane marker enzymes. Crude outer membrane fractions, from which are derived the gradient fractions CB1, CB2 and CB3, accounted for 1.5% of the original mitochondrial protein and 8.0% of the mitochondrial monoamine oxidase activity. In agreement with the findings of Comte and Gautheron (40) CB1, the uppermost band obtained by sucrose density gradient centrifugation, was the most purified preparation of outer membranes obtained. Although this fraction accounted for only 0.2% of the total mitochondrial protein, it was enriched 14.8-fold in monoamine oxidase activity from mitochondria and represented approximately 3.0% of the original mitochondrial monoamine oxidase activity. In contrast to the results of others using rat tissue mitochondria (19-21), the outer membrane fractions of heavy bovine heart mitochondria were not enriched in OR-ATPase activity. CB3, representing only 0.5% of the starting mitochondrial protein, exhibits the best enrichments for both OR-ATPase and oligomycin-sensitive ATPase activities (5.0 and 3.3-fold respectively from heavy mitochondria). Lysosomal acid phosphatase and sarcolemmal 5'-nucleotidase activities represented only 0.2% and 1.5% respectively of the total units of beef heart homogenate in our heavy mitochondrial preparations. These contaminants were not significantly enriched in any of the sub-mitochondrial fractions.

Initial experiments with this fractionation scheme used mixed and light preparations of bovine heart mitochondria.

As demonstrated earlier these mitochondrial preparations exhibit much higher specific activities for OR-ATPase than do heavy mitochondrial preparations (see Table 1). Table 7 shows that OR-ATPase activity is approximately 2-fold enriched in outer membrane fractions (CB1) when mixed and light populations of mitochondria were used as the starting material. The ATPase activity of CB1 from mixed mitochondrial populations was inhibited equally as well by azide as by oligomycin, suggesting that the resistant ATPase of these outer membrane fractions was not a different form of the energy-transducing ATPase. Well-known inhibitors of ATPases of sarcolemma and sarcoplasmic reticulum (81-84) had negligible effects on the OR-ATPase activity of outer membrane fractions of mixed mitochondria (Table 8). Monoamine oxidase activity in CB1 was enriched 10.3-fold from mixed mitochondrial populations and 8.0-fold from light mitochondria. These mitochondrial preparations, however, were heavily contaminated with lysosomal material; light mitochondria accounted for 1.0% of the units of acid phosphatase in the bovine heart homogenates and mixed populations for as much as 1.8%. Acid phosphatase activity was also enriched in outer membrane fractions (5.2 and 12.7-fold from mixed and light mitochondria). That OR-ATPase activity enriches with acid phosphatase activity in CB1 when mixed and light populations of mitochondria are the starting material suggests a lysosomal origin for the OR-ATPase

TABLE 7

Specific Activities of Enzymes in Outer Membrane Fractions

Of Mixed and Light Populations of Mitochondria

Procedure 1 was used to prepare outer membrane fractions (CB1). Values

for mixed mitochondria are the means of three experiments. Values for

light mitochondria are the means of two experiments.

* Recovery of acid phosphatase in mixed and light populations from

bovine heart homogenate was 1.8% and 1.0% respectively.

	Specific Activities (μmol/min/mg $\pm$ S.E.M.)			
Enzyme	Mixed mitochondria	Outer membranes	Light mitochondria	Outer -membranes
Monoamine oxidase	2.4 $\pm$ 1.0	24.8 $\pm$ 7.2	2.1 $\pm$ 0.4	16.8 $\pm$ 4.1
Acid phosphatase	0.009 $\pm$ 0.002*	0.047 $\pm$ 0.013	0.017 $\pm$ 0.005*	0.216 $\pm$ 0.050
OR-ATPase	0.15 $\pm$ 0.04	0.36 $\pm$ 0.07	0.24 $\pm$ 0.06	0.48 $\pm$ 0.10
Cytochrome oxidase	3.6 $\pm$ 0.7	0.8 $\pm$ 0.1	4.1 $\pm$ 1.0	1.0 $\pm$ 0.30

TABLE 8

Effect of Inhibitors on ATPase Activity of Outer Membranes

From Mixed Heavy and Light Mitochondria

Inhibitor	Concentration	ATPase activity µmol/min/mg protein ± S.E.M.	Inhibition %
None	---	0.88 ± 0.08	0
Sodium azide	5.0 mM	0.38 ± 0.10	57.4
Oligomycin	10 µg/mg of protein	0.37 ± 0.07	58.3
Oligomycin *	10 µg/mg of protein		
+ vanadate	10.0 mM	0.37 ± 0.07	58.0
+ EGTA	1.0 mM	0.37 ± 0.07	58.0
+ ouabain	0.1 mM	0.36 ± 0.07	59.1

Note: Results are the means of three experiments.

 * Enzyme fraction was preincubated with oligomycin and then the other
    inhibitors were added separately to the final incubation medium.

activity in these outer membrane fractions. Therefore, further work on mitochondrial OR-ATPase activity used heavy mitochondrial preparations to avoid problems with contaminating lysosomal ATPase activity.

b)   Cyclic AMP-Dependent Protein Kinase

Table 9 shows the specific activities and recoveries of cyclic nucleotide-dependent and independent (basal) protein kinase activities in fractions derived by fractionation procedure 1 from heavy bovine heart mitochondria. The protein kinase activity of all fractions exhibits an activation of approximately 2-fold by cyclic AMP. The C-mitoplast fraction is not enriched in protein kinase activity although it does account for approximately 63% of the starting mitochondrial units of cyclic nucleotide-dependent protein kinase and 62% of the basal protein kinase activities. This fraction is not enriched in adenylate kinase or malate dehydrogenase (Table 6) although it does account for 58% and 42% of the heavy mitochondrial units of these soluble mitochondrial marker enzymes.

Mitochondrial protein kinase activity may exist in soluble forms as evidenced by the enrichment of basal protein kinase (4.1-fold) and cyclic AMP-dependent protein kinase (2.5-fold) in C-int fractions (Table 9). Of the original protein kinase activity in mitochondria, 30.8% of the basal activity and 19.1% of the cyclic nucleotide-dependent activity are found in these fractions. C-int fractions are enriched 3.7-fold in adenylate kinase and

TABLE 9

Specific Activities of Protein Kinases in Fractions Prepared

From Heavy Mitochondria of Bovine Heart by Procedure 1

Enzyme	Specific Activities (pmol/min/mg protein ± S.E.M.)						Recovery
	Mitochondria	C-mitoplast	C-int	CB_1	CB_2	CB_3	%
Basal kinase	60.7 ± 19.2	42.1 ± 10.1	249 ± 36	319 ± 43	423 ± 34	416 ± 83	101.3
Basal kinase + cAMP-dependent protein kinase	131 ± 23	75.5 ± 15.1	427 ± 46	599 ± 64	1039 ± 159	949 ± 101	85.0
cAMP-dependent protein kinase	69.8 ± 22.0	33.4 ± 11.0	178 ± 26	280 ± 34	616 ± 72	533 ± 63	70.9

Note: All data are the means of three preparations. The mean protein (%) in the fractions was: mitochondria (100), C-mitoplast (91.3), C-int (7.5), CB_1 (0.2), CB_2 (0.4), and CB_3 (0.5). Basal protein kinase activity was assayed in the absence of cAMP, and basal kinase + cAMP-dependent protein kinase activity was assayed in the presence of 0.5 μM cAMP. Samples were treated with 0.1% Triton X-100 prior to addition to the assay system.

represent 28% of the recoverable units of this enzyme
activity (Table 6). The 5.2-fold enrichment of malate
dehydrogenase in this fraction suggests that damage to the
inner membrane occurs during this fractionation procedure.

Basal and cyclic nucleotide-dependent protein kinases
are enriched in all gradient fractions, most notably CB2
(7.0-fold for basal activity and 8.8-fold for cyclic AMP-
dependent activity) and to a lesser extent in CB3
(Table 9). CB2 and CB3 are mixed outer and inner membrane
fractions, enriched in monoamine oxidase (6.4 and 3.2-fold,
respectively) and to a lesser extent in cytochrome oxidase
activities (2.2 and 1.5-fold, respectively). Adenylate
kinase and malate dehydrogenase were not enriched in any of
the gradient fractions (Table 6).

IV   Localization of OR-ATPase and Cyclic AMP-Dependent
     Protein Kinase by Fractionation Procedure 2
a)   OR-ATPase

The inclusion of trypsin to mitochondrial fractionation
methods may increase outer membrane yields by cleaving the
putative connections that exist between the outer and inner
membranes of heart mitochondria (36). Table 10 shows
mitochondrial marker enzymes and ATPase activities in
fractions derived by procedure 2 using heavy bovine heart
mitochondria. SB1 was the most enriched outer membrane
fraction (4.9-fold enrichment in monoamine oxidase activity
from mitochondria). OR-ATPase was most enriched in SB3 and
SB2 (2.3-fold in terms of specific activities from

TABLE 10

Specific Activities of Marker Enzymes and ATPases in

Fractions Prepared from Heavy Mitochondria by Procedure 2

Enzyme	Specific Activities (μmol/min/mg protein ± S.E.M.)					Recovery
	Mitochondria	S-mitoplast	SB_1	SB_2	SB_3	%
Monoamine oxidase	1.8 ± 0.4	1.3 ± 0.5	8.8 ± 2.8	5.6 ± 2.1	3.3 ± 1.6	87.0
Cytochrome oxidase	4.2 ± 0.8	4.7 ± 0.9	5.3 ± 1.0	3.4 ± 1.1	5.6 ± 0.6	76.5
Malate dehydrogenase	1.2 ± 0.1	1.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	54.7
Adenylate kinase	0.20 ± 0.02	0.05 ± 0.01	0.07 ± 0.02	0.12 ± 0.02	0.10 ± 0.02	22.5
OR-ATPase	0.12 ± 0.03	0.10 ± 0.02	0.19 ± 0.01	0.23 ± 0.05	0.23 ± 0.05	78.4
CS-ATPase	0.89 ± 0.20	0.75 ± 0.10	1.15 ± 0.16	2.01 ± 0.35	1.60 ± 0.31	79.0
Acid phosphatase	0.005 ± 0.001	0.002 ± 0.001	0.007 ± 0.002	0.006 ± 0.003	0.002 ± 0.001	32.1
5' nucleotidase	0.016 ± 0.004	0.011 ± 0.004	0.010 ± 0.002	0.011 ± 0.003	0.014 ± 0.007	62.2

Note: All data are the means of six preparations. Recovery of protein (%) from starting heavy mitochondrial material (100%) was: SB_1 (2.4), SB_2 (6.1), SB_3 (12.8), and S-mitoplast (45.8).

mitochondria). In agreement with fractionation scheme 1, OR-ATPase activity did not co-localize with outer membrane fractions when heavy bovine heart mitochondria were used as the starting material for the fractionation methods.

It is apparent that no clear correlation between OR-ATPase activity and the mitochondrial marker enzymes exists. Oligomycin-sensitive ATPase activity also exhibits the best enrichments in SB2 (2.3-fold from mitochondria) and to a lesser extent in SB3. Cytochrome c oxidase activity is marginally enriched in SB3 (1.3-fold from mitochondria) but not in SB2. Comparison of Table 6 and Table 10 suggests that the specific activities for oligomycin-sensitive and -resistant ATPase activities are somewhat elevated (1.6 and 1.7-fold respectively) following treatment of heavy mitochondria with trypsin in this procedure. Acid phosphatase and 5'-nucleotidase are not enriched in SB2 or SB3 suggesting that lysosomal and sarcolemmal contamination are not contributing to the enrichment of mitochondrial OR-ATPase activity in these fractions.

b)   Cyclic AMP-Dependent Protein Kinase Activity.

Treatment of heavy mitochondria with trypsin and soybean trypsin inhibitor containing solutions (fractionation procedure 2) resulted in a 60% loss in both basal and cyclic AMP-dependent protein kinase activities. In three experiments, basal protein kinase activities in heavy mitochondria (72.0 $\pm$ 15 pmol/min/mg) were found to decrease following treatment with trypsin-antitrypsin

containing solutions, to 30.5 ± 5.0 pmol/min/mg. Cyclic
AMP-dependent protein kinase activity was similarly
affected; activities of 74.0 ± 15 pmol/min/mg decreasing to
31.3 ± 8.0 pmol/min/mg.

Table 11 demonstrates the specific activities of
protein kinases in fractions prepared from heavy
mitochondria of bovine heart by procedure 2. Basal and
cyclic AMP- dependent protein kinase activities exhibit the
best enrichment in SB2 (2.6 and 3.5-fold respectively from
mitochondria). Comparison of Tables 10 and 11 demonstrates
that there is no obvious correlation between the
distribution of the mitochondrial protein kinases and the
mitochondrial marker enzymes employed in these studies.
Monoamine oxidase is the only marker enzyme enriched in SB2
(3.1-fold from mitochondria), although greater enrichments
of this outer membrane marker are obtained in SB1 (Table 10)

From the starting mitochondrial material, recoveries of
cyclic AMP-dependent protein kinase activity were 56.5%.
This closely parallels the 54.7% recovery of malate
dehydrogenase from mitochondria (Table 10). The recovery of
adenylate kinase was found to be substantially lower than
these values (22.5% from mitochondria). It is likely that
during this procedure, a portion of the mitochondrial cyclic
AMP-dependent protein kinase activity was lost with matrix
or intermembrane space material.

S-mitoplast fractions, although not significantly
enriched in malate dehydrogenase activity, retain

TABLE 11

Specific Activities of Protein Kinases in Fractions Prepared

From Heavy Mitochondria of Bovine Heart by Procedure 2

Enzyme	Specific Activities (pmol/min/mg protein $\pm$ S.E.M.)					Recovery
	Mitochondria	S-mitoplast	SB1	SB2	SB3	%
Basal kinase	30.5 $\pm$ 5.0	16.8 $\pm$ 5.1	34.6 $\pm$ 5.2	79.6 $\pm$ 10.3	42.5 $\pm$ 7.1	61.7
Basal kinase + cAMP-dependent protein kinase	61.8 $\pm$ 16.0	26.1 $\pm$ 4.3	71.5 $\pm$ 8.1	189.5 $\pm$ 31.0	89.4 $\pm$ 20.3	59.0
cAMP-dependent protein kinase	31.3 $\pm$ 8.0	9.6 $\pm$ 3.0	36.9 $\pm$ 4.0	109.9 $\pm$ 29.0	46.9 $\pm$ 10.2	56.5

Note: All data are the means of three preparations. The mean protein (%) in the fractions was: mitochondria (100), S-mitoplast (47.1), SB1 (2.1), SB2 (5.9), SB3 (12.6). Basal protein kinase activity was assayed in the absence of cAMP, and basal kinase + cAMP-dependent protein kinase activity was assayed in the presence of 0.5 μM cAMP. Samples were treated with 0.1% Triton X-100 prior to addition to the assay system.

approximately 50% of this enzymatic activity from the
original mitochondrial material. These fractions, however,
account for only 14% of the cyclic AMP-dependent protein
kinase activity in the starting mitochondrial preparations.
S-mitoplast fractions were not enriched in adenylate kinase
and recoveries of adenylate kinase in these fractions (11.5%
from mitochondria) more closely paralleled the recovery of
cyclic AMP-dependent protein kinase. It is evident from
these results that no conclusions regarding a matrix or
intermembrane space localization for mitochondrial cyclic
AMP-dependent protein kinase activities can be reached. It
is possible that, like liver mitochondria (45), this
enzymatic activity exists in both of these compartments. It
is likely that a portion of the cyclic AMP-dependent protein
kinase activity exists in a membrane-bound state as
suggested by the enrichments in CB2 and CB3 of fractionation
procedure 1 (Table 9).

V   Characterization of Mitochondrial OR-ATPase Activity.

It was decided that SB3 (Table 10) was a convenient
fraction in which to study mitochondrial OR-ATPase activity.
Starting with heavy mitochondrial material, this fraction
exhibited a 2-fold enrichment in OR-ATPase activity and
protein yields of 15%. CB3 (Table 6) although more
enriched, represented only 0.5% of the starting
mitochondrial protein. Outer membrane fractions (CB1)
prepared from light mitochondria by fractionation method 1
(Table 7) were also examined to compare the OR-ATPase

activity of lysosomal enriched material to that of SB3. This was necessary to rule out the possibility that the OR-ATPase activity in SB3 was the result of lysosomal contamination.

a)   Stability and Activation by 2-Mercaptoethanol

The resistant ATPase activity of SB3 was found to be unstable even after storage at -70 °C in EDTA-containing sucrose. Addition of 1-5 mM 2-mercaptoethanol to the reaction mixture increased resistant ATPase activity by about 1.3-fold. SB3 was then preincubated in the presence of 50 mM 2-mercaptoethanol at 25 °C. There was a 2-fold increase in SB3 resistant ATPase after 2 hours of pre-incubation (Fig. 10). Preincubated controls lacking 2-mercaptoethanol were not activated. In contrast, resistant ATPase activity of CB1 of light mitochondria (probably representing lysosomal ATPase) was not stimulated by preincubation with 2-mercaptoethanol. Oligomycin-sensitive ATPase activity of SB3 was not affected by preincubation with 2-mercaptoethanol.

b)   Solubilization of SB3 Azide-Resistant ATPase Activity

Solubilization of mitochondrial protein with Triton X-100 was found to eliminate the inhibitory effects of oligomycin on mitochondrial ATPase activity (see Fig. 13, panel 2). Consequently, in results dealing with Triton X-100 solubilized preparations, resistant ATPase refers to that activity seen in the presence of 5 mM sodium azide. Fig. 11 demonstrates that recovery of azide-sensitive ATPase

Figure 10 <u>Activation of oligomycin-resistant ATPase of</u>

<u>membrane fractions of bovine heart mitochondria</u>

<u>by preincubation with 2-mercaptoethanol</u>

Samples were preincubated in 0.25 M sucrose 20 mM

Tris-HCl buffer, 1 mM EDTA (pH 7.5) containing

50 mM 2-mercaptoethanol. Aliquots were withdrawn

as specified for assay of ATPase activity in the

presence of 10 µg oligomycin / mg protein.

Inner membranes (SB3) of heavy mitochondria were

prepared by procedure 2 (●——●); outer membranes

(CB1) of light mitochondria were prepared by

procedure 1 (O——O). Each point represents the

mean of two experiments.
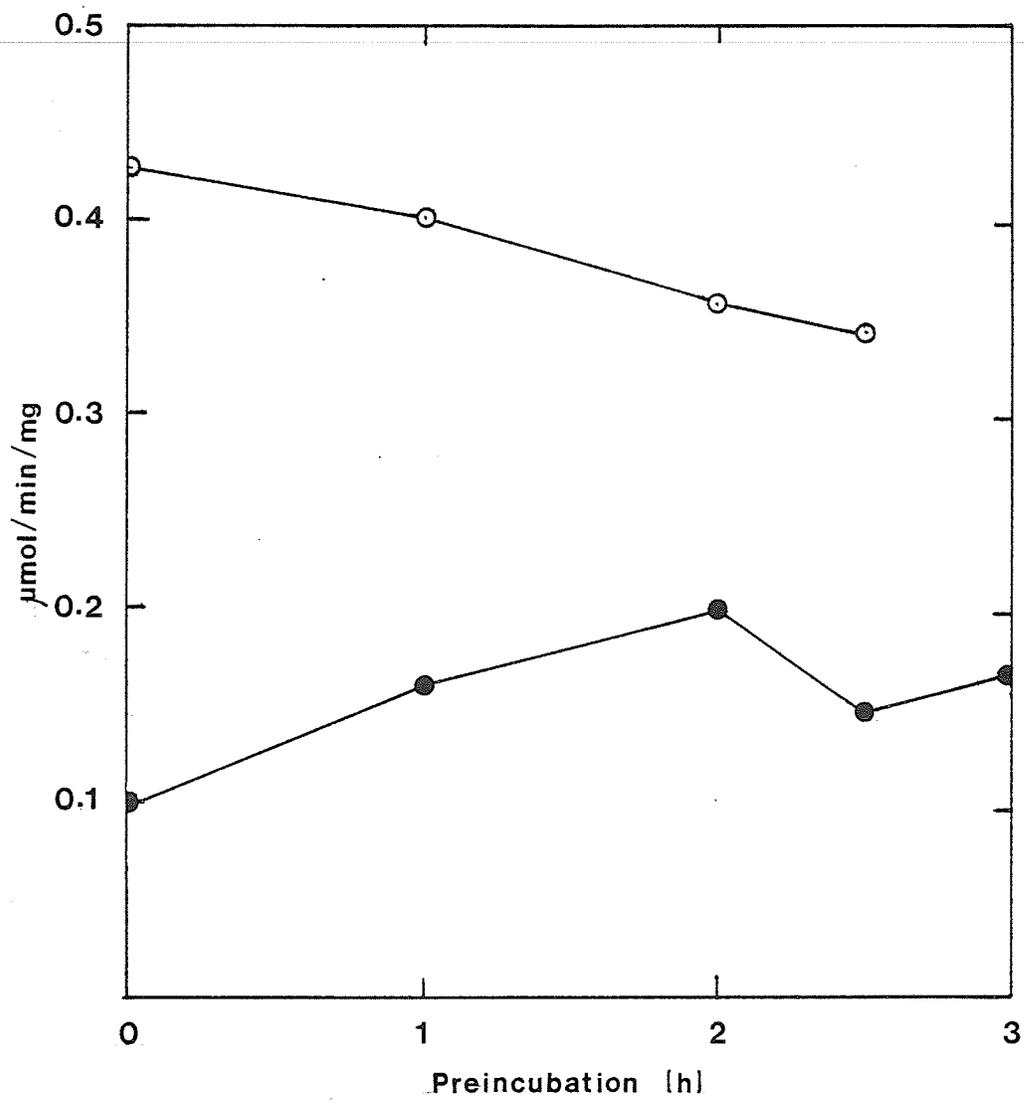
Figure 11   <u>Solubilization by Triton X-100 of SB3 ATPase</u>

<u>from heavy bovine heart mitochondria</u>

Procedure 2 was used to prepare SB3 which was

then diluted to 1 mg protein / ml with 20 mM

Tris-HCl, pH 7.5, and 1 mM EDTA.   Triton X-100

(%, v/v) was added as specified.   Fractions

were then incubated at 20° C for 30 min and

centrifuged at 105,000 g for 1 hour.

Supernatant and pellets were analyzed for

ATPase activity.   Solubilized azide-sensitive

ATPase (O—O); solubilized, azide-resistant

ATPase (□—□), azide-sensitive ATPase of the

insoluble fraction (●—●); azide-resistant

ATPase of the insoluble fraction (■—■).
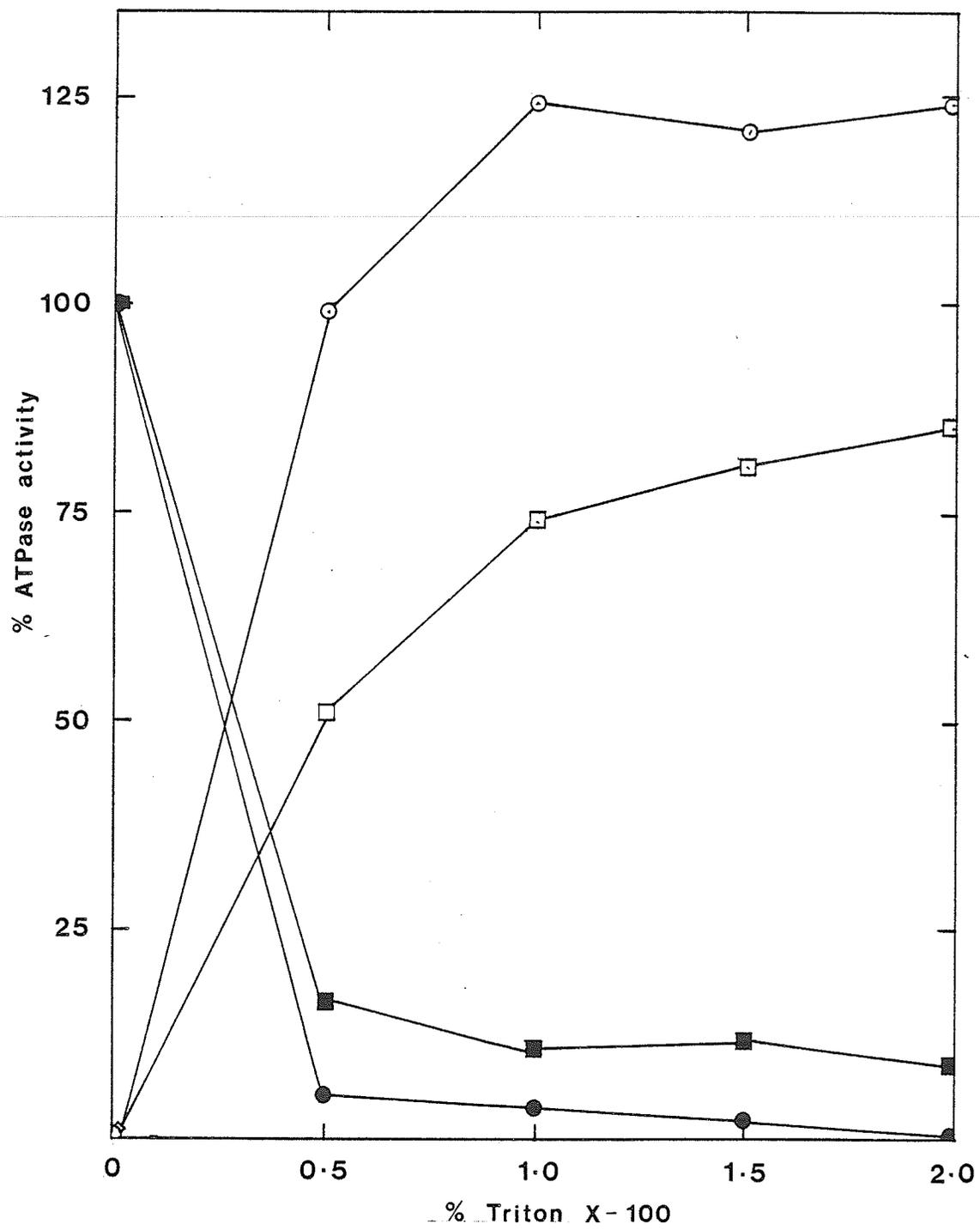
activity in the extract solubilized with Triton X-100 was about 130%, with little change in the specific ATPase activity as compared to unsolubilized SB3. In contrast, recovery of azide-resistant ATPase averaged about 80% (varied from 70% - 84% in four experiments) when solubilized with 2.0% Triton X-100. Azide-resistant ATPase activity of SB3 (0.234 $\pm$ 0.065 μmol/min/mg) dropped approximately 35% after solubilization. The activity of resistant ATPase of solubilized samples of SB3 was stimulated about 2-fold upon preincubation with 50 mM 2-mercaptoethanol as was described for the original SB3 fractions (Fig. 10).

c)   Electrophoresis of Solubilized Extracts of SB3

Fig. 12 shows three major bands in SB3 from heavy mitochondria that stained for ATPase activity in the absence of sodium azide (lane A). Band 1 was present, but staining was much less intense when sodium azide was added to the staining mixture (lane D). Band 2 and band 3 did not stain in the presence of sodium azide. Azide-resistant ATPase staining was unaffected by the inclusion of ouabain, EGTA or sodium orthovanadate (results not shown). CB1 derived from light mitochondria by fractionation scheme 1 was also stained for ATPase in the absence and presence of sodium azide (lanes B and E respectively). A band of resistant ATPase activity, migrating further than the resistant ATPase of SB3, was noted in this lysosomal enriched fraction

Figure 12   <u>Electrophoresis of solubilized membrane</u>

   <u>fractions of bovine heart mitochondria</u>

   The gels contained 5% (w/v) acrylamide and

   0.1% Triton X-100. Tris/glycine buffer

   (pH 8.3) of the upper chamber contained 0.1%

   Triton X-100. Pre-electrophoresis was for

   20 min at 20 °C at 2 mA/tube (internal

   diameter, 5 mm, 75 mm in length). Samples

   were applied and electrophoresis was at

   2 mA/tube at 20 °C for 20 min followed by 1 h

   and 15 min at 3 mA/tube. The staining

   solution contained 5 mM magnesium acetate,

   1 mM lead nitrate, 0.1 M Tris/acetate buffer

   (pH 7.5) and 3 mM ATP. Gels were stained for

   2 h at 30 °C. Sodium azide (5 mM) was added

   to the staining solution as specified.

   TD tracking dye

   Lane A: 70 μg of SB3 fraction from heavy

   mitochondria; Lane B: 70 μg of CB1 fraction

   from light mitochondria; Lane C: combined CB1

   and SB3 fractions, 70 μg of each; Lanes D, E

   and F: same as Lanes A, B and C, respectively

   but stained with azide present. The major

   bands of Lane A are marked 1, 2 and 3.
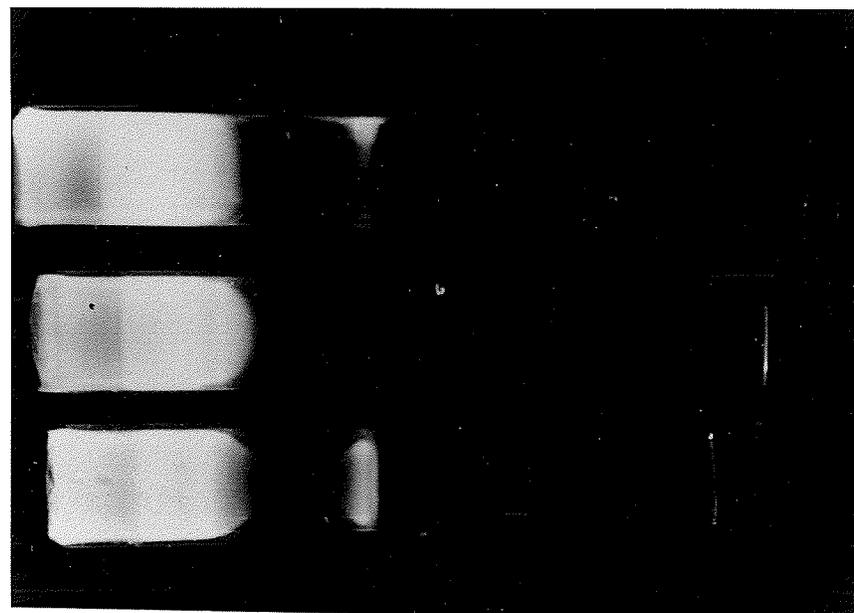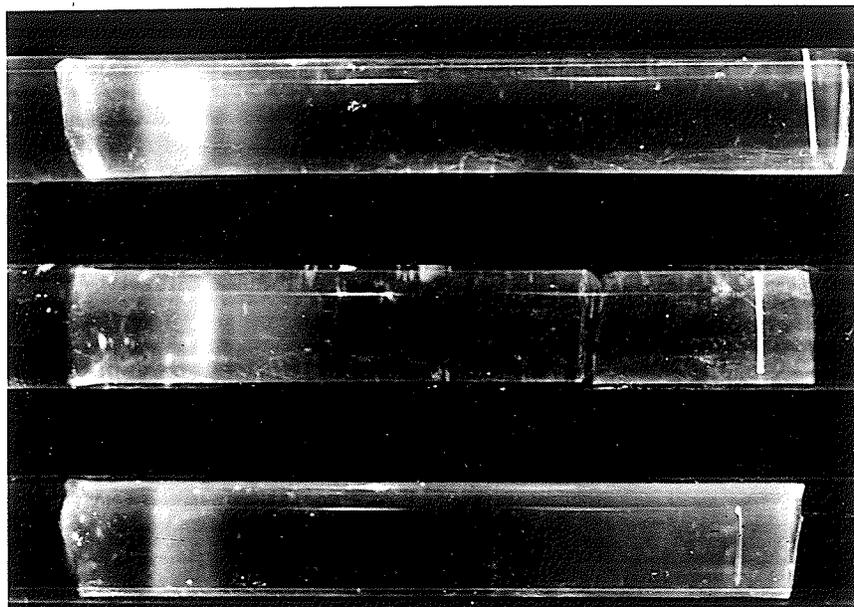
(lane E). The two azide-resistant bands were discernible when CB1 from light mitochondria and SB3 were added together and then stained with azide present (lane F). In the absence of azide, CB1 as well as CB1 plus SB3 gave wide bands of ATPase activity in which there was no discernible separation of band 1 and band 2 ATPase staining bands (lanes B and C respectively).

d) Effect of Inhibitors on SB3 ATPase Activity

Table 12 shows that azide-resistant ATPase activity of SB3 was unaffected by vanadate, ouabain and EGTA indicating that sarcolemmal and sarcoplasmic reticulum contamination were not a contributing factor to this activity. SB3 ATPase activity was inhibited equally by azide or oligomycin, suggesting that resistant ATPase of this fraction was not the result of soluble F1-ATPase derived from the inner mitochondrial membrane. It was noted, however, that the low ATPase activity in the presence of azide was mostly susceptible to inhibition by oligomycin; in this sense the two inhibitors act almost additively. With both inhibitors present, inhibition of SB3 ATPase was 97.4% complete.

e) Investigation of the Additive Effects of Oligomycin
and Azide

To investigate the additive effects of oligomycin and azide on SB3 ATPase activity method 2 was used so that the time course of the ATPase reaction could be followed continuously. SB3 ATPase activity in the absence or presence of oligomycin is linear with respect to time with

TABLE 12

Effect of Inhibitors on SB$_3$ ATPase Activity

Enzyme fractions were preincubated with oligomycin as described in 'Materials and Methods". All other inhibitors were added directly to the assay. ATPase assay method 1 was used. Values are the means of three experiments.

Addition	Concentration	S.A. (µmol/min/mg) ± S.E.M.	% Inhibition
None	--	1.773 ± 0.276	0
Azide	1 mM	0.234 ± 0.065	86.8
Oligomycin	10 µg/mg	0.213 ± 0.033	88.0
Azide + oligomycin	1 mM + 10 µg/mg	0.046 ± 0.007	97.4
Azide + vanadate	1 mM + 10 µM	0.213 ± 0.025	88.0
Azide + ouabain	1 mM + 1 mM	0.213 ± 0.030	88.0
Azide + EGTA	1 mM + 1 mM	0.209 ± 0.029	88.2

specific activities of 1.99 and 0.209 μmol/min/mg

respectively, in agreement with activities derived from the

set time (10 min) assay (Fig. 13, panel 1). In the presence

of azide, however, a biphasic time course, with fast and

slow phases of ATP hydrolysis is evident. It is notable

from examination of the time course results, that a set time

interval assay would give approximately the same activities

in the presence of oligomycin or azide. Preincubation of

SB3 with azide (5 min at 30 °C) had no effect on the time

course of the ATPase reaction suggesting that interaction of

the enzyme with azide occurs after the ATPase reaction has

started. When used in combination, oligomycin and azide

produce a slightly biphasic time course. It is apparent

from the inhibition curves that oligomycin has little effect

on the azide-induced slow phase of ATPase hydrolysis as

reaction rates are very similar whether azide or a

combination of azide and oligomycin are used. The azide-

induced fast phase is largely inhibited by oligomycin and

this may account for the much lower specific activities seen

in the timed interval assay when these inhibitors are used

together.

Fig. 13 (panel 2) shows the effects of oligomycin and

azide on the time course of the ATPase reaction when

Triton X-100 solubilized preparations of SB3 are used.

Oligomycin has little effect on ATPase activity following

solubilization, and time courses in the presence and absence

of this inhibitor are linear. Azide again induces a

Figure 13 <u>Effect of oligomycin and azide on the time</u>

<u>course of the ATPase reaction</u>

Method 2 was used to determine ATPAse

activity. No inhibitor (O—O); 10 μg

oligomycin / mg protein preincubated with

sample prior to addition to the assay (▲—▲);

5 mM azide added directly to the assay (■—■);

10 μg oligomycin / mg protein and 5 mM azide

in combination (●—●).

SB3 (panel 1)

Triton X-100 solubilized SB3 (panel 2)
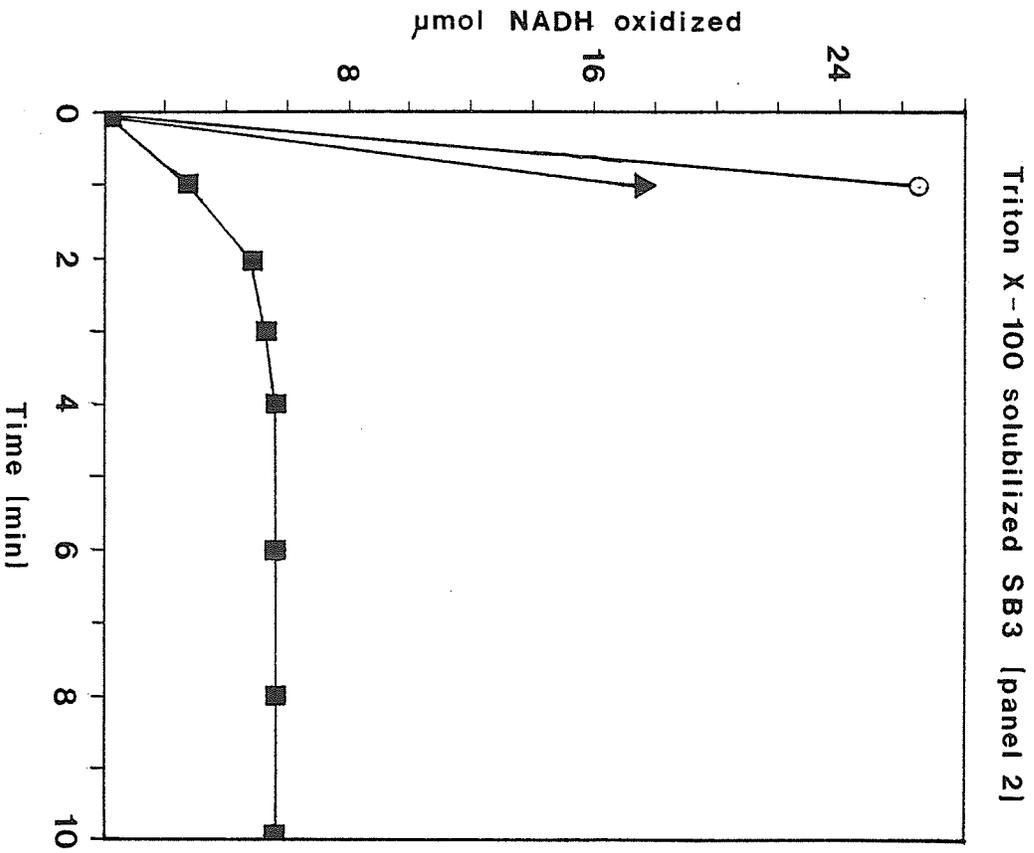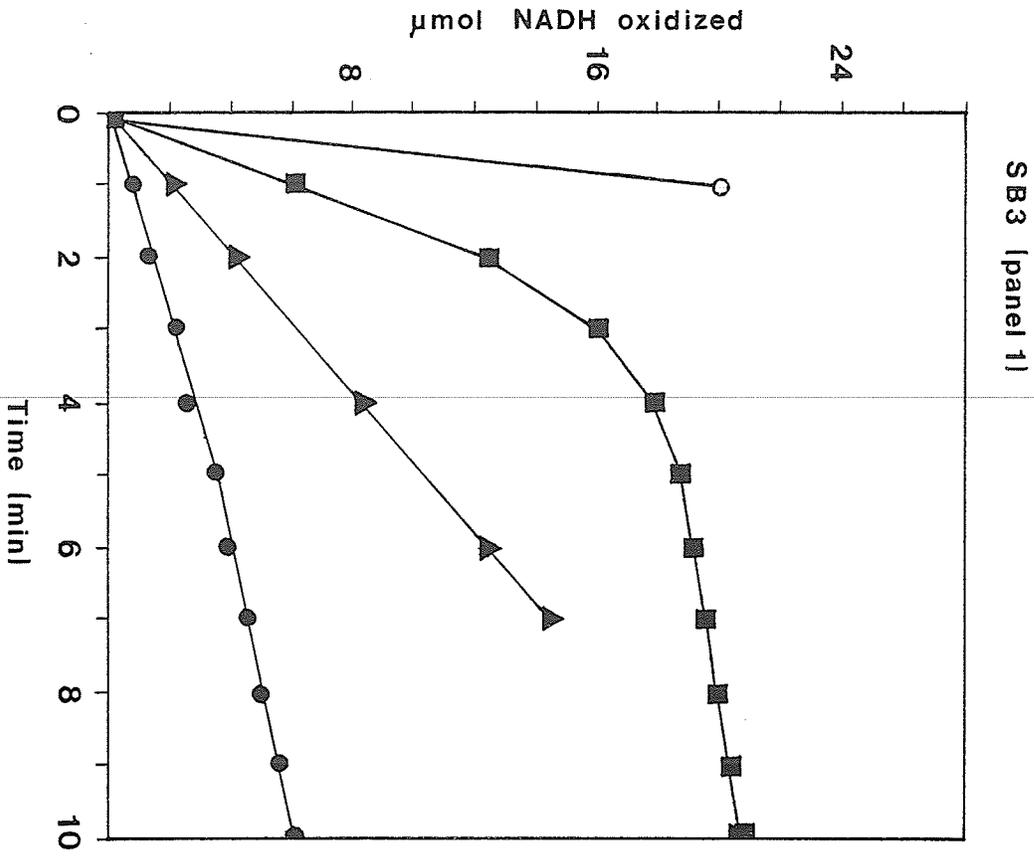
biphasic time curve, with complete inhibition of ATPase activity in the latter portions of the time course.

The azide sensitivity of OR-ATPase activity and the complete inhibition of ATPase activity seen in the azide-induced slow phase of ATP hydrolysis suggest that OR-ATPase activity results from incomplete inhibition of the mitochondrial energy-transducing ATPase. I have found no evidence suggesting the existence of an unique oligomycin-resistant ATPase activity in bovine heart mitochondria. Accordingly, no further attempts were made to study this enzymatic activity or to implicate it in calcium efflux from heart mitochondria.

VI   Partial Purification of Two Mitochondrial Cyclic AMP -
Dependent Protein Kinases

Table 13 shows the separation and purification of two cyclic AMP-dependent protein kinases from bovine heart mitochondrial supernatant fractions following DEAE-cellulose chromatography. Ultracentrifugation (105,000 g for 1 hour) of mitochondrial sonicates resulted in a 4-fold purification of kinase activity in the supernatant fraction with recoveries of 85%. Although recovery is good, it was noted that as much as 97% of the activity (3-fold enriched) could be recovered at this step if higher ionic strength buffers (50 mM Tris-HCl, 10 mM NaCl, 5 mM 2-mercaptoethanol, pH 7.5) were used as the sonication medium. Fig. 14 shows that two peaks of protein kinase activity were obtained upon DEAE-cellulose chromatography of the soluble mitochondrial

TABLE 13

Purification of Mitochondrial Cyclic AMP-dependent Protein Kinases

Step	Total Protein (mg)	Specific Activity pmol/min/mg	Total Units	Recovery %
Sonicated mitochondria	9072	158	1429800	100
Ultracentrifugation	2022	601	1215830	85
DEAE-cellulose PK I	238	231	46586	3.4
PK II	206	2570	529420	37.0
Type I-m* purification				
Sephacryl S-200	88.3	368	32459	2.3
Hydroxylapatite	11.1	524	5812	0.4
Sephacryl S-300	4.3	715	3075	0.2
Type II-m* purification				
Sephacryl S-200	83.3	5046	420332	29.4
Sephacryl S-300	41.4	6114	253120	17.7
Hydroxylapatite	7.0	11645	81515	5.7

Note: * See text for clarification of these designations.

0.5 μM cyclic AMP was present in all assays.

Figure 14   <u>Separation of isozymes of mitochondrial</u>

<u>protein kinase on DEAE-cellulose</u>

The column was run and the fractions

analyzed for protein kinase activity as

described under 'Experimental Methods'.
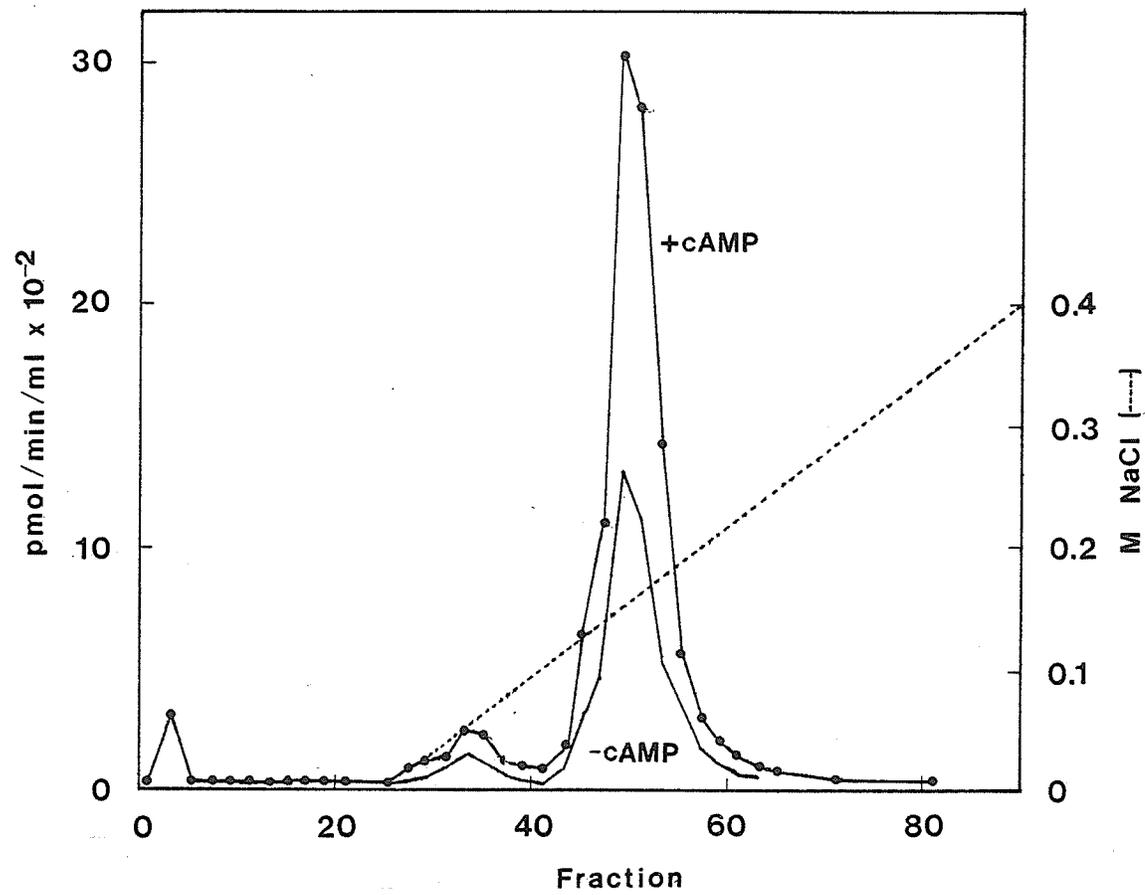
extracts. Both peaks were activated by cyclic AMP to extents found in other tissues and eluted at positions in the NaCl gradient expected for Type I and II enzymes (53). For this reason the bovine heart mitochondrial isozymes will be referred to as Type I-m and Type II-m.

Types I and II isozymes of the cytosol were shown before (52, 53) to have similar catalytic subunits but different regulatory subunits. The heptapeptide, Kemptide (leu-arg-arg-ala-ser-leu-gly) was shown to be a substrate of high affinity for the free catalytic subunit (85, 86). It is apparent from Table 14 that Kemptide inhibited the phosphorylation of our histone substrate by both isozymes from bovine heart mitochondria.

Chromatography on Sephacryl columns in the absence of EDTA-containing solutions resulted in multiple elution peaks for both kinases. This effect was not noted when EDTA-containing buffers were used. Chromatography of Type II-m kinase on Sephacryl S-200 resulted in a kinase peak with a shoulder corresponding to the peak elution fraction of Type I-m (Fig. 15).

It is evident from the ion-exchange elution profile that the peak corresponding to cyclic AMP-dependent protein kinase Type I-m is a relatively minor component of mitochondrial supernatant fractions. The most purified preparations of Type I-m were 3.1-fold enriched from the DEAE-cellulose chromatography step. Further purification of Type II-m resulted in an enzyme preparation that was 74-fold

TABLE 14

Effect of Kemptide on Phosphorylation of Histone H2B

By Isozymes From Bovine Heart Mitochondria

Fraction	Kemptide (3 mM)	Kinase activity (pmol/min/mg)	Inhibition %
Peak I	—	231.0	0
	+	6.5	97.3
Peak II	—	2570.0	0
	+	100.0	97.8

Note: The peak fractions were pooled fractions from Fig 14. The

assay mixture contained 0.5 µM cyclic AMP and histone H2B

with or without kemptide as specified.

Figure 15 <u>Sephacryl S-200 chromatography of Type I-m</u>

<u>and Type II-m protein kinases</u>

The column was run and the fractions

analyzed in the presence of cyclic AMP as

described under 'Experimental Methods'.

Type I-m (●---●); Type II-m (●——●).
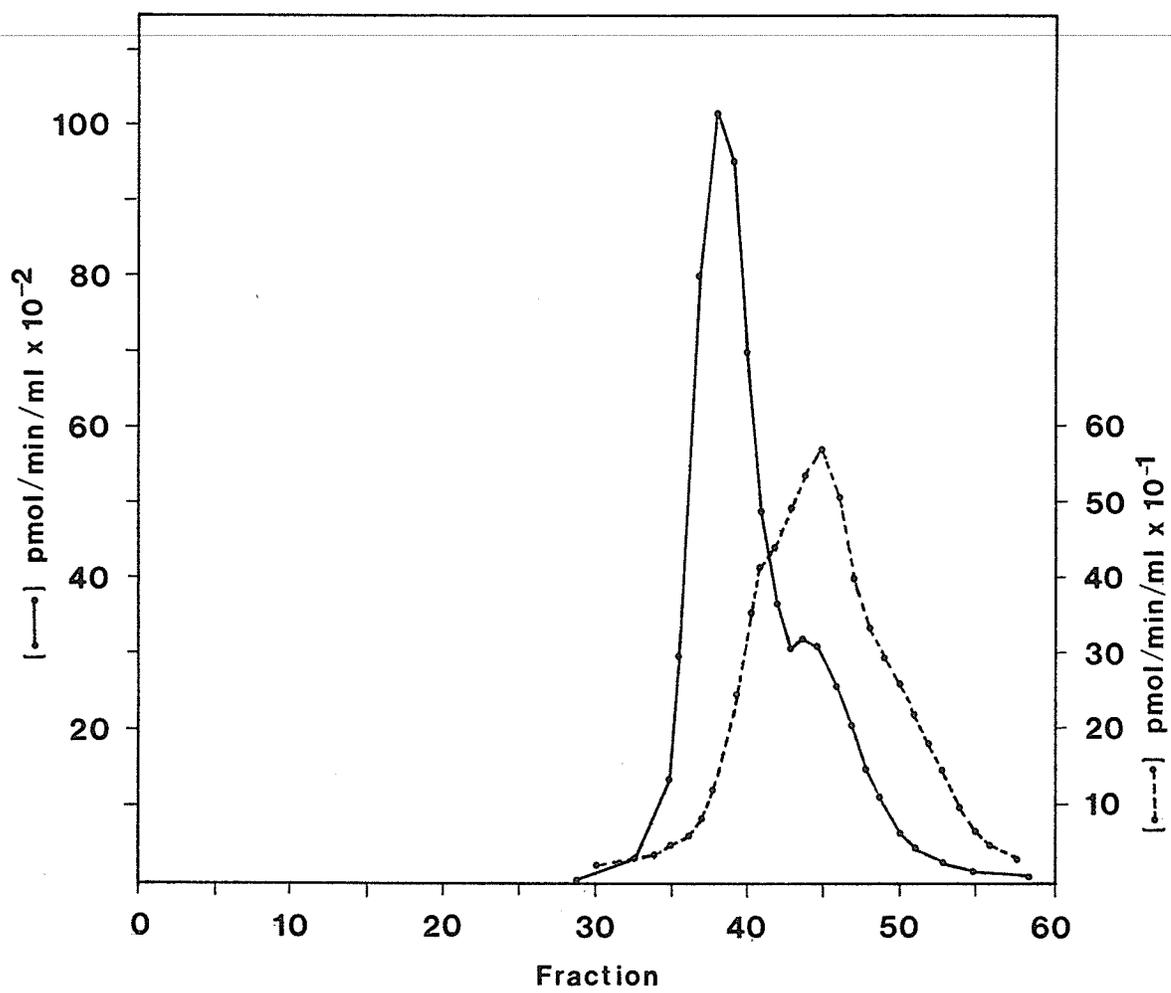
enriched from mitochondria with a yield of 5.7% of the total

mitochondrial histone kinase activity (Table 13).

VII Properties of Type I-m and Type II-m Preparations

a) Purity

SDS-gel electrophoresis of the isozyme preparations at

each stage of the purification is shown in Fig. 16. The

most purified preparations of Type I-m (lane 7) and

Type II-m (lane 11) are not pure, but do exhibit a Coomassie

blue staining band in the 40,000-42,000 dalton range,

corresponding to reported molecular weights of the catalytic

subunit (52, 53). Type I-m preparations show two intensely

stained bands with molecular weights of about 44,000 and

46,000 daltons that approximate reported values for the R I

subunit (53). It is not known at this time if bands in the

34,000 and 44,000 dalton region represent degradation

products of the 46,000 dalton band. Impurities with

molecular weights of approximately 53,000 and 69,000 are

also present in Type I-m preparations. Type II-m

preparations (lane 11) show several poorly stained bands in

the 50,000-55,000 dalton region, which could represent

different isoforms of the R II-m subunit (87) although

proteolytic degradation of one form can not be ruled out.

Poorly resolved bands in the 44,000-45,000 dalton range in

Type II-m fractions may represent contaminating amounts of

R I. Several other unidentified bands are evident

suggesting the impurity of Type II-m preparations.

Figure 16   <u>SDS-gel electrophoresis of mitochondrial</u>

<u>protein kinase fractions</u>

The purification fractions listed in Table 14

were analyzed in 10% polyacrylamide mini slab

gels as described under 'Experimental Methods'.

Lane 1, molecular weight marker proteins from top

to bottom; albumin (67,000 Da), ovalbumin

(45,000 Da), chymotrypsinogen (25,000 Da),

myoglobin (17,800 Da); Lane 2, 15 μg of sonicated

mitochondrial protein; Lane 3, 8.5 μg of the

105,000 g supernatant.   Lanes 4 to 7 represent

further purification fractions of Type I-m

protein kinase from the 105,000 g supernatant.

Lane 4, 9.0 μg from the DE-52 step; Lane 5,

5.6 μg from the Sephacryl S-200 step; Lane 6,

4.3 μg from the hydroxylapatite step; Lane 7,

1.8 μg from the Sephacryl S-300 step.   Lanes 8 to

11 represent further purification steps of

Type II-m protein kinase from the 105,000 g

supernatant.   Lane 8, 7.7 μg from the DE-52 step;

Lane 9, 4.9 μg from the Sephacryl S-200 step;

Lane 10, 4.2 μg from the Sephacryl S-300 step;

Lane 11, 3.4 μg from the hydroxylapatite step.
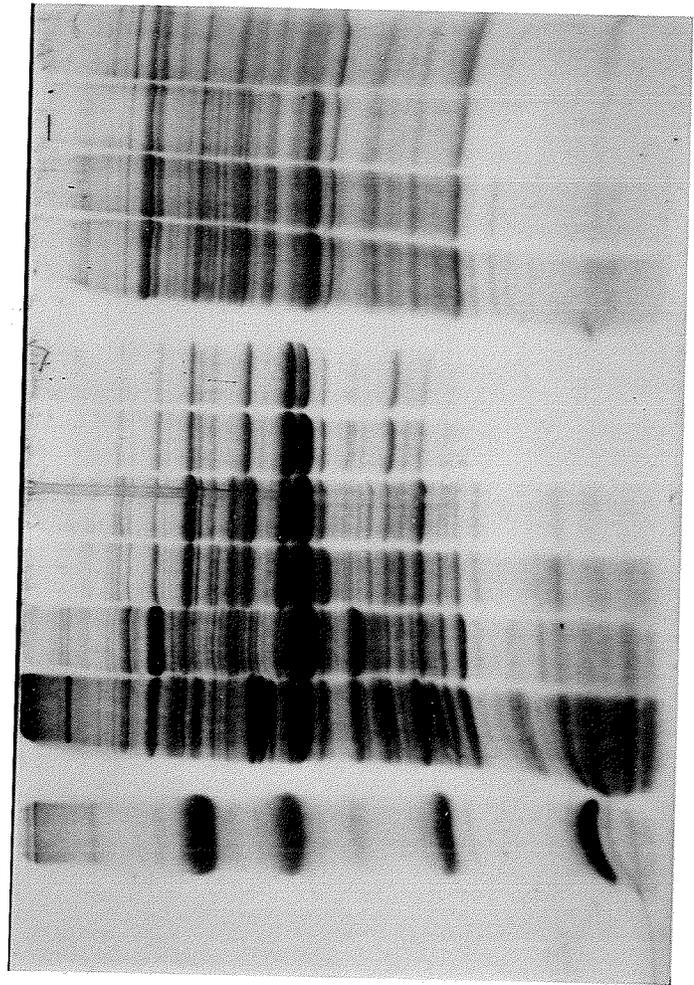
67 kDa —
45 kDa —
25 kDa —
17.8 kDa —

1 2 3 4 5 6 7 8 9 10 11

b) Stability

Activities of both isozymes measured in the presence of cyclic AMP were stable over prolonged periods when the preparations were stored in 5 mM Tris-HCl, 1 mM EDTA and 5 mM 2-mercaptoethanol, pH 7.5 at -70 °C with protein concentrations greater than 3 mg/ml. Activities of both isozyme preparations, measured in the absence of cyclic AMP, as much as doubled over a one month period. This suggested that the regulatory subunits of both isozyme preparations were unstable when stored under these conditions.

c) ATPase Activities

The most purified preparations of Type I-m and II-m exhibited cyclic AMP-dependent ATPase activity. In the presence of cyclic AMP, rates for the ATPase reaction (method 3) were constant for 45 minutes with 50 μg of Type I-m or 10 μg of Type II-m (Fig. 17). Histone phosphorylation and ATPase activities of Type I-m were activated 2.5-fold by the inclusion of cyclic AMP to assays; activation of Type II-m activities were 3.3-fold (Fig. 18). ATPase activities represented only a small fraction of the protein phosphorylation activities (12.0% and 1.9% for Types I-m and II-m respectively). Maximum kinase activation for both enzymes was at 0.5 μM cyclic AMP when 10 μg of Type I-m and 0.2 μg of Type II-m were used in the assays. Increased amounts of enzyme were needed to readily detect ATPase activities and for this reason considerably higher

Figure 17   <u>Response of ATPase activity of Type I-m and</u>

<u>Type II-m protein kinases to incubation time</u>

All values are the mean of two experiments done

in the presence of cyclic AMP.   ATPase activity

of Type I-m (▲—▲) and Type II-m (●—●).   Time

points were determined with 10 µg of Type II-m

(in the presence of 27.5 µM cAMP) or 50 µg of

Type I-m (in the presence of 2.5 µM cAMP).
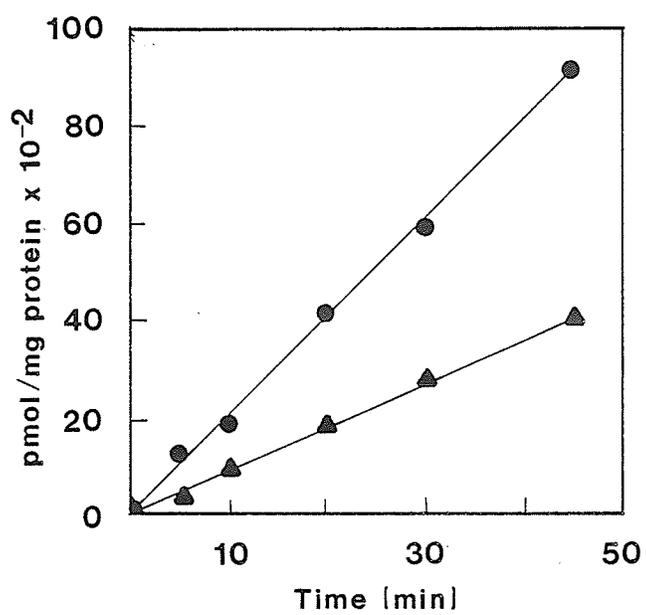
Figure 18 Cyclic AMP-dependent ATPase and protein kinase

activities of the mitochondrial isozymes

Protein kinase activity (●—●) and ATPase

activity (▲—▲) of Type I-m. Protein kinase

activity (O—O) and ATPase activity (△—△)

of Type II-m. Protein kinase activity was

assayed as described in 'Experimental Methods'

with 10 µg of Type I-m and 0.2 µg of Type II-m.

ATPase activity was assayed by method 3 with

50 µg of Type I-m and 10 µg of Type II-m.
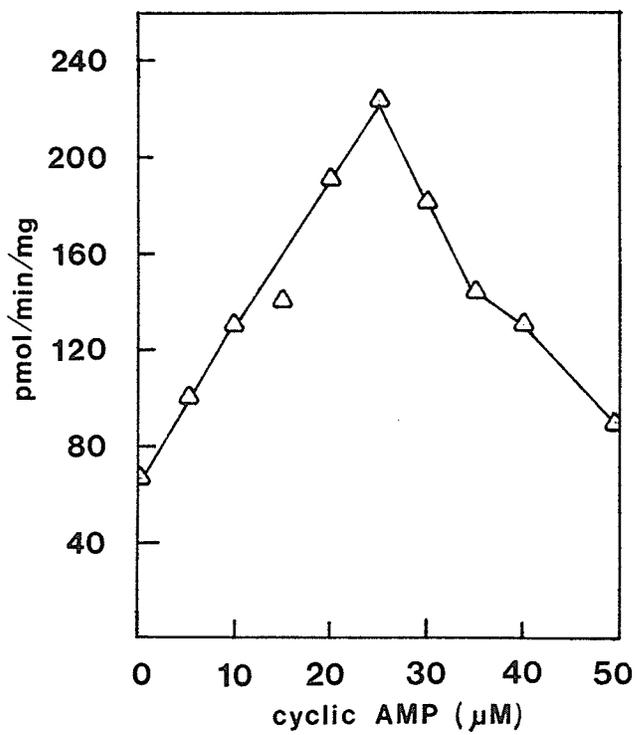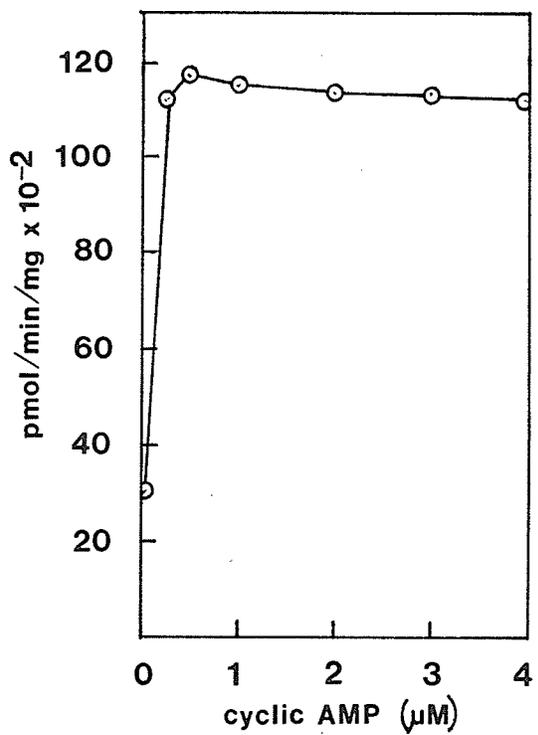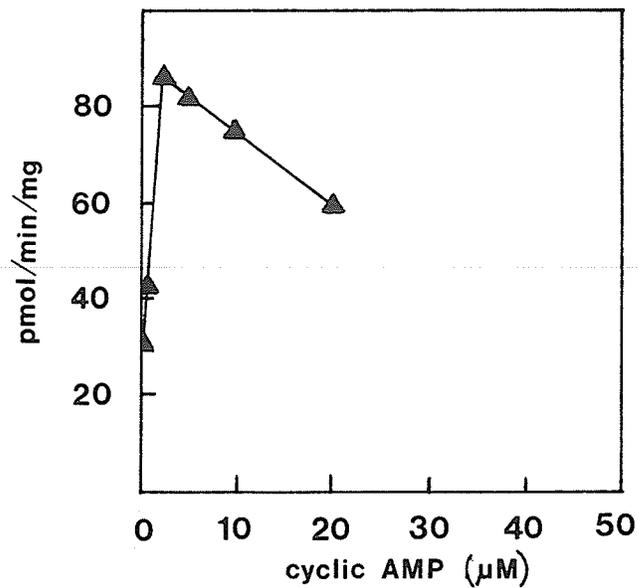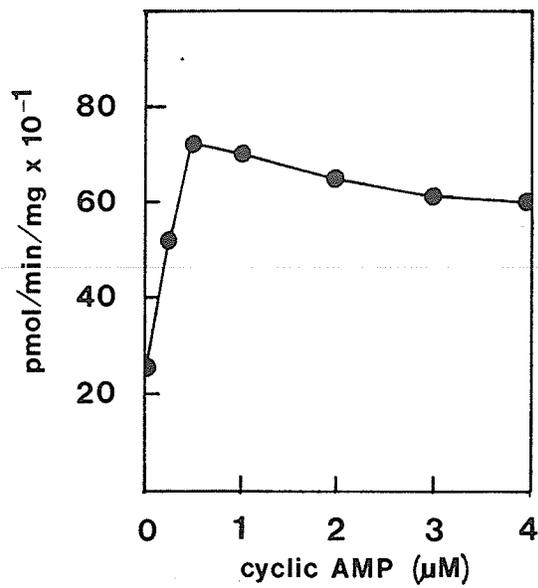
concentrations of cyclic AMP were needed to maximally

activate this activity in both isozyme preparations.

d) pH Characteristics

In the presence of cyclic AMP the pH for maximal

protein phosphorylation activity was estimated to be 7.0 for

Type I-m (Fig. 19), and 7.5 for Type II-m (Fig. 20). In the

absence of cyclic AMP protein phosphorylation activity of

isozyme Type II-m exhibited a broad pH-activity profile with

an optimum range between pH 7.0 and pH 8.0. The protein

kinase activity of Type I-m exhibited a more basic pH

optimum of 7.5 in the absence of cyclic AMP. ATPase

activities for both isozyme preparations demonstrated

optimal pH values similar to those described for kinase

activities (Fig. 19 and Fig. 20). Type II-m exhibited

optimal ATPase activity at pH 7.5 in the presence of

cyclic AMP, and at pH 8.0 in the absence of cyclic AMP. The

shift to a more basic pH optimum in the absence of cyclic

AMP suggests that the association between the catalytic and

regulatory subunits of mitochondrial protein kinase is

pH-sensitive. Unfortunately a similar analysis of Type I-m

ATPase activity in the absence of cyclic AMP was not

possible due to limiting amounts of this preparation.

e) Effects of Magnesium

The kinase and ATPase activities of isozyme Type II-m

were absolutely dependent on the presence of added magnesium

ions to assays and demonstrated maximal activities at 2.5 mM

Mg2+ (Fig. 21). Protein kinase activity of Type I-m was

Figure 19   Response of protein kinase and ATPase

activities of Type I-m to pH

Each point represents the mean of two

experiments. Protein kinase activity in the

presence (●——●) and in the absence (○——○) of

cyclic AMP. ATPase activities in the presence

(▲——▲) of cyclic AMP. Protein kinase and ATPase

activities were determined as described in the

legend to Figure 18 with the exception that the

buffers used were imidazole-HCl for pH 6.5 and

7.0, Tris-HCl for pH 7.5 - 8.5, and glycine-KOH

for pH 9.0 - 10.0.
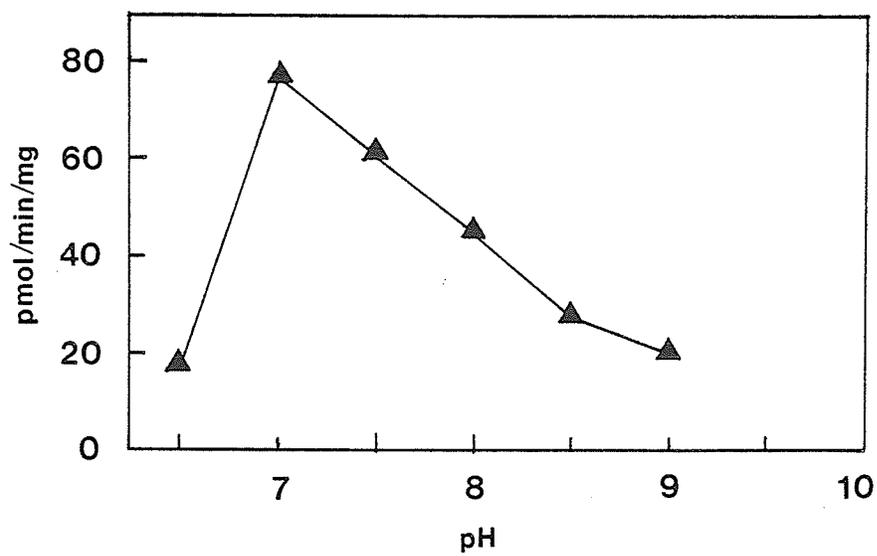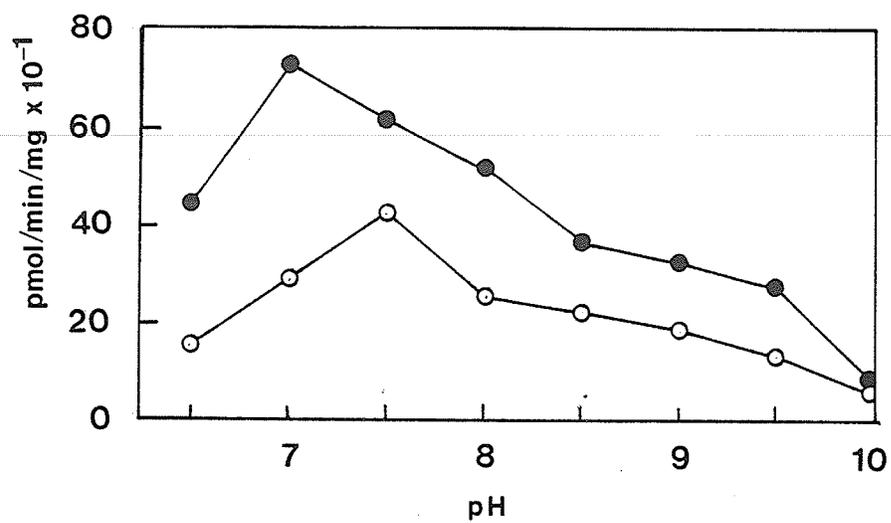
Figure 20   Response of protein kinase and ATPase

activities of Type II-m to pH

Each point represents the mean of two

experiments.   Protein kinase activities in the

presence (●—●) and absence (○—○) of cyclic AMP.

ATPase activities in the presence (▲—▲) and

absence (△—△) of cyclic AMP.   Protein kinase and

ATPase activities were determined as described in

the legend to Figure 18.   Assay buffers were as

described in the legend to Figure 19.
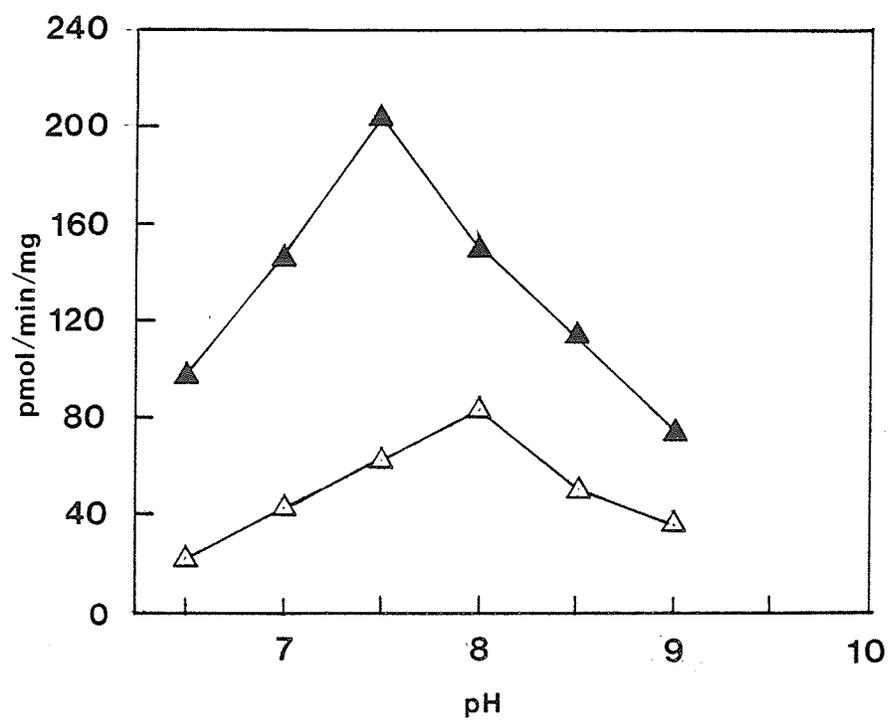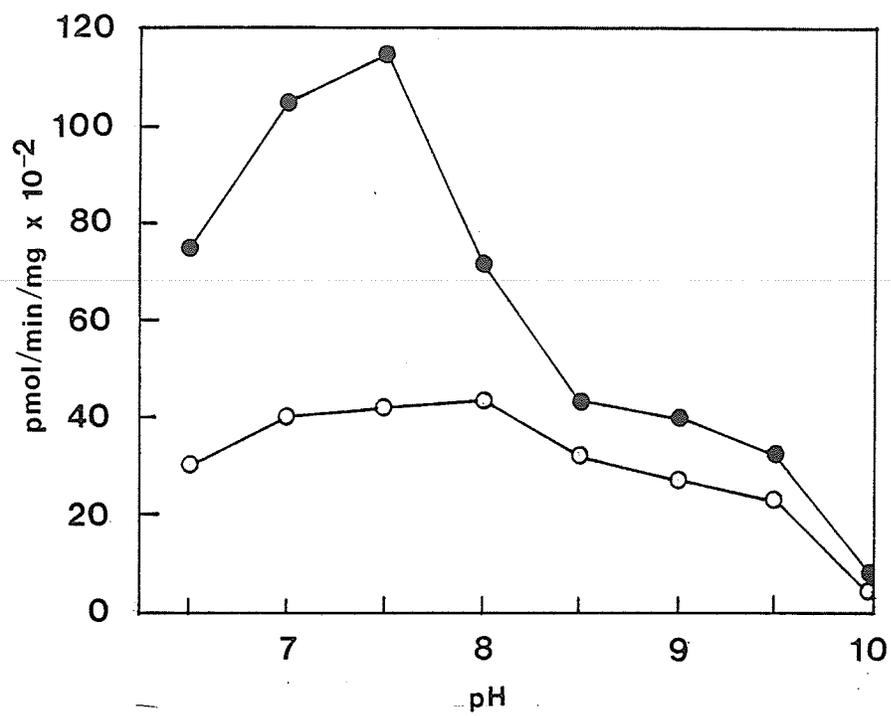
Figure 21   Effect of magnesium concentration on protein

kinase and ATPase activities of Type II-m

Protein kinase (●—●) and ATPase (▲—▲)

activities were determined as described in

the legend to Figure 18.   Each point represents

the mean of two experiments in the presence
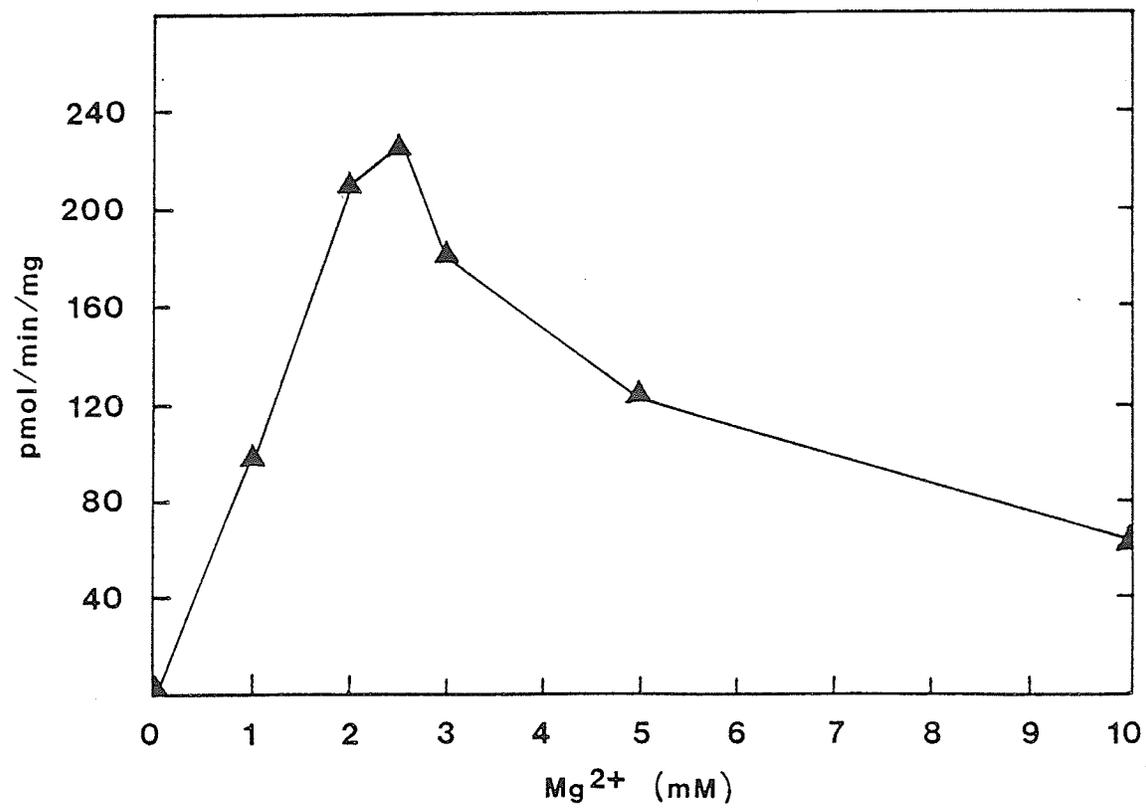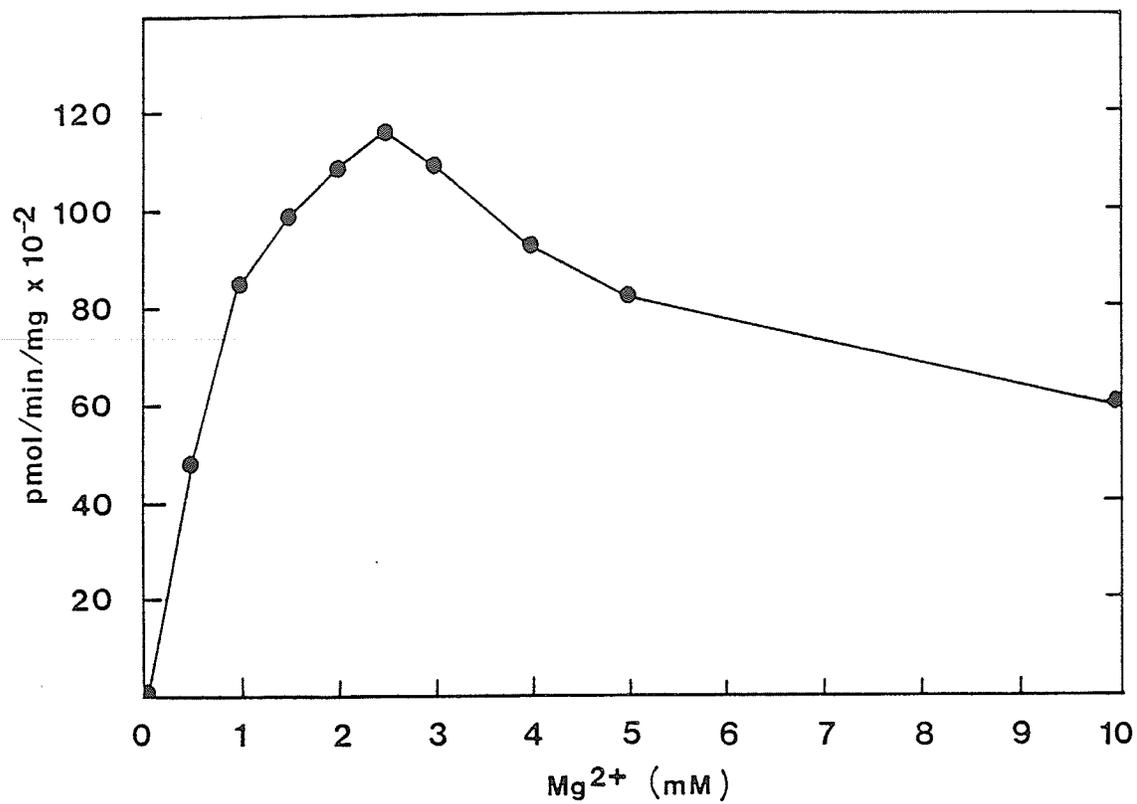
of cyclic AMP.

Figure 22   Effect of magnesium concentration on Type I-m
            protein kinase activity

            Protein kinase activity was determined as

            described in the legend to Figure 18.   Each point

            represents the mean of two experiments in the

            presence of cyclic AMP.
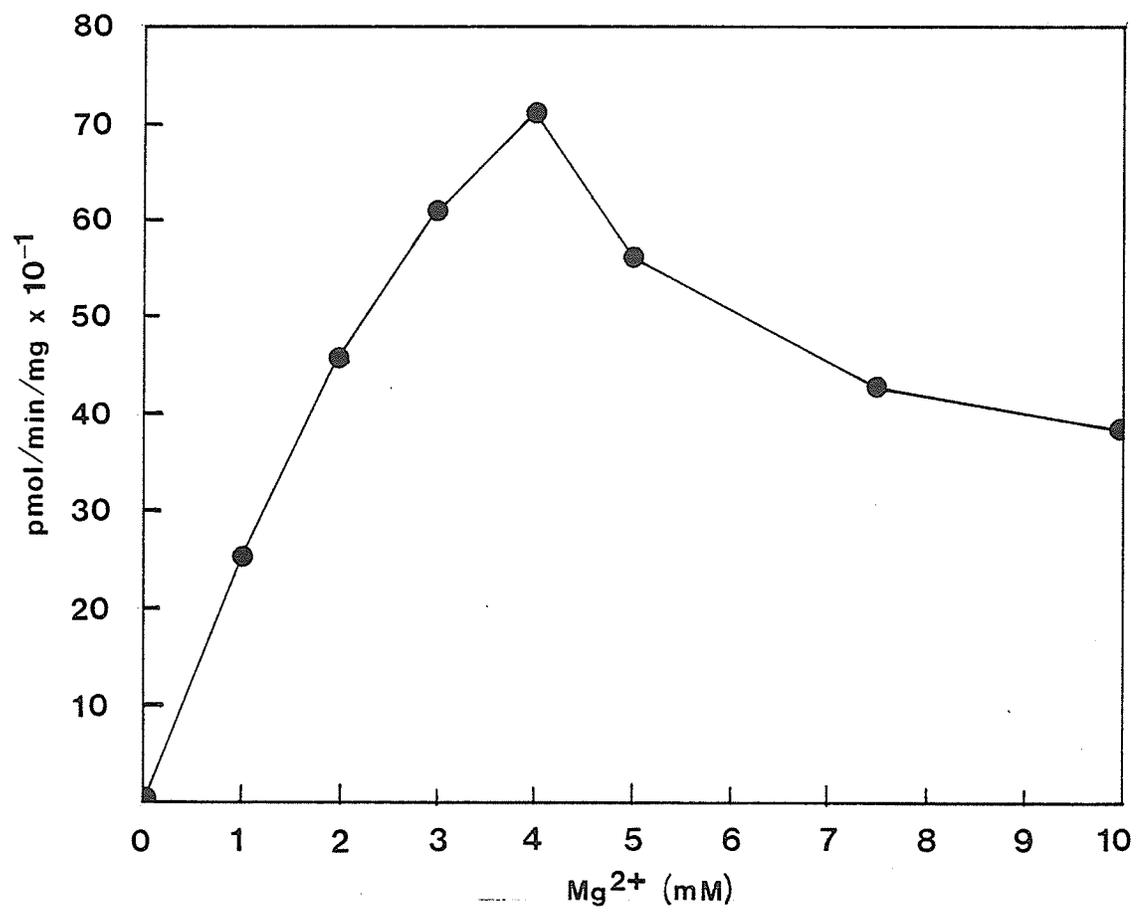
optimal at 4 mM Mg2+ (Fig. 22).   The effects of magnesium on

the ATPase activity of Type I-m were not examined.

f)   Effects of Inhibitors on Isozyme ATPase Activity

Oligomycin (20 µg/mg protein) or azide (5 mM) alone or

in combination had no effect on the ATPase activity of the

most purified preparations of Type I-m and II-m.   This

suggests that mitochondrial energy-transducing ATPase was

not contributing to isozyme ATPase activity.   Inhibitors of

sarcolemmal ATPase activity (1.0 mM ouabain or 10 µM

vanadate), or sarcoplasmic reticulum ATPase activity (0.5 mM

EGTA) were also without effect (data not shown).

VIII Can Oligomycin-Azide-Resistant ATPase Activity

Serve As a Marker for Cyclic AMP-Dependent Protein

Kinase Activity?

It was of interest to determine if the distribution of

protein kinase activity in mitochondrial fractions

correlated with ATPase activity that was resistant to a

combination of oligomycin and azide.   Table 15 shows the

distributions of oligomycin-azide-resistant ATPase and basal

and cyclic AMP-dependent protein kinase activities in

fractions derived from procedures 1 and 2.   C-fractions

derived from fractionation procedure 1 show very poor

correlation between the ATPase and protein kinase activities

in all fractions other than the C-mitoplast fraction.

Protein kinase activity is much more enriched in CB2 and CB3

than is ATPase activity.   There is much better correlation

in the S-fractions of procedure 2, however it was noted that

TABLE 15

Oligomycin-azide resistant ATPase and Protein Kinase Activities

Of Fractions From Bovine Heart Mitochondria

These results are the means of three experiments. ATPase activity
was measured in the presence of 10 μg oligomycin/mg protein and 5 mM azide.
Protein kinase activity was measured in the presence of Triton X-100.
The C-fractions were produced by procedure 1 and the S-fractions by
procedure 2. Relative specific activity (R.S.A.) is the % recovered
activity/% recovered protein in each fraction.

Fraction	Oligomycin-azide-resistant ATPase R.S.A.	Protein Kinase Activity	
		cAMP-dependent R.S.A.	basal R.S.A.
C-mitoplast	0.8	0.8	0.7
C-int	0.8	2.4	4.2
CB 1	3.4	4.9	5.2
CB 2	3.2	14.1	6.8
CB 3	2.4	12.1	6.7
S-mitoplast	0.7	0.4	0.7
SB 1	4.6	1.5	1.3
SB 2	6.0	5.3	3.1
SB 3	1.9	2.5	1.6

inclusion of either 0.5 µM or 25 µM cyclic AMP to the assay

had no stimulating effect on the ATPase activity of these

fractions. This suggests that ATPase activity in the

presence of oligomycin and azide is not the result of the

ATPase activity resulting from mitochondrial protein

kinases.

DISCUSSION

I   OR-ATPase Activity of the Outer Mitochondrial Membrane

Earlier reports have described the existence of a mitochondrial OR-ATPase activity that was localized to the outer membrane of rat kidney cortex (19) and spleen mitochondria (20, 21). OR-ATPase activity was also described in rat heart mitochondria (20). It was suggested that outer membrane OR-ATPase activity may act as a mediator of mitochondrial calcium efflux (19).

In the present studies OR-ATPase was found to exhibit the highest specific activities in bovine heart mixed (heavy and light) and light mitochondrial preparations. These mitochondrial preparations were found to be heavily contaminated with lysosomal material. Lysosomal ATPase activity, that is resistant to oligomycin and azide, has recently been described (23-26). I have found that following fractionation of lysosomal contaminated mitochondrial preparations, OR-ATPase as well as acid phosphatase co-localized with outer membrane fractions. Heavy mitochondria were much less contaminated with lysosomal material and when used as the starting material for either fractionation method, OR-ATPase activity did not localize to the outer mitochondrial membrane. This suggested that in outer membrane fractions derived from mixed populations of bovine heart mitochondria, much of the observed OR-ATPase activity resulted from lysosomal contamination. It should be noted that previous reports detailing the existence of an outer membrane OR-ATPase

activity in mitochondria from rat tissues made no attempts to assess the lysosomal contamination of their mitochondria or submitochondrial fractions.

II   OR-ATPase Activity in Subfractions of Heavy
     Mitochondria

a)   Evidence that OR-ATPase Activity Results from
     Incomplete Inhibition of the Energy-transducing ATPase

Fractionation of heavy bovine heart mitochondria by either of the procedures employed in these studies have demonstrated that OR-ATPase activity enriches in fractions that are not enriched in outer membrane material. The findings from several different experiments suggest that this mitochondrial OR-ATPase activity (at least in bovine heart tissue) results from incomplete inhibition of the energy-transducing ATPase. It was noted in both fractionation techniques employed in these studies that fractions enriched in OR-ATPase activity (CB3 of fractionation method 1 and SB2 and SB3 of fractionation method 2) were also enriched in oligomycin-sensitive activity. Additional evidence results from a comparison of Tables 6 and 10 which demonstrate that treatment of heavy mitochondria with trypsin resulted in a co-activation of the oligomycin-sensitive and resistant ATPase activities. It was reported that the F1-ATPase inhibitor protein is sensitive to proteolytic degradation by trypsin (31, 88). This could readily explain the co-activation of resistant and sensitive activities by trypsin assuming that both

derive from the energy-transducing ATPase. In agreement with the results of others (33), oligomycin and azide when used in combination result in almost complete inhibition of mitochondrial ATPase activity. This suggests that mitochondrial OR-ATPase activity is sensitive to the effects of a second specific inhibitor of the energy-transducing ATPase. Electrophoresis in a non-denaturing system of Triton X-100 solubilized mitochondrial subfractions enriched in OR-ATPase activity demonstrated that azide-resistant ATPase activity co-migrates with a band of azide-sensitive ATPase activity. Azide-resistant ATPase activity from fractions enriched in the lysosomal marker acid phosphatase, did not co-migrate with azide-resistant or -sensitive ATPase activity derived from heavy mitochondria. This suggests that lysosomal ATPase activity was not contributing to azide-resistant ATPase activity in subfractions from heavy mitochondria.

Azide inhibition of bovine heart mitochondrial ATPase activity was non-linear with respect to time; an initial fast phase followed by a slow and almost completely inhibited phase of ATP hydrolysis. Similar findings have previously been described (89) and have led to the suggestion that azide stabilizes the inactive E*-ADP complex formed during ATP hydrolysis. If the slow phase is considered to represent maximal inhibition of mitochondrial ATP hydrolysis by azide (98-100% of the ATPase activity is inhibited in this phase) then very little activity is left

to account for an ATPase activity that could be involved in mitochondrial calcium efflux.

b)   Effects of 2-Mercaptoethanol on Mitochondrial

OR-ATPase Activity

Preincubation of enzyme preparations with 2-mercapto-ethanol resulted in a 2-fold activation of ATPase activities in the presence of oligomycin. These results are puzzling in that no activation of the oligomycin-sensitive ATPase activity was observed. The ATPase activity of fractions enriched in lysosomal material was not activated by preincubation with 2-mercaptoethanol suggesting that contamination of SB3 with lysosomal ATPase is an unlikely explanation for the observed effect. Thiol reagents, such as pCMB, do inhibit the ATPase activity of submitochondrial particles but have no effect on the activity of soluble F1 (28, 90, 91). These findings have led to the suggestion that thiol groups may be important in proton translocation by the Fo subunit of the energy-transducing ATPase (91). It is possible that reduction of thiol groups by 2-mercaptoethanol alters the binding of oligomycin to the oligomycin sensitivity conferring protein of the Fo complex. It has been reported that trypsin (used in the preparation of SB3) results in proteolytic alterations of the Fo complex (88, 92). This may also affect the inhibition of the energy-transducing ATPase by oligomycin. Therefore, activation of mitochondrial OR-ATPase activity by reducing

agents does not necessarily suggest an enzymatic activity distinct from the energy-transducing ATPase.

III Mitochondrial Cyclic AMP-Dependent Protein Kinase Activity

a) Evidence for an Intramitochondrial Location

The present study has described the separation of two cyclic AMP-dependent protein kinases from mitochondrial soluble fractions. Sonication or treatment of mitochondrial preparations with Triton X-100 results in an almost 2-fold activation of the protein kinase activity, suggesting an intramitochondrial localization for this activity. Cyclic AMP-dependent protein kinase of the Type I variety has not previously been described in soluble or particulate fractions of bovine heart (54). It is possible that mitochondrial membranes in these studies were simply too contaminated to allow the detection of the Type I mitochondrial isozyme.

b) Substrate Specificity and Cyclic AMP Dependence

The protein kinases of bovine heart mitochondria described in these studies are unique among the mitochondrial protein kinases studied so far in their dependence for cyclic-AMP and substrate preference. Protamine which was the substrate preferred over histones for the protein kinases of rat liver mitochondria (45), was a very poor substrate for the kinase of heart mitochondria. The acidic proteins, casein and phosvitin, were also poor substrates which, along with their cyclic AMP-dependence,

distinguish the kinases of present studies from the
mitochondrial protein kinases studied by others (41-43).
Kemptide, a substrate of high affinity for the free
catalytic subunit of cyclic AMP-dependent protein kinases
(81, 82), inhibited the phosphorylation of our histone
substrate by both isozymes from bovine heart mitochondria.
In this case, phosphorylation of Kemptide itself would not
be detected since the peptide is not retained on Whatman
3 MM disks and special methods are required for its recovery
(81, 82). These results suggest that the isozymes of bovine
heart mitochondria have catalytic subunits similar to those
of protein kinases studied earlier (85, 86). However, until
the regulatory subunits of the mitochondrial isozymes are
characterized further, the mitochondrial isozymes are
tentatively called Type I-m and Type II-m.

It is puzzling that these kinases have not previously
been described in bovine heart mitochondria. Vardanis (44)
has described a cyclic nucleotide-independent histone kinase
in mouse liver and heart as well as in bovine heart
mitochondria. This enzyme exhibited optimal activity at pH
8.5. My studies on partially purified preparations of
bovine heart mitochondrial isozymes suggest that at this pH
much of the cyclic AMP-dependence would be lost. The
exclusion of azide from assays for the cyclic nucleotide-
independent activity may also be a contributing factor. I
have found that mitochondrial membranous fractions exhibited
little protein phosphorylation ability in assays containing

only oligomycin as the inhibitor of mitochondrial ATPase activity. I felt a likely explanation for this observation would be substrate depletion since ATP can be readily hydrolyzed by mitochondrial preparations even in the presence of oligomycin. Inclusion of azide, in addition to oligomycin, greatly stimulated protein kinase activity in mitochondrial membrane fractions. This effect is most likely mediated by a more complete inhibition of the mitochondrial energy-transducing ATPase. The lack of 2-mercaptoethanol, and the use of different phosphorylation substrates in mitochondrial protein kinase assays of previous reports (41, 44) may also explain why cyclic AMP-dependent protein kinases have not previously been described in heart mitochondria.

c)   Intramitochondrial Location

In fractionation scheme 1, basal and cyclic AMP-dependent protein kinase activities were enriched in C-int fractions. These fractions lacked detectable amounts of inner and outer membrane marker enzymes but were enriched in intermembrane space material as suggested by the enrichment of adenylate kinase. Cyclic AMP-dependent protein kinase activity eluting as a Type II enzyme from DEAE-cellulose has been described in rat liver mitochondria (45). Of the total activity in these mitochondria, 90% was localized to the intermembrane space and the remainder to the matrix. It is not unusual for mitochondrial enzymes to be distributed to more than one mitochondrial compartment (93). Unfortunately

damage to the inner membrane occurred during fractionation method 1, resulting in the contamination of C-int fractions with matrix material as suggested by the enrichment of malate dehydrogenase. It was therefore not possible to assign an intermembrane space or matrix localization to cyclic AMP-dependent protein kinase activity in bovine heart mitochondria.

Localization of mitochondrial protein kinase activity by fractionation procedure 2 was complicated by the finding that treatment of heavy mitochondria with trypsin and soybean trypsin inhibitor resulted in a 60% loss of both basal and cyclic AMP-dependent protein kinase activities. A possible explanation for this loss of activity derives from the finding that soybean trypsin inhibitor is capable of inhibiting the catalytic subunit of cyclic AMP-dependent protein kinases (55, 56). It has been reported that trypsin, although it has no apparent effect on enzymatic activity (47, 54), promotes solubilization of membrane-bound forms of cyclic AMP-dependent protein kinases (54). This may complicate the localization of these enzymes in heart mitochondria when trypsin-utilizing fractionation methods are employed.

Protein and enzyme recoveries in fractionation scheme 2 are somewhat lower than those described by Scholte (36). This probably results from differences in harvesting the sucrose gradient fractions. We drew off the gradient bands with a J-shaped hypodermic needle, pelleted the membranous

material by centrifugation and discarded the supernatant material. This would explain the poor recoveries of adenylate kinase (22.5% from the starting mitochondrial material) and other intermembrane space material following removal of the outer mitochondrial membrane. The recoveries of malate dehydrogenase (54.7%) and cyclic AMP-dependent protein kinase from mitochondria (57.5%) are also relatively low suggesting that a portion of the cyclic AMP-dependent protein kinase activity may be lost with soluble matrix or intermembrane space material. An alternative explanation could involve the loss of membrane-bound cyclic AMP-dependent protein kinase following trypsin solubilization.

A good percentage of the heart mitochondrial protein kinase activity probably exists in a membrane-bound state. During fractionation procedure 1, approximately 31% of the mitochondrial basal protein kinase activity and 19.1% of the cyclic AMP-dependent protein kinase activity associated with a soluble fraction (C-int); the remainder may be associated with mitochondrial membranes. Cyclic AMP-dependent protein kinases bind to membranes via the regulatory sub-unit, which may remain membrane-bound following release of the catalytic subunit (54). It is notable that enrichment of cyclic nucleotide-independent kinase activity (presumably representing free catalytic sub-unit) was greater in C-int fractions than was cyclic-AMP dependent activity. This suggests disproportionate amounts of free catalytic subunit and the holoenzyme in soluble fractions; some of the

holoenzyme may remain membrane-bound. Basal and cyclic AMP-dependent activities are most enriched in the gradient fractions of both fractionation methods, most notably CB2 and SB2. These gradient bands are mixed outer and inner membrane fractions, enriched in monoamine oxidase and to a lesser extent in cytochrome c oxidase. Adenylate kinase and malate dehydrogenase are not enriched in these gradient fractions, suggesting that vesicular entrapment of soluble mitochondrial enzymes is an unlikely explanation for the enrichment of protein kinase activity in these fractions. It is more likely that at least a portion of the cyclic AMP-dependent activity exists in a membrane-bound form. Apparently sonication can result in the release of membrane-bound holoenzyme as evidenced during the first step of the purification procedure. It was also noted that use of a higher ionic strength sonication buffer can improve the yield of protein kinase activity in the 105,000 g supernatant.

d)   Purification of the Mitochondrial Isozymes

The purification scheme described in the present study results in the partial purification of the mitochondrial isozymes. Type II-m was purified approximately 74-fold from mitochondria. Type I-m was found to be a minor component of the kinase activity in mitochondrial soluble fractions. SDS-gel electrophoresis suggests that Coomassie-blue staining bands, corresponding to the reported molecular weights of the C and R subunits of the cytosolic enzymes,

are components of the mitochondrial isozyme preparations. In the absence of cyclic AMP, activities of both isozyme preparations increased substantially with prolonged storage suggesting an instability of the regulatory subunits.

Several features have emerged during the partial purification of the mitochondrial isozymes which may prove useful in subsequent purification attempts. Chromatography on Sephacryl columns in the absence of EDTA-containing solutions resulted in multiple elution peaks for both isozymes. This effect was not observed when EDTA-containing buffers were used. It was previously noted that prolonged storage of cyclic AMP-dependent protein kinases may result in their conversion to multiple molecular forms but that this conversion can be minimized by storing the enzymes in EDTA-containing buffers (94). This may explain the multiple elution peaks noted in the absence of EDTA. Chromatography of Type II-m on Sephacryl resulted in a kinase peak with a shoulder corresponding to the peak elution fraction of Type I-m. Co-elution of Type I with Type II cyclic AMP-dependent protein kinase during DEAE-cellulose chromatography has been noted (95), and may explain the shoulder on Type II-m elution profiles. Alternatively, different isoforms of the R II subunit, differing in molecular weights, have been described (87) and could explain the shoulder on elution profiles of Type II-m. Since Type I-m and Type II-m both elute with 0.15 M potassium phosphate during chromatography on hydroxylapatite

it is possible that Type II-m preparations are contaminated to some extent by Type I-m.

e)   ATPase Activity of the Mitochondrial Isozymes

Type I-m and Type II-m isozyme preparations exhibit ATPase activity that is unaffected by the inclusion in assays of inhibitors of the mitochondrial energy-transducing ATPase, sarcolemmal ATPase or the sarcoplasmic reticulum ATPase. That this activity is cyclic AMP-dependent and shares similar properties with the protein phosphorylation ability of both isozyme preparations suggests that both activities derive from the C subunit. It is unlikely that cyclic AMP-dependent protein kinases account for any significant amount of mitochondrial OR-ATPase activity. The ATPase activity of the isozyme preparations is several orders of magnitude lower than mitochondrial activities even when oligomycin and azide are both included in the assay. There is also little correlation between ATPase and kinase distributions in the mitochondrial fractionation procedures. Like the cytosolic enzymes (59, 61) it is possible that ATPase activities of the mitochondrial isozymes be used in future to help elucidate the catalytic mechanisms of these enzymes.

f)   Possible Roles For Mitochondrial Cyclic AMP-
      Dependent Protein Kinases

There are several potential roles for these enzymes in mitochondria. Many membrane-associated processes in liver mitochondria, including the transport of calcium and

phosphate (96-101), oxidative phosphorylation and electron transport (97, 102, 103) and ATPase activity (100, 101, 103), are affected by pre-treatment with hormones. It is possible that some of these effects are mediated by mitochondrial protein phosphorylation. Cyclic AMP-dependent phosphorylation of the b-subunit of the mitochondrial F1-ATPase enhances proteolytic degradation of the protein in the cytosol of S49 mouse lymphoma cells (104). In mitochondria phosphorylated proteins could be eliminated, possibly by the ATP-dependent protease that has been implicated in the rapid degradation of incomplete mitochondrial proteins (105, 106). Other possibilities stem from the finding that the mitochondrial F1-ATPase inhibitor protein can be phosphorylated by cyclic AMP-dependent protein kinase activity (31). ATP hydrolysis by the F1-ATPase can power mitochondrial Ca2+ uptake through the electrogenic uniport system (107). It is possible that phosphorylation-dephosphorylation of the inhibitor protein could indirectly regulate mitochondrial Ca2+ uptake by affecting the activity of the F1-ATPase.

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