

THE UNIVERSITY OF MANITOBA

STRUCTURE - FUNCTION RELATIONS IN THE
CALCIUM-DEPENDENT PROTEIN MODULATOR
OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

by

Michael Walsh

B.Sc. (Hons.) University College, Dublin 1974

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

WINNIPEG, MANITOBA

MAY 1978

MAR 22 1978

THE UNIVERSITY OF MANITOBA
FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to
the Faculty of Graduate Studies for acceptance, a Ph.D. thesis
entitled: "STRUCTURE - FUNCTION RELATIONS IN THE CALCIUM-
DEPENDENT PROTEIN MODULATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE"

.....
.....
submitted by Michael P. Walsh,
in partial fulfilment of the requirements for the Ph.D. degree.

A. Moran
.....
Advisor
Harry W. Duckworth
.....
Jerry L. ...
.....

.....
Dr. C. M. Kay (University of Alberta)
External Examiner
.....
.....
.....

Date of oral examination: March 21, 1978
The student has satisfactorily completed and passed the Ph.D.
oral examination.

A. Moran
.....
Advisor
Harry W. Duckworth
.....
Jerry L. ...
.....

John D. Belsky
.....
Chairman of Ph.D. Oral*
Dr. Felix D. Bantel
John D. Kenje

(*The signature of the Chairman does not necessarily signify that
the Chairman has read the complete thesis.)

STRUCTURE - FUNCTION RELATIONS IN THE
CALCIUM-DEPENDENT PROTEIN MODULATOR
OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

BY

MICHAEL WALSH

A dissertation submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

© 1978

Permission has been granted to the LIBRARY OF THE UNIVER-
SITY OF MANITOBA to lend or sell copies of this dissertation, to
the NATIONAL LIBRARY OF CANADA to microfilm this
dissertation and to lend or sell copies of the film, and UNIVERSITY
MICROFILMS to publish an abstract of this dissertation.

The author reserves other publication rights, and neither the
dissertation nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.



To Mary, Katy and Emma.

"An experiment is never a failure solely because it fails to achieve predicted results. An experiment is a failure only when it also fails adequately to test the hypothesis in question, when the data it produces don't prove anything one way or another".

Robert M. Pirsig in "Zen and the Art of Motorcycle Maintenance", (1974), William Morrow and Company, Inc., New York, N.Y. 10016.

ACKNOWLEDGEMENTS

I am most grateful to my thesis supervisor, Dr. Frits C. Stevens, for his continual interest and encouragement in every aspect of my graduate education, particularly relating to the investigations described herein.

I am most appreciative of the efforts of Drs. J.H. Wang and H. Duckworth, as members of my Ph.D. Advisory Committee, in particular for their considerable interest in my research progress and for many helpful discussions.

My thanks go to Ms. Kathleen Haacke and Mr. Philip Ngai for their expert technical assistance.

I am very grateful to Dr. Cyril Kay at the University of Alberta for providing the opportunity for me to spend a very profitable ten days in his laboratory, and to Mr. Kim Oikawa for his untiring efforts during the course of the circular dichroism studies we performed. My thanks go to Mr. Max Hincke for his computerized analyses of portions of the CD data obtained.

I gratefully acknowledge receipt of a Medical Research Council of Canada Studentship which enabled me to carry out these investigations.

ABSTRACT

Three aspects relating to the structure and function of the Ca^{2+} -dependent protein modulator of cyclic nucleotide phosphodiesterase were investigated: (1) the mode of interaction between the modulator and phosphodiesterase, (2) the conformational changes induced in the modulator by the binding of Ca^{2+} , and (3) the structural homology between the modulator and troponin C, the calcium-binding subunit of the muscle troponin complex.

Chemical modification of selected functional groups of the protein modulator indicated that the site for interaction with phosphodiesterase is located between the second and third calcium-binding regions and is on the surface of the molecule. The integrity of both methionine and lysine residues is essential for the expression of phosphodiesterase-stimulating activity. It is proposed that interaction with phosphodiesterase occurs via an initial site recognition involving Lys 75 and Lys 77, and possibly other neighbouring charged side chains, followed by formation of a strong binding interaction through hydrophobic interactions involving Met 71, 72 and 76. Chemical modification of histidine, tyrosine and arginine residues, on the other hand, did not affect the stimulation of phosphodiesterase - none of these residues are located in the vicinity of the proposed phosphodiesterase binding domain. Similar studies indicated that the site for interaction with phosphodiesterase is distinct from the site for interaction with troponin I.

A combination of circular dichroism, chemical modification and controlled enzymatic digestion of the protein modulator in the presence and absence of Ca^{2+} provided insight into the conformational changes

occurring as the modulator binds Ca^{2+} . Removal of Ca^{2+} results in a substantial loss of secondary structure: the helical content decreases from 49% to 40%, whereupon the molecule becomes much more susceptible to tryptic digestion and urea denaturation. On a more refined level, His 107, for example, becomes more accessible as the modulator binds Ca^{2+} ; as revealed by kinetic analysis of carbethoxylation of this residue. Similar studies revealed altered reactivity of the other functional groups of the modulator upon binding of Ca^{2+} .

The protein modulator and troponin C exhibit approximately 80% sequence homology, taking conservative replacements into account (Vanaman et al., 1977). Circular dichroism studies of the protein modulator indicated the overall secondary structure of the molecule to be very similar to that of troponin C. Furthermore, controlled tryptic digestion of the modulator with structural and functional characterization of the resultant fragments revealed that cleavage occurred at positions homologous to those previously observed (Drabikowski et al., 1977a) in troponin C. The two calcium-binding proteins, therefore, exhibit similar tertiary structures. In addition, they undergo similar conformational changes upon binding of Ca^{2+} ions. The structural differences between the modulator and troponin C are small, but, in functional terms, these subtle differences have profound effects.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
ABSTRACT	vi
TABLE OF CONTENTS	viii
ABBREVIATIONS	xiii
MATERIALS	xiv
INTRODUCTION	1
I. Purposes of this study	2
III. Strategy	3
LITERATURE REVIEW	5
I. Introduction	5
II. Discovery of protein modulator	5
III. Occurrence	7
IV. The modulator in transformed cells	10
V. Mechanism of activation of PDE	11
VI. Calcium binding properties	13
VII. Stoichiometry	15
VIII. Conformational change accompanying Ca ²⁺ binding	16
IX. Modulator-induced conformational change in PDE	19
X. Physical properties	20
A. Molecular weight and isoelectric point	20
B. Ultraviolet absorption spectrum	21
XI. Chemical structure	21
A. Amino acid composition	21
B. Amino terminal analysis	22
C. Amide determination	22
D. Amino acid sequence	23
E. Presence of an unusual amino acid	24
XII. Application of the modulator protein in enzyme purification	25
A. Ion-exchange chromatography	25
B. Affinity chromatography	26
XIII. A family of homologous calcium-binding proteins produced by gene replication	28
A. Parvalbumins	28
B. Troponin C	29
C. Myosin light chains	31
D. Ca ²⁺ -dependent protein modulator	32
XIV. Evolutionary studies	35

	Page
XV. Other activities of the Ca ²⁺ -dependent protein modulator . . .	37
A. Are the modulator and TN-C functionally interchangeable?	37
1. Substitution of modulator for troponin C	37
2. Effect of TN-C on PDE activity	38
B. Activation of brain adenylate cyclase	38
C. Activation of (Ca ²⁺ -Mg ²⁺) ATPase	41
D. Stimulation of Ca ²⁺ transport	42
E. Activation of myosin light chain kinase	43
F. Modulator binding protein	45
XVI. Physiological roles	47
A. Regulation of cyclic nucleotide metabolism	47
1. Tissue distribution	47
2. Developmental changes	48
3. Subcellular distribution	49
4. Possible mechanism of regulation	50
B. Regulation of smooth muscle contraction	53
C. Mediation of Ca ²⁺ -regulated processes in general	54
XVII. Other effectors of modulator-dependent PDE	55
A. Lipids	55
B. Pharmacological agents	57
C. Others	57
GENERAL EXPERIMENTAL PROCEDURES	59
I. Purification of bovine brain protein modulator	59
II. Preparation of modulator-deficient phosphodiesterase	71
III. Assay of cyclic nucleotide phosphodiesterase	74
IV. Assay of protein modulator activity	76
V. Electrophoretic procedures	80
A. 15% PAGE	80
B. Urea-PAGE	82
C. SDS-PAGE	83
D. Isoelectric focusing	85
VI. Cyanogen bromide cleavage	87
VII. Performic acid oxidation	87
VIII. Acid hydrolysis and amino acid analysis	87
IX. Protein and peptide determination by the ninhydrin method after alkaline hydrolysis	88
X. Circular dichroism	88
XI. Calcium ion concentration	89
XII. Analysis of Ca ²⁺ titration data	89
EXPERIMENTAL	91
I. Comparison of bovine heart and bovine brain modulator protein	91
A. Introduction	91
B. Experimental procedure	91
C. Results and discussion	92

II.	Histidine modification	95
	A. Introduction	95
	B. Experimental procedure	95
	C. Results and discussion	96
III.	Arginine modification	99
	A. Introduction	99
	B. Experimental procedure	101
	C. Results and discussion	101
IV.	Tyrosine modification	102
	A. Introduction	102
	B. Experimental procedure	103
	1. Nitration	103
	2. Occurrence of intermolecular cross-linking	104
	C. Results and discussion	104
	1. Effects of nitration	104
	2. Occurrence of intermolecular cross-linking	107
	3. Circular dichroism	111
	3.1 Effect of Ca ²⁺	111
	3.1.1 Native modulator	111
	3.1.2 Nitrotyrosyl modulator	113
	3.2 Interaction with troponin I	116
	3.2.1 Native modulator	116
	3.2.2 Nitrotyrosyl modulator	118
V.	Triple modification: tyrosine, arginine, and histidine	121
	A. Introduction	121
	B. Experimental procedure	121
	1. Tyrosine modification	121
	2. Arginine modification	122
	3. Histidine modification	122
	C. Results and discussion	123
	1. Characterization	123
	2. Effect on PDE-stimulating activity	125
	3. Effect on troponin C-like activities	125
	3.1 Effect on Ca ²⁺ -dependent change in electrophoretic mobility	126
	3.2 Effect on interactions with troponin I	128
	4. Effect of EGTA on PDE stimulation	129
VI.	Carboxyl group modification	132
	A. Introduction	132
	B. Experimental procedure	135
	1. Determination of total free carboxyl content	135
	2. Carboxyl group modification in the native protein	135
	C. Results and discussion	136
	1. Total free carboxyl content	136
	2. Carboxyl group modification in the native protein	137
VII.	Lysine modification	145
	A. Introduction	145

1.	Carbamoylation	145
2.	Guanidination	146
B.	Experimental procedure	147
1.	Carbamoylation	147
2.	Guanidination	147
C.	Results and discussion	148
1.	Carbamoylation	148
2.	Guanidination	153
VIII.	Methionine modification	155
A.	Carboxymethylation	155
1.	Introduction	155
2.	Experimental procedure	158
2.1	Modification: effect on activity	158
2.2	Characterization	159
2.2.1	Carboxymethylation	159
2.2.2	Tryptic digestion	159
2.2.3	Autoradiography	159
2.2.4	Elution of labeled peptides	160
3.	Results and discussion	160
3.1	Effect on modulator activity	160
3.2	Urea-PAGE	162
3.3	Characterization	162
B.	Mild oxidation	166
1.	Introduction	166
2.	Experimental procedure	168
2.1	Mild oxidation	168
2.1.1	Analytical scale	168
2.1.2	Preparative scale	168
2.2	⁴⁵ Ca ²⁺ -binding studies	169
2.3	Isolation of modified cyanogen bromide peptide	170
3.	Results and discussion	171
3.1	Oxidation in the presence of Ca ²⁺	171
3.1.1	Effect on PDE-stimulating activity	171
3.1.2	Urea-PAGE	173
3.1.3	Phosphodiesterase- and Ca ²⁺ -binding properties of oxidized modulator	173
3.1.4	Effect on troponin C-like activities	178
3.1.5	Identification of oxidized methionine residues	180
3.1.6	Circular dichroism studies	183
3.1.6.1	Effect of Ca ²⁺ on CD spectra	183
3.1.6.2	CD titration studies	186
3.1.6.3	Interactions with troponin I	190
3.2	Oxidation in the absence of Ca ²⁺	191
3.2.1	Effect on PDE-stimulating activity	191
3.2.2	PAGE	194
3.2.3	Phosphodiesterase- and Ca ²⁺ -binding properties of oxidized modulator	194
3.2.4	Comparison of cyanogen bromide peptides	196