Kinetic comparison of the calcium-calmodulin sensitive cyclic nucleotide phosphodiesterases from bovine brain, heart and placental tissues.

Nadarajah, Karuppiah

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KINETIC COMPARISON OF THE CALCIUM-CALMODULIN SENSITIVE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM BOVINE BRAIN, HEART AND PLACENTAL TISSUES

BY

Nadarajah Karuppaiah

A DISSERTATION

Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada

1987
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ABSTRACT

The selective adsorption of the anthraniloyl- (ANT) and methylantraniloyl-
(MANT) \( \text{S}^\text{'} \)-derivatives of adenosine monophosphate (AMP) and guanosine
monophosphate (GMP) onto diethylaminoethyl- (DEAE) Sephadex, in the presence
of zinconyl chloride, was adapted to the direct fluorescent discontinuous assay
of cyclic nucleotide phosphodiesterases (PDEs). In this assay the enzyme is
incubated with either of the 2'-0-anthraniloyl and 2'-0-methylantraniloyl cyclic
AMP or cyclic GMP derivatives. After quenching of the reaction, zinconyl chloride
is added and the product is separated from the substrate on a column (0.4 mL)
of DEAE-Sephadex. The product is then eluted with sodium chloride (2M) and
quantitated spectrophotometrically.

Calmodulin-dependent cyclic nucleotide phosphodiesterases from bovine
brain, heart and placenta, purified either by calmodulin-Sepharose or monoclonal
affinity chromatography, have been compared with respect to their steady-state
kinetic parameters \( K_m \) and \( V_{\max} \) for the hydrolysis of the fluorescent
2'-0-anthraniloyl- or 2'-0-methylantraniloyl- derivatives of cAMP and cGMP.

The calmodulin-Sepharose purified enzymes from all the tissue sources displayed
the catalytic properties of the "high \( K_m \)" phosphodiesterases. The effect of
calmodulin on the kinetic parameters was substrate dependent: with cAMP, \( K_m \)
was lowered (1.65-2.5 fold) and \( V_{\max} \) increased (2.5-4 fold). However, with cGMP
as the substrate, calmodulin increased both the \( K_m \) (1.65-2.5 fold) and \( V_{\max} \) (2.5-4
fold).

The calmodulin-phosphodiesterase conformation specific monoclonals (1)
enabled the isolation of 63 Kd and 60 Kd isozymes from the brain as well as
57-59 Kd species from the heart and the placenta. In addition, the use of the
two derivatives of cGMP and cAMP permitted the detection of subtle kinetic differences between the phosphodiesterases. All the enzymes displayed the lowest $K_m$ for 3'-O-methylothraniloyl-cGMP. The largest variation in $K_m$ values between the phosphodiesterases was observed with 3'-O-anthraniloyl-cAMP, the $K_m$ of values of 133 $\mu$M and 156 $\mu$M were obtained for the brain 60 Kd and the placenta enzymes respectively, whereas the heart and the brain 63 Kd enzymes displayed $K_m$s that were approximately 3-fold lower. The 2'-O-methylothraniloyl- derivative of cAMP yielded $K_m$s that were approximately 3-fold lower for the brain 60 Kd and the placenta enzymes, whereas that of the brain 63 Kd and the heart enzymes increased slightly (1.2-1.5 fold). In terms of $V_{max}$, all the enzymes studied displayed comparable values with 2'-methylothraniloyl-cGMP as the substrate. The brain 63 Kd isozyme had larger $V_{max}$ values than the 60 Kd isozyme with all four pseudo substrates. The largest $V_{max}$s were obtained with 2'-anthraniloyl-cGMP. These results are discussed in terms of differences in the catalytic sites of the enzymes.

A comparison of the catalytic properties of calmodulin-Sepharose and monoclonal affinity purified enzymes indicate that the enzymes obtained by the two methods are kinetically distinct. The heart and brain PDEs purified by CI monoclonal affinity were found to differ in the oncomodulin dose dependant activation of cAMP hydrolysis. Oncomodulin was shown to activate the heart enzyme to the same extent as calmodulin. The study indicates that the heart phosphodiesterase has approximately 25-fold higher affinity for oncomodulin than the brain enzyme. These results suggest significant differences between the heart and brain enzymes with respect to activator protein-binding domains.

Bovine heart and placenta-PDE purified by calmodulin-Sepharose have been demonstrated with respect to their stimulation by the monomer and dimer form
of recombinant oncomodulin. The heart enzyme had a 10-fold higher affinity for the dimer form with 2'-O-anthrancyl-cGMP as substrate. The steady state kinetic parameters $K_m$ and $V_{max}$ for the hydrolysis of the fluorescent 2'-O-anthrancyl-cGMP have also been compared to the effect of calmodulin and recombinant (monomer and dimer) oncomodulin.
DEDICATION

To My Family
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my heartfelt appreciation and gratitude to Dr. B. Mutus my research advisor, for his continuous guidance and encouragement in directing the present study to its understanding.

I would also like to thank other members of my committee: Dr. D. Thomas, Dr. N.F. Taylor and Dr. E.L. Stephan for their cooperation and advice given to me throughout my entire work.

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This work has been supported by The Natural Sciences and Engineering Research Council (Canada) and the University of Windsor.
INTRODUCTION

1. Historical Background

2. Postulated Role of Cyclic Nucleotides

3. Multiple Molecular Forms of Phosphodiesterases
   A. cGMP-Binding Phosphodiesterase
      i. Light-Activated Cyclic GMP Phosphodiesterase
   B. "Km = Lpm" Cyclic AMP Phosphodiesterases
   C. "Lo = Km" 3',5'-Cyclic AMP and Cyclic GMP Phosphodiesterases
   D. Ca²⁺/calmodulin Dependent Phosphodiesterase

4. Activator Proteins
   A. Calmodulin: The Ubiquitous Ca²⁺-Dependent Regulatory Protein
      i. Calmodulin Dependent Enzymatic and Cellular Processes
      ii. Calmodulin Binding Domain

B. Onconudulin

5. Cyclic Nucleotide Derivatives as Probes for Phosphodiesterases
   Catalytic and Regulatory Sites

6. Methods of Isolation and Purification of Phosphodiesterase

7. Phosphodiesterase Assay Methods

8. Summary of Objectives

(ix)
MATERIALS AND METHODS

1. Materials
2. Apparatus
3. Reagents
4. Methods

A. Synthesis of Fluorescent Analogs
B. Determination of Standard Curve with Authentic ANT-AMP, MANT-AMP, ANT-GMP and MANT-GMP
C. Direct Enzymatic Fluorescent Assay
D. Purification of Calmodulin
E. Preparation of Calmodulin-Sepharose 4B
F. Preparation of Affi-Blue Sepharose 4B
G. Preparation of Monoclonal Affinity Columns with ACC-1 (antibody directed against bovine calmodulin) and ACAP-1 (antibody against bovine heart calmodulin activated phosphodiesterase)

H. Purification of Bovine Brain, Heart and Placental Phosphodiesterase by Calmodulin Sepharose Affinity Chromatography
I. Purification of Bovine Brain and Heart Phosphodiesterase Using ANTI-Phosphodiesterase Monoclonal Antibody Affinity Chromatography
J. Separation of Phosphodiesterase from Calmodulin Using Organomercurial Sepharose Affinity Chromatography
L. Assay of Cyclic Nucleotide Phosphodiesterase
M. Gel Electrophoresis
N. Protein Estimation
RESULTS

1. Characterization of the Fluorescent Cyclic Nucleotide Derivatives

2. Development of a Direct Discontinuous Fluorescent Assay


5. Investigations of the Kinetic Effects of Reduced Recombinant Oncomodulin (r-ONC) and Oxidized recombinant Oncomodulin (r-ONC-d) on heart and placenta cyclic Nucleotide Catalyzed Hydrolysis of ANT–cGMP

6. Dose–dependant Activation of Heart and Placenta Phosphodiesterases by Recombinant Oncomodulin and Calmodulin

7. Differential Stimulation of Bovine Brain and Heart Cyclic AMP Phosphodiesterases by Oncomodulin

(xii)
LIST OF FIGURES

Figure 1  Sequence of Calmodulin  13
Figure 2  Stereo drawing of a-C backbone of calmodulin  16
Figure 3  Comparison of the aminoacid sequence of rat parvalbumin  21
Figure 4  Chemical structures of cAMP and cGMP derivatives  24
Figure 5  Structures of ANT and MANT derivatives of cAMP and cGMP  26
Figure 6  Absorption spectra of cyclic-AMP and AMP derivatives  47
Figure 7  Absorption spectra of cyclic-GMP and GMP derivatives  49
Figure 8  Fluorescence emission spectra of cyclic-AMP and AMP derivatives  51
Figure 9  Fluorescence emission spectra of cyclic-GMP and GMP derivatives  53
Figure 10 Titration curve of 1:1 mixture of ANT-AMP/ANT-cAMP with zirconyl chloride  55
Figure 11 Elution profile of ANT-AMP and ANT-cAMP on DEAE-Sephadex  57
Figure 12 Speculative structure of citrate-Zr4+-ANT-AMP complex  59
Figure 13 Standard curve of ANT-AMP obtained with 8:2 mixture of ANT-cAMP and ANT-AMP  61
Figure 14 Rate of hydrolysis of ANT-cAMP by bovine brain cyclic nucleotide phosphodiesterase  64
Figure 15 Standard curves of AMP derivatives obtained by the modified procedure

Figure 16 Standard curves of GMP derivatives obtained by the modified procedure

Figure 17 SDS Page electrophoresis of calmodulin-Sepharose affinity column purified enzymes

Figure 18 Plots of enzyme velocity as a function of substrate concentration with bovine brain cyclic nucleotide phosphodiesterase purified by calmodulin-Sepharose affinity chromatography

Figure 19 Plots of $v^{-1}$ versus $s^{-1}$ of the rates of hydrolysis of MANT-cAMP and MANT-cGMP by bovine cyclic nucleotide phosphodiesterase

Figure 20 Plots of $v^{-1}$ versus $s^{-1}$ on the rates of hydrolysis of MANT-cAMP and MANT-cGMP by bovine heart cyclic nucleotide phosphodiesterase

Figure 21 Plots of $v^{-1}$ versus $s^{-1}$ on the rates of hydrolysis of MANT-cAMP and MANT-cGMP by bovine placenta cyclic nucleotide phosphodiesterase

Figure 22 SDS PAGE gel electrophoresis of Monoclonal (ACAP-1 and ACC-1) affinity column purified enzymes
Figure 23 Double reciprocal plots of velocity as a function of substrate
(ANT-cAMP and MANT-cAMP) concentration with bovine brain
(60-61) Kd cyclic nucleotide phosphodiesterase purified by
monoclonal affinity chromatography

Figure 24 Double reciprocal plots of velocity as a function of substrate
(ANT-cGMP and MANT-cGMP) with bovine brain (60-61) Kd cyclic
nucleotide phosphodiesterase purified by monoclonal affinity
chromatography

Figure 25 Double reciprocal plots of velocity as a function of substrate
(ANT and MANT-cGMP) with 63 Kd bovine brain cyclic
nucleotide phosphodiesterase purified by monoclonal affinity
chromatography

Figure 26 Double reciprocal plots of velocity as a function of substrate
(ANT and MANT-cAMP) concentration with bovine heart cyclic
nucleotide phosphodiesterase purified by monoclonal affinity
chromatography

Figure 28 Double reciprocal plots of velocity as a function of substrate
(ANT and MANT-cAMP) concentration with bovine heart cyclic
nucleotide phosphodiesterase purified by monoclonal affinity
chromatography
Figure 29 Double reciprocal plots of the velocity as a function of substrate (ANT-cAMP and MANT-cAMP) concentration with the bovine placenta cyclic nucleotide phosphodiesterase

Figure 30 Double reciprocal plots of the velocity as a function of substrate (ANT-cGMP and MANT-cGMP) concentration with the bovine placenta cyclic nucleotide phosphodiesterase

Figure 31 SDS PAGE analysis of reduced and oxidized recombinant oncomodulin from rat hepatoma

Figure 32 The effect of calmodulin on bovine heart cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

Figure 33 The effect of reduced recombinant oncomodulin on bovine heart cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

Figure 34 The effect of oxidized recombinant oncomodulin on bovine heart cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

Figure 35 The effect of calmodulin on bovine placenta cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

Figure 36 The effect of reduced recombinant oncomodulin on bovine placenta cyclic nucleotide phosphodiesterase activity with ANT-cGMP as substrate
Figure 38 Differential stimulation of calmodulin-Sepharose affinity purified bovine heart cyclic nucleotide phosphodiesterase by reduced and oxidized recombinant oncomodulin

Figure 39 The effect of reduced concentrations recombinant oncomodulin with calmodulin-Sepharose affinity purified bovine placenta phosphodiesterase

Figure 40 The effect of calmodulin concentrations on the stimulation of heart and placenta phosphodiesterase purified (ACAP-1) by monoclonal (ACAP-1) affinity column chromatography

Figure 41 SDS PAGE gel electrophoresis of bovine brain and heart enzymes purified by monoclonal (C1) affinity column chromatography

Figure 42 The reverse HPLC purification of oncomodulin free of calmodulin contamination

Figure 43 Use of sulphydryl affinity chromatography to obtain oncomodulin free of possible contamination

Figure 44 Similar stimulation of bovine heart phosphodiesterase by oncomodulin purified by reverse phase HPLC and organomercurial resin

Figure 45 Differential stimulation of brain and heart phosphodiesterase by oncomodulin

Figure 46 Bar graphs comparing the kinetic constants of bovine phosphodiesterases in the presence and absence of calmodulin.
Figure 47 Bar graph comparing the kinetic constants of monoclonal affinity purified bovine phosphodiesterases. Upper row display $K_m$s. Lower row display $V_{max}$s.

Figure 48A Model for the interactions of cGMP with the A site of the cyclic nucleotide phosphodiesterase. (A) Part of an amino acid functioning as a hydrogen donor; (B) part of an amino acid functioning as a hydrogen acceptor, x: positively charged amino acid side-chain.

Figure 48B Model for the possible intramolecular H-bonding between the nitrogen and the delocalized negative charge of cyclic phosphate.
LIST OF TABLES

Table 1 Multiple forms of cyclic nucleotide phosphodiesterase

Table 2 Calmodulin-dependent enzymes and calmodulin regulated cellular processes

Table 3 Methods of purification of phosphodiesterase

Table 4 Rf values of fluorescent cyclic nucleotide derivatives

Table 5 Absorption and fluorescent properties of cyclic nucleotide derivatives

Table 6 Purification of bovine placenta phosphodiesterase

Table 7 Calmodulin activation of CaM-Sepharose affinity purified phosphodiesterases

Table 8 Effect of calmodulin on the kinetic parameters of cyclic nucleotide phosphodiesterases

Table 9 Specific activities of ACAP-1, ACC-1 monoclonal affinity column purified cyclic nucleotide phosphodiesterases

Table 10 Kinetic constants of bovine brain, heart and placenta phosphodiesterase purified by monoclonal affinity column chromatography

Table 11 Kinetic constants of the effect of calmodulin/calmodulin on heart and placenta phosphodiesterases

Table 12 Heart and placenta phosphodiesterase activities of CaM-Sepharose fraction ACAP-1 eluate breakthrough fraction ACAP-1
Table 13. The Effect of Anti-CaM IgG on Calmodulin and Oncomodulin
Stimulated Heart-PDE Activity

Table 14. Kinetic Parameters for the PDE cited in the literature
LIST OF APPENDICES

Appendix

I. Steady-state kinetic parameters
II. Definition of Rf
III. Diagrammatic representation of the semi-automated kinetic analysis of phosphodiesterases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANT</td>
<td>anthraniloyl</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cNMP</td>
<td>cyclic nucleotide monophosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol</td>
</tr>
<tr>
<td>Kd</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>MANT</td>
<td>methylaniloyl</td>
</tr>
<tr>
<td>max</td>
<td>maximum</td>
</tr>
<tr>
<td>min</td>
<td>minimum</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>Mr</td>
<td>relative molecular weight</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>mM</td>
<td>millimetre</td>
</tr>
<tr>
<td>ONC</td>
<td>oncomodulin</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methane sulfonyl fluoride</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>r-ONC</td>
<td>recombinant oncomodulin</td>
</tr>
</tbody>
</table>
TEMED

TLCK

TPCK

μg

μM

N,N,N',N'-Tetramethylethylenediamine

N-α-P Tosyl-L-lysine Chloromethyl ketone

L-1-Tosylamide-2-phenyl-Ethylchloromethylketone

microgram

micromolar
INTRODUCTION

Enzymes are proteins specialized to catalyze biological reactions. They are among the most remarkable biomolecules known because of their extraordinary specificity and catalytic power, which are far greater than those of man-made catalysts.

1. Historical Background

The enzyme phosphodiesterase (E.C. 3.1.4.17) which catalyzes the hydrolysis of 3',5'-monophosphate nucleosides (cyclic AMP and cyclic GMP) was originally discovered by Sutherland and Rall (1,2). Since then the existence of this enzyme has been reported in a wide variety of eukaryotic cells and tissues (3-10).

Cyclic AMP was discovered during studies of hormonal regulation of glycogenolysis in the liver by Rall and Sutherland in 1957 (2,11). They elucidated the profound effect of hormones which can raise the levels of cyclic AMP in different tissues and also the high affinity binding proteins of cyclic AMP (cyclic AMP-dependent protein kinase and phosphodiesterase).

2. Postulated Role of Cyclic Nucleotides

It is well established that many hormones (e.g. epinephrine, norepinephrine) and neurotransmitters (e.g. acetylcholine, norepinephrine) exert their action on target cells by elevating the intracellular concentration of cyclic nucleotides (cyclic AMP and cyclic GMP). This is achieved by increasing the activity of cyclic nucleotide cyclase (adenylate cyclase and guanylate cyclase) which catalyzes the formation of 3',5'-nucleosides (3',5'-cyclic AMP and 3',5'-cyclic GMP) from adenosine and guanosine triphosphate (ATP and GTP) as shown below (Eqn. 1).
The cyclic AMP (secondary messenger) formed mediates various cellular metabolic pathways (Scheme 1). In the liver, epinephrine stimulates glycogen breakdown and inhibits glycogen synthesis. The epinephrine released by the adrenal medulla binds to the liver cell and stimulates adenylate cyclase to form cyclic AMP.

Cyclic AMP then promotes a chain of events causing the breakdown of glycogen to yield glucose. As long as epinephrine is present, liver adenylate cyclase remains activated maintaining cyclic AMP levels. However, if the secretion of epinephrine stops, adenylate cyclase is no longer stimulated, thus preventing the formation of cyclic AMP. The remaining cyclic AMP is then destroyed by phosphodiesterase as shown below (Eqn. 2).

\[
\text{3',5'-cAMP} \xrightarrow{\text{Mg}^{2+} \text{Phosphodiesterase}} \text{5'-AMP}
\]

As a result, the cell returns to its resting stage. Thus, it can be seen that cyclic nucleotide cyclases and phosphodiesterases, which are fundamental components of the intracellular regulatory system, act in concert to determine the intensity and duration of cyclic nucleotide response.

Since the discovery of cyclic AMP by Sutherland and Rall (2,11) there has been continued interest in the possible involvement of cyclic AMP with other cellular events. It has been implicated with ion transport (8,12), muscular contraction (9,12) and enzyme synthesis (13).

Cyclic nucleotides have also been reported in maintaining the normal cell phenotype (14,15). The specific manner in which they act is not yet clear. Cyclic AMP concentrations have been observed to be very high with respect to cyclic GMP in certain systems. This difference is believed, by many investigators, to inhibit abnormally rapid cell proliferation (16,17). On the other hand, high levels
Scheme 1: Interrelation of the two intracellular messenger systems is shown by this diagram. PDE (Phosphodiesterase), PG (prostaglandin) (165).
Scheme 1

NEUROTRANSMITTER OR HORMONE

ADENYLIATE CYCLASE

CYCLIC AMP

PROTEIN KINASE

ATP

5'-AMP

PDE

PROTEIN KINASE

ATP

CALMODULIN

EFFECOR ENZYMES

SIOCHEMICAL RESPONSE

Nerve impulse or other stimulus

CELL MEMBRANE

Ca2+

CALMODULIN

EFFECOR ENZYMES

SIOCHEMICAL RESPONSE
of cyclic GMP to cyclic AMP enhances cell proliferation (15,19,19). From such observations it has been proposed that an increase in the cyclic AMP to cyclic GMP ratio inhibits cell division whilst an elevation in the cyclic GMP to cyclic AMP ratio promotes cell proliferation. In fact a change in the concentration of the two cyclic nucleotides in favour of cyclic GMP has been reported in the expression of the malignant phenotype (20-24) and in the hyperproliferative disease state of psoriasis (25).

Thus it can be seen that cyclic nucleotides mediate a variety of cellular events with their formation and degradation regulated by adenylate cyclase and phosphodiesterase respectively.

3. Multiple Molecular Form of Phosphodiesterases

Since the discovery of cyclic nucleotide phosphodiesterase in tissue extracts and with the advent of different isolation techniques, it is quite clear that a number of different enzyme forms exist. These multiple forms are being distinguished on the basis of substrate specificity, electrophoretic chromatographic behaviour and responses to effectors (Table 1).

A. cGMP-Binding Phosphodiesterase

Phosphodiesterase which binds cGMP with high affinity are classified into three categories having different kinetic, regulatory and physical properties.

1. Light-Activated Cyclic GMP Phosphodiesterase

The light activated phosphodiesterase found in the outer rod segments of the retina has been purified to homogeneity from both amphibian and bovine retina (26,27). Electrophoretic analysis of this form purified from frog and bovine eye shows a doublet of MW 108,000–110,000 daltons and 84,000–88,000 daltons.
Table 1. Multiple forms of cyclic nucleotide phosphodiesterases

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Monomer</th>
</tr>
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<tbody>
<tr>
<td>[Table not visible]</td>
<td>mol wt.</td>
</tr>
<tr>
<td></td>
<td>Km (μM)</td>
</tr>
<tr>
<td></td>
<td>Vmax (μmoles/min^-1mg^-1)</td>
</tr>
<tr>
<td></td>
<td>Km (μM)</td>
</tr>
<tr>
<td></td>
<td>Vmax (μmoles/min^-1mg^-1)</td>
</tr>
<tr>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>3.A. cGMP-binding cyclic nucleotide phosphodiesterases</td>
<td></td>
</tr>
<tr>
<td>i) Light-activated cGMP phosphodiesterase</td>
<td></td>
</tr>
<tr>
<td>a) bovine eye 84/88000</td>
<td>200</td>
</tr>
<tr>
<td>b) frog eye 108/110000</td>
<td></td>
</tr>
<tr>
<td>ii) cGMP-stimulated</td>
<td></td>
</tr>
<tr>
<td>a) bovine adrenal 105000</td>
<td>40</td>
</tr>
<tr>
<td>b) rat liver (cystolic)</td>
<td>40</td>
</tr>
<tr>
<td>(particulate)</td>
<td>34</td>
</tr>
<tr>
<td>iii) Platelet and lung type</td>
<td>1</td>
</tr>
<tr>
<td>3.B. &quot;Low Km&quot; cyclic AMP phosphodiesterase</td>
<td></td>
</tr>
<tr>
<td>a) Insulin-sensitive 52000</td>
<td>0.7</td>
</tr>
<tr>
<td>b) cAMP-specific 60,000</td>
<td>2.2</td>
</tr>
<tr>
<td>3.C. &quot;Low Km&quot; cAMP and cGMP phosphodiesterases</td>
<td>106,000</td>
</tr>
</tbody>
</table>
### 3. D. Ca\(^{2+}\)/calmodulin-stimulated phosphodiesterase

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>a) Heart</td>
<td>59,000</td>
<td>220</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>b) Brain</td>
<td>(i) 64,000</td>
<td>150</td>
<td>300</td>
<td>2.5-2.8</td>
</tr>
<tr>
<td></td>
<td>(ii) 60,000</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Ca(^{2+})/CaM</td>
<td>28.0-38.5</td>
<td>166.0</td>
<td>2.5-2.8</td>
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<tr>
<td></td>
<td>EGTA</td>
<td>68.0-70.0</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(iii) 63,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca(^{2+})/CaM</td>
<td>10.0-12.5</td>
<td>10.0</td>
<td>1.1-1.3</td>
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<tr>
<td></td>
<td>EGTA</td>
<td>66.0-70.0</td>
<td>1.7</td>
<td>6.0-7.0</td>
</tr>
<tr>
<td>c) &quot;Low Km&quot;</td>
<td>~2</td>
<td></td>
<td></td>
<td>~1</td>
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</table>
respectively. Evidence has been presented to indicate that the high affinity cyclic GMP binding site is distinct from the catalytic site (28). The activity of the enzyme has been shown to be controlled by the degree of bleaching of rhodopsin. The mechanism by which light-activated rhodopsin causes an increase in phosphodiesterase activity is not yet clear.

iii. cGMP-Stimulated Cyclic Nucleotide Phosphodiesterases

This category of enzymes are characterized by low concentrations of cyclic GMP being able to stimulate the hydrolysis of cyclic AMP (29,30). Cyclic GMP is believed to achieve this effect by interacting at a regulatory site, specific for cyclic GMP, which is distinct from the substrate binding site. The enzyme isolated and purified from bovine adrenal tissue showed a single band of about 105,000 MW, while that isolated from rat liver revealed a dimer of 134,000 MW employing SDS gel electrophoresis (30). The pure forms of the enzyme catalyze the hydrolysis of both cyclic AMP and cyclic GMP with a Km of 40 and 10 μM for the bovine adrenal tissue. The enzymes isolated from rat liver (the particulate and the cytosolic) had similar kinetic constants Km = 34 μM and Km = 40 μM, respectively.

iii. cGMP-Binding Phosphodiesterases from Platelet and Lung

The enzyme appears to be the major type of cyclic GMP binding protein in platelets (41). Except for the distinction between the catalytic and cyclic GMP binding site, little is known about this enzyme form (41). In the lung tissue, the major receptors for cyclic GMP are cyclic GMP-dependent protein kinase and a cyclic GMP-binding phosphodiesterase. It appears to be similar or identical to the platelet enzyme (42). At low concentrations of cyclic GMP the enzyme does not increase the rate of cAMP hydrolysis. The Km is 4 μM for cyclic GMP
and it is immunologically shown that this form of the enzyme is different from that of the bovine adrenal, heart or the outer rod segment (43).

B. "Low Km" Cyclic AMP Phosphodiesterases

This low Km cyclic AMP or "negative cooperative" form of enzyme has been reported in many mammalian tissues (44-48). It has a high affinity for cAMP in the range (10^{-7} to 10^{-6}M) and about 100-200 μM for cyclic GMP. The enzyme has been reported to be particulate in nature in many tissues.

It has been shown that the particulate forms are very susceptible to proteolysis. Thompson et al (44) originally described the soluble phosphodiesterase from dog kidney having high specificity for cAMP. The protein had a molecular weight of about 60,000 daltons with a Km of 2.2 μM and a V_max of 0.09 μMoles/min/mg. It was suggested that perhaps the enzyme had low intrinsic velocity or had been subjected to proteolysis during purification. An enzyme prepared from rat liver membranes, having the same substrate specificity has a higher V_max for cAMP (~ 9 μMoles/min/mg) (45). Particularly interesting is that it can undergo insulin and cAMP-dependent phosphorylation with subsequent increase in the V_max. Recently, it was shown that cAMP specific phosphodiesterases of fat cells reveal two peaks of activity when fractionated using DEAE-cellulose chromatography; however only one of the peaks is increased in its activity by insulin.

C. "Low Km" 3',5'-Cyclic AMP and Cyclic GMP Phosphodiesterases

This type has been recently reported in a variant cell line (X30a) of 549 lymphoma cells (49). The Km reported for these enzymes are many times lower than the Km for other phosphodiesterases. They are on the order of 100 and 40 nM for cGMP and cAMP respectively.
D. Ca$^{2+}$/calmodulin Dependent Phosphodiesterase

This form of the enzyme has been extensively investigated in heart and brain tissues (3,50–56,58–61). It has two classes of binding sites, the regulatory and the catalytic. The activation of the enzyme is mediated by calmodulin which binds allosterically at the regulatory site of the enzyme (51,62–63). The native enzyme is thought to be a dimeric species capable of binding a calmodulin molecule per subunit.

Originally, it was thought that only one form of the calmodulin dependent phosphodiesterase existed in mammalian tissues. Recently, different molecular forms of the enzyme have been detected in bovine brain, 60000 MW and 63000 MW (64) and bovine heart 59000 MW (65,66). The brain enzymes which have 60 kDa and 63 kDa, appear to be different with respect to $K_m$, $V_{max}$ and their ability to act as substrates for protein kinase, their affinity towards calmodulin in the phosphorylated form and their rates of dephosphorylation by calcineurin phosphatase (65). The brain and heart enzymes have only been compared with respect to their affinity for calmodulin (66). The heart enzyme was shown to have an affinity ($0.1$ nM) ~ 10 fold higher than either of the brain isoforms. These enzymes have yet to be compared kinetically.

Another form of the calmodulin dependent phosphodiesterase has been reported in the chicken heart muscle, liver membrane and bovine lung tissue. This has relatively high affinity for cAMP (low $K_m$) (67–69). No cross reactivity of this form has been noted with the monoclonal antibody raised to the major heart or brain calmodulin dependent phosphodiesterase (70).
4. Activator Proteins

A. Calmodulin: The Ubiquitous Ca\textsuperscript{2+}-Dependent Regulatory Protein

Calmodulin was originally discovered by Cheung (71) as an activator of cyclic nucleotide phosphodiesterase. The protein is one member of a family of low molecular weight acidic calcium binding proteins (72). It has no intrinsic enzymatic properties but an extensive body of evidence supports the premise that Ca\textsuperscript{2+}-calmodulin is an intracellular regulator of diverse physiological processes (62). It is now established that calmodulin is widely distributed, found in mammalian tissues (62,71,73-76), in the one-cellular organisms (77-78), invertebrates (79-81), pea plants, mungbeans, wild carrots (82), fungus (83) and in cancerous tissues (84-86).

The Ca\textsuperscript{2+}-binding protein calmodulin is a small, heat stable, acidic polypeptide. It contains four Ca\textsuperscript{2+}-binding sites with affinities for Ca\textsuperscript{2+} in the micromolar range, and mediates a large number of enzyme activities. Bovine brain calmodulin was the first chemically homogenous calmodulin purified and made available for its complete amino-acid sequence (76). Bovine brain calmodulin protein contains 148 amino-acid residues and has a molecular mass of 16,700 daltons (Fig. 1). The amino-acid sequence of bovine brain calmodulin have been compared to amino-acid sequences of calmodulin isolated from other sources. Invertebrate calmodulin have approximately seven differences in their amino acid sequence while the protozoan has approximately twelve differences from the bovine brain sequence. The only amino-acid sequence known for a plant calmodulin is from spinach (87). This plant calmodulin has approximately eight differences in its amino acid sequence and particularly interesting is the presence of a cysteine residue at position 26.
Figure 1 Sequence of calmodulin: The sequence of bovine brain calmodulin is shown utilizing the one-letter code for amino acid residues. A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr. The four proposed Ca$^{2+}$-binding domains with the stretches of $\alpha$-helix (darker circles) are indicated. Domains 3 and 4 contain the two tyrosyl residues; the single trimethyllysyl residue lies between domains 3 and 4. Domain 3 also contains the single histidyl residue. Note the homology especially between domains 1 and 3 and between domains 2 and 4 (76).
Recently, Babu et al (88) determined the three dimensional crystal structure of rat testes calmodulin. They describe the protein as consisting of two globular lobes connected by a long eight-turn α helix and no contacts between the lobes. Each of the lobes bind two calcium ions as illustrated in Figure 2. The calcium binding segments consist of residues 20-31, 56-67, 93-104 and 129-140, with each binding segment containing twelve residues.

The molecular mechanism of calmodulin activation was first elucidated with phosphodiesterase (89-92). It has been proposed that the mechanism of phosphodiesterase activation involves the binding of Ca^{2+} to calmodulin. This induces a conformational change towards a more helical structure, which in turn associates with the enzyme to form the Ca^{2+}-activated calmodulin enzyme complex (Scheme 2).

\[
\text{Ca}^{2+} + \text{CaM}_{\text{inactive}} \xrightarrow{\text{iii}} \text{Ca}^{2+}\cdot\text{CaM}^*_{\text{active}}
\]
\[
\text{Enz}_{\text{inactive}} + \text{Ca}^{2+}\cdot\text{CaM}^*_{\text{active}} \xrightarrow{\text{iv}} \text{Ca}^{2+}\cdot\text{CaM}^*\cdot\text{Enz}_{\text{activated}}
\]

From the above scheme, it is evident that the activity of phosphodiesterase is dependent upon the presence of calcium ions in the cell. Calmodulin is activated when the intracellular Ca^{2+} reaches and exceeds micromolar concentrations. But as soon as Ca^{2+} returns to a steady state level via cell membrane extrusion or mitochondrial uptake, the active calmodulin enzyme dissociates thus decreasing enzyme activity to its basal level.

1. **Calmodulin Dependent Enzymatic and Cellular Processes**

The widespread occurrence of calmodulin which lacks tissue and species specificity and high concentrations of calmodulin found therein implicates its fundamental role in cellular function.
Figure 2  Stereo drawing of the α-C backbone of calmodulin. The four Ca ions are represented by dotted circles (88).
As an intracellular receptor of Ca\(^{2+}\), calmodulin is a potential regulator of the rate of cyclic nucleotide degradation by its action on cyclic nucleotide phosphodiesterase (93–95). Various calmodulin-dependent enzymes and cellular processes regulated by calmodulin are summarized in Table 2.

ii. Calmodulin Binding Domain

The calmodulin-dependent enzymes are thought to contain a common site for interacting with calmodulin (CaM). This postulate is based on two lines of experimental evidence. First, these enzymes have similar mechanisms of calmodulin-dependent activation (112). Second, several of these enzymes have been shown to interact with the same monoclonal antibody form (113). Significant progress has been made in identifying the structural features of this so-called "CaM-binding domain" with the discovery that CaM interacts with a variety of small peptide hormones and toxins, in a Ca\(^{2+}\)-dependent manner, with equal or greater affinity than observed with target enzymes (114–116). In addition, these peptides appear to interact at the CaM target protein-binding domain as they competitively block target-protein binding. Several of these studies have used mellitin or mastoparan which are peptide components of bee and wasp venoms respectively. These peptides which interact with CaM with KDs in the nM range have common structural features: an \(\alpha\)-helical region ~ 15 nm in length of target protein which is basic and amphiphilic. Hence, the CaM-binding domain would likely possess these minimum structural features. A further implication of these results is that the target protein binding domain of CaM is acidic and amphiphilic, likely comprising a region which extends from the C-terminal lobe to mid-section of the \(\alpha\)-helical segment connecting the C- and N-terminal lobes (117).
<table>
<thead>
<tr>
<th>Regulatory Processes</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Activates adenylate cyclase</td>
<td>Reference 96-98</td>
</tr>
<tr>
<td>Activates Ca$^{2+}$-ATPase</td>
<td>Reference 99,100</td>
</tr>
<tr>
<td>Activates myosin light chain kinase</td>
<td>Reference 101-102</td>
</tr>
<tr>
<td>Activates NAD kinase</td>
<td>Reference 103</td>
</tr>
<tr>
<td>Activates guanylate cyclase</td>
<td>Reference 104</td>
</tr>
<tr>
<td>Activates glycogen synthase kinase</td>
<td>Reference 105</td>
</tr>
<tr>
<td>Enhances microtubule assembly</td>
<td>Reference 106</td>
</tr>
<tr>
<td>Regulates the synthesis and release of neurotransmitter</td>
<td>Reference 107-109</td>
</tr>
<tr>
<td>Regulation of glycogen breakdown</td>
<td>Reference 110</td>
</tr>
<tr>
<td>Regulation of Ca$^{2+}$ fluxes</td>
<td>Reference 111</td>
</tr>
</tbody>
</table>
B. Oncomodulin

Oncomodulin was first detected and reported by MacManus (118,119) in rat Morris hepatomas. It has since been detected in solid tumors of pig, mice, human neoplastically transformed cells in vitro, human and rodent placenta (120-124). It is a 11,700 MW acidic calcium binding protein containing 100 amino-acid residue and is not a fragment of CaM. It binds two moles of calcium per molecule with very high affinity.

The primary sequence of oncomodulin (ONC) is similar to that of β-parvalbumins with nearly 100% sequence homology in the two Ca^{2+}-binding domains and 50% overall sequence homology (125,126) (Figure 3). The physiological role of ONC is as yet, unclear. However, several lines of evidence suggest a CaM-like Ca^{2+}-dependent regulatory role of this tumor protein. These include the stimulation of DNA synthesis in Ca^{2+} starved non-neoplastic liver cells, the activation of nuclear protamine kinase (127) and rat heart cyclic nucleotide phosphodiesterase (128). Oncomodulin exhibits a calcium-specific conformational change and can mimic calmodulin-like property (130). Recently, Mutus and Flohr (130A) observed that ONC can dimerize in vitro via its Cys-18 thiol. The disulphide-linked dimer ONC appears to possess a more CaM-like target-protein binding domain in comparison to ONC as determined by a mellitin displacement assay. ONC and ONC-dimer have yet to be compared to CaM with respect to their dose-dependance and kinetic effects in the regulation of cyclic nucleotide phosphodiesterase.
Figure 3 Comparison of the amino acid sequence of rat parvalbumin with rat oncomodulin. Amino acid residues (A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr). Solid boxes indicate identity of parvalbumin with oncomodulin. The coordinates X,Y,Z,−Y,−X, and −Z refer to the calcium-binding ligands established by Krebsinger (125).
Cyclic Nucleotide Derivatives As Probes for Phosphodiesterase Catalytic and Regulatory Sites

The existence of multiple forms of phosphodiesterases raise the question of whether these enzymes have unique binding or catalytic and allosteric sites. In order to gain an insight to the existence of distinct activating and hydrolytic sites on the phosphodiesterase many investigators have reported the use of native and derivatized cyclic nucleotides (131-134) (Figure 4). The demonstration of different sites could provide convenient pharmacological tools to study their role in the intact cell. Kesbeke et al (133) demonstrated the existence of two distinct sites on cGMP-stimulated phosphodiesterase isolated from Dictyostelium discoideum with cGMP derivatives. In a similar study Erneux et al (131-133) have demonstrated stronger evidence for the existence of catalytic and allosteric sites on cGMP stimulated phosphodiesterase by making a careful choice of analogues of cGMP and cAMP. Coushie et al (132) have demonstrated and compared the catalytic sites of two purified phosphodiesterase forms from mammalian tissue: the calmodulin-sensitive (bovine brain) and the cGMP-stimulated (rat liver) forms. They concluded that the catalytic sites of the two enzymes have similar domains by observing the similar and specific inhibitor potencies of the substrate analogues.

Fluorescent analogues of cAMP and cGMP have been synthesized and utilized by Hiratsuka (135) to study the bovine heart cyclic nucleotide phosphodiesterase. Although no attempt was made to use the analogs as probes for cyclic nucleotides, it has been suggested that these analogues can be used as probes to investigate the catalytic domain of cyclic nucleotide requiring enzymes (Figure 5).
Figure 4 Chemical structures of cAMP, cGMP derivatives. r-CP = ribose 3',5'-monophosphate. Left: cAMP-test kit. Mapping of H-bond interactions. Middle: cAMP/cGMP-test kit. Mapping of dipole-induced interactions. Right: cGMP-test kit. Mapping of H-bond interactions. Numbers in parentheses refer to the following compounds: (1) cAMP, adenosine 3',5'-monophosphate; (2) cGMP, guanosine 3',5'-monophosphate; (3) c6cIMP, 6-chloropurine 3',5'-monophosphate; (4) c7cAMP, 7-deazaadenosine 3',5'-monophosphate; (5) cAn3MP, 3'-amino-3'-deoxyadenosine 3',5'-monophosphate; (6) cAn5MP, 5'-amino-5'-deoxyadenosine 3',5'-monophosphate; (7) c7cGMP, 7-deazaguanosine 3',5'-monophosphate; (8) dcGMP, 2'-deoxyguanosine 3',5'-monophosphate; (9) cGnp2MP, 2'-0-(2,4-dinitrophenox)-guanosine 3',5'-monophosphate; (10) cGnp3MP, 3'-amino-3'-deoxyguanosine 3'-5'-monophosphate; (11) cIMP, benzimidazole 3',5'-monophosphate; (12) cFMP, purine 3',5'-monophosphate (131).
Figure 4
Figure 5 Structures of Ant and Mant derivatives of cAMP and cGMP.
Figure 5

Ant-cAMP  R=H
Mant-cAMP  R=CH₃

Ant-cGMP  R=H
Mant-cGMP  R=CH₃
6. Methods of Isolation and Purification of Phosphodiesterase

Some of the methods of isolation and purification of phosphodiesterase are summarized in Table 3. The purification methods used by various investigators have mainly involved ion-exchange affinity, gel filtration and monoclonal affinity chromatography.

7. Phosphodiesterase Assay Methods

The most popular assay for cyclic nucleotide phosphodiesterases employ the conversion of radioactive substrates to the radioactive products which then requires the separation of the radioactive 5'-nucleotide phosphate product from the unhydrolyzed substrate. Many chromatographic procedures have been reported for this purpose (135-142). Separation of a cyclic nucleotide from the corresponding nucleoside is generally easier than separation of the corresponding 5'-monophosphate, and hence most assays incorporated the principle introduced by Butcher and Sutherland (136). This involved converting all the 5'-monophosphate product to the corresponding nucleoside with snake venom nucleotidase and subsequent separation on batch or column anion exchange resin. The batch procedures have advantages over column or paper chromatography; however, in some systems, this procedure can yield erroneous results of incomplete removal of the nucleotide, partial removal of diprophosphorylated product or both (144-149). Column anion-exchange chromatography can ensure complete removal of the unhydrolyzed cyclic nucleotide and also, can be easily automated for a continuous run (150). Selective precipitation of the substrate or product have been employed by some investigators (151-153). Krishna et al (153) employed the addition of ZnSO₄ and Ba(OH)₂ which results in the quantitative precipitation of 5'-AMP and leaving cAMP in the supernatant. Norman et al (154) monitored
Table 3: Methods of Purification of Phosphodiesterase

<table>
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</tr>
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<td>Crude Extract</td>
<td>100%</td>
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<td>DEAE-cellulose chromatography</td>
<td>52</td>
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<td>3</td>
<td>Affi-Gel Blue Chromatography</td>
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### III. Bovine Heart Muscle (39)

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### IV. Bovine Heart (38)

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<td>3</td>
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### V. Bovine Heart (65)

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<td>3</td>
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**Bovine Brain**

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the appearance of product in the precipitate. The procedure involved trapping
the precipitate on a glass fibre filter, washing the precipitate, and then counting
the radioactivity. The potential problem encountered with the precipitation, assay
is the instability of the precipitate under conditions which results in the
trapping of the unreacted nucleotide substrate in the precipitate. Thin layer
chromatography has been employed as a means to separate the product from
the substrate and does not require the use of snake venom 5'-nucleotidase (135),
however the latter technique is tedious and must be applied cautiously.

As described earlier, the snake venom nucleotidase has been employed to
generate the nucleoside from the 5'-monophosphate following hydrolysis of the
cyclic nucleotide. At this stage, equal amounts of inorganic phosphate are also
released. The amount of phosphate formed can be determined colorimetrically
by the addition of ammonium molybdate and α-naphthol sulfonic acid to form
the chromogen that can be measured at 660 nm (155).

Johnson et al (156) recently reported a direct continuous assay with the
use of fluorescent 2'-methylothraniloyl derivative of cyclic GMP (Mant-cGMP)
that undergoes a large decrease in its fluorescence as it is hydrolyzed by
phosphodiesterase. Although this derivative provides a sensitive and continuous
assay, it can only be performed with Mant-cGMP and not the other derivatives,
as Hiratsuka (135) had used in a discontinuous assay for the heart
phosphodiesterase.

8. Summary of Objectives

The bovine brain and heart Ca²⁺/calmodulin dependent phosphodiesterases
have been isolated and their physical and chemical properties determined. The
reports have indicated the discrepancy between the two enzymes in terms of
their molecular weight (58,59). More recently, Hansen and Beavo (65) have purified
three distinct forms of phosphodiesterase with the aid of monoclonal antibodies
specific to the phosphodiesterase induced conformation of Ca\(^{2+}\)-calmodulin. They
have indicated the differential affinity of heart and brain phosphodiesterase
towards calmodulin. No further work has been reported indicating the differential
properties of the enzymes. Sharma et al (58) have detected two forms of brain
phosphodiesterase, 60 kD and 63 kD and in subsequent studies with monoclonal
antibodies, have established that although the isoforms are immunologically
related, they are not identical. In fact, the isoforms were shown to be quite
distinct with respect to their kinetic properties, their affinity for calmodulin,
their ability to serve as a substrate for protein kinase, altered affinity towards
calmodulin subsequent to phosphorylation and their rates of dephosphorylation
(64). The catalytic properties of these enzymes have yet to be studied in detail.
Such studies would indicate whether these enzymes are functionally distinct
from other calmodulin-dependent phosphodiesterases in tissues other than those
of the heart and brain.

The rat heart phosphodiesterase has been found to be stimulated by
oncomodulin (the calmodulin-like modulator protein). Since oncomodulin is found
in rodent and human placenta, it is possible that the protein may also affect
the activity of bovine placenta phosphodiesterase.

It was the aim of the present study to investigate the properties of
calmodulin dependent phosphodiesterases from bovine brain, heart and a
previously unexplored source, the placenta, purified by calmodulin-Sepharose or
monoclonal affinity chromatography. The steady-state kinetic (Appendix 1)
properties are to be compared for the hydrolysis of two related cAMP and cGMP
fluorescent analogues which have been reported by Hiratsuka (135) to be useful
as substrates and probes for cyclic nucleotide phosphodiesterases.
MATERIALS AND METHODS

1 A. Materials

5'-Adenosine Monophosphate
Ammonium Sulfate
Calcium Chloride
3',5'-Cyclic Adenosine Monophosphate
3',5'-Cyclic Guanosine Monophosphate
DEAE-Cellulose
DEAE-Sephadex
Ethylene Diamine Tetraacetic Acid (EDTA)
Ethylene Glycol Bis(g-Aminoethyl ether) N,N,N',N'-Tetraacetic Acid (EGTA)
5'-Guanosine Monophosphate
β-mercaptoethanol
Molecular Weight Markers
5'-Nucleotidase EG 3.1.3.5 from Crotalus Atrox Venom
Organomercurial Sepharose
Phenylmethane sulfonyl fluoride
Sepharose 4B
Sodium Dodecyl Sulfate
L-1-Tosylamide-2-phenyl-Ethyl-Chloromethylketone (TPCK)

Supplier:
Sigma Chemical Company,
St. Louis, Missouri,
USA
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>Location</th>
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<td>Milwaukee, Wisconsin, USA</td>
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<td>Zirconyl Chloride</td>
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<tr>
<td>Isatoic Anhydride</td>
<td>BDE Chemicals</td>
<td>Toronto, Ontario, Canada</td>
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<td>Methyl Isatoic Anhydride</td>
<td></td>
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</tr>
<tr>
<td>Magnesium Acetate</td>
<td>Bio-Rad Laboratories</td>
<td>32nd &amp; Griffin, Richmond, USA</td>
</tr>
<tr>
<td>Sodium Chloride</td>
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<td>Windsor, Ontario, Canada</td>
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<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>Dr. Dennis Banville/</td>
<td>Ottawa, Ontario, Canada</td>
</tr>
<tr>
<td>Glycine</td>
<td>Biomedical Technologies</td>
<td>Cambridge, Massachusetts, USA</td>
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<tr>
<td>Anticalmodulin-IgG</td>
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<tr>
<td>Bovine Brain, Heart and</td>
<td>Dr. J.P. MacManus</td>
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<tr>
<td>Placental Tissues</td>
<td>Animal/Cell Physiology Group</td>
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<td>Division of Biological Sciences</td>
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<td>Anti-Phosphodiesterase Monoclonal Antibody (C1) Immobilized on Sepharose 4B</td>
<td>Department of Medical Biochemistry</td>
<td>Calgary, Alberta, Canada</td>
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</table>
ACC-1: monoclonal antibody directed against bovine calmodulin
ACAP-1: monoclonal antibody directed against bovine heart calmodulin activated phosphodiesterase

Dr. J.A. Beavo
Department of Pharmacology
University of Washington
Seattle, Washington, USA

All other reagents used were of analytical grade.

B. Apparatus

Econo Columns (15 x 0.9 cm) were obtained from Bio-Rad Laboratories.

Fluorescent measurements were recorded at 25°C in a Model RF 540, Shimadzu fluorescence spectrophotometer. Ultra Violet spectrophotometric measurements were performed with a Shimadzu UV 160 double beam spectrophotometer. pH measurements were carried out with a Fischer Accumet model 800. Centrifugation was done with an IEC centrifuge Model PR-5000.

C. Reagents

All solutions were prepared using double distilled deionized water.

2. Methods

A. Synthesis of Fluorescent Analog:

The Ant and Mant derivatives of cyclic AMP and cyclic GMP were synthesized according to Hiratsuka (135). 2 mmol of cAMP and cGMP were dissolved in 30 mL of water at 38°C. The pH was adjusted to 9.6 with 2N NaOH. To this solution, isatoic anhydride or methyl isatoic anhydride was added with continuous stirring. The pH was maintained at 9.6 for 2 hr. The reaction products were then lyophilized overnight and reconstituted in about 10 mL of water. The solution containing the reaction products was divided into two batches and each batch was subjected to Sephadex LH-20 column (2.5 x 55 cm) chromatography, pre-
equilibrated with 30% ethanol. The column was then eluted with the same solvent at a flow rate of 40 mL/hr collecting 5 mL fractions with a Gilson microfractionator. The fractions containing the desired product were visualized by their deep blue fluorescence, observed with a hand-held UV lamp. Thin layer chromatography was performed with 1-propanol: Ammonium hydroxide: water (5:1 v/v/v, containing 0.5 g/L of EDTA). Fractions containing the derivatized analogs characterized by their Rf (See Appendix II) values were pooled and lyophilized. If lyophilized fractions after thin layer chromatography revealed more than one spot they were rechromatographed to obtain the pure form of the fluorescent analogs. Further characterization of the synthesized analogs were performed by obtaining their absorption and fluorescence spectra.

B. Determination of Standard Curve with Authentic Ant-AMP, MAnt-AMP, Ant-GMP and MAnt-GMP

To determine the standard curve, the fluorescent analogs, 3',5'-derivatives and 5'-derivatives were dissolved in buffer (40 mM Tris-HCl pH 8) in a ratio of 8:2. This was done to mimic an enzymatic reaction. The total volume was 200 μL.

C. Direct Enzymatic Fluorescent Assay

The enzymatic assay of crude and purified phosphodiesterase was carried out in the following manner: the standard reaction mixture contained in a total volume 0.2 mL, 36 mM Tris-HCl, 1mM Mg(CH₂COO)₂ pH 7.5, absence or presence of activator protein (calmodulin, reduced or oxidized oncomodulin), imidazole and Ant or MAnt cyclic nucleotide derivatives. After incubation for 15 to 30 min at 30°C, the reaction was terminated by boiling for 1 min after which 100 μL of 100 mM zirconyl chloride (in 150 mM sodium citrate pH 7.0) was added. The
mixture was allowed to incubate for 10 min and then added to DEAE-Sephadex column (0.5 x 0.9 cm) pre-equilibrated with 10 mM sodium citrate containing 2 mM morpholinoethanesulfonic acid (MES) chloride pH 7. The column was washed with about 10-20 mL of the same buffer. Prior to elution of the bound product, the wash was checked fluorimetrically for complete removal of unhydrolyzed fluorescent cyclic nucleotide. The column was then eluted with 2 M NaCl salt in 10 mM sodium citrate pH 7.0. The eluted product was monitored fluorimetrically and quantitated by comparison to a standard curve of authentic Ant or Mnt of nucleotide phosphate derivative obtained in a similar manner.

D. Purification of Calmodulin

Calmodulin was purified according to the procedure of Sharma (155) with minor modifications. 1 kg brain tissue was cut into small pieces and homogenized with buffer A (20 mM Tris-HCl, 1 mM Mg(CH3COO)2, containing 0.1 mM EGTA, 1 mM EDTA pH 7.5). The homogenate was centrifuged at 4200 rpm for 30 min and the supernatant was passed through glass wool. The filtrate was then applied to a DEAE-cellulose column (12 x 9 cm) pre-equilibrated with buffer A. The column was washed with 2-3 bed volumes of Buffer A containing 0.05 M NaCl, 0.1 mM EGTA, 1 mM EDTA, pH 7.5 and then eluted with 2-3 bed volumes of the same buffer containing 0.5 M NaCl.

The eluate was then dialyzed and subjected to heat treatment by incubating in a boiling water bath, and then cooled on ice. The cooled sample was centrifuged at 4200 rpm for 1 hr to remove the precipitate. The supernatant was pooled and CaCl2 added to 1 mM final concentration and then applied onto a 20 mL phenyl Sepharose column pre-equilibrated with buffer A containing 1 mM CaCl2 pH 7.5. After application of sample, the column was washed with
2 to 3 bed volumes of buffer A containing 0.05 mM CaCl$_2$, 0.1 M NaCl pH 7.5
and then eluted with buffer A containing 0.1 mM EGTA, 0.2 M NaCl at pH 7.5.
Fractions containing the protein were pooled, dialyzed against water and then
lyophilized. Lyophilized samples were reconstituted in buffer (40 mM Tris-HCl,
150 mM KCl pH 7.0) and then subjected to high pressure liquid chromatography.
Fractions containing the activator protein characterized by their retention time
on the column were pooled and concentrated and stored at -20°C until further
use.

F. Preparation of Calmodulin-Sepharose 4B

Sepharose 4B was activated by cyanogen bromide following the protocol
of March et al (159) and purified calmodulin was coupled to the activated cyanogen
bromide Sepharose 4B.

G. Preparation of Monoclonal Affinity Columns With ACC-1 (antibody
directed against bovine calmodulin) and ACAP-1 (antibody directed
against bovine heart calmodulin activated phosphodiesterase)

Solid phase ACC-1 or ACAP-1 was prepared by non-covalent coupling to
Protein-A-Sepharose with minor modifications. Purified absorbants (ACC-1 or
ACAP-1) 2 mg were dissolved in buffer B (40 mM Tris-HCl, 1 mM Mg(CH$_3$COO)$_2$)
containing 0.1 mM CaCl$_2$ and 0.1 M NaCl pH 7.5. The solution was then mixed
with 1 mL of Protein A-Sepharose in a 5 mL plastic vial and allowed to stir
gently at 4°C for about 1 hr. The stirred mixture was then packed in an Econo
column (15 x 1.2 cm) and washed with the same buffer to remove unbound absorbants. The monoclonal antibody affinity prepared column was then stored in phosphate buffered saline (PBS) at 4°C until further use.

H. Purification of Bovine Brain, Heart and Placental Phosphodiesterase by Calmodulin Sepharose Affinity Chromatography

The isolation procedure used was a modification of a protocol by Sharma et al (155). 1 kg of tissue is partially thawed and homogenized with 3 L of cold buffer B containing 0.1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol pH 7.5. The homogenization was carried out in a Waring blender for 15 seconds at low speed and thirty seconds at high speed. The homogenate was centrifuged at 4200 rpm for 45 min using an IEC PR 600 centrifuge. The mixture was passed through glass wool and solid ammonium sulfate was added (277 g/L) with continuous stirring to make the filtrate 45% saturated. Mixture was allowed to stir for 30 min and then centrifuged at 4200 rpm for 45 min. Supernatant was discarded and the precipitate was redissolved in about 300 ml of homogenizing buffer containing 20 mM Tris–HCl, pH 7.5 and then dialyzed overnight against the same buffer. Dialyzed sample was applied onto Protein A-Sepharose 4B equilibrated with the same buffer. The column was washed with 2–3 bed volumes of buffer B containing 0.1 mM EGTA, 0.15 M NaCl, 10 mM BME, pH 7.5 and then eluted with 2–3 bed volumes of the same buffer containing high salt (1 M NaCl) instead. The eluate was dialyzed overnight against buffer B containing 0.1 mM EGTA and 10 mM BME, pH 7.5. The dialyzed eluate was then made 1 mM CaCl₂ and stirred for another half hour before application onto a CaM-Sepharose affinity chromatography column pre-equilibrated with buffer B containing 1 mM CaCl₂ and 10 mM BME pH 7.5. The column was washed with buffer B containing 0.1 M NaCl, 0.05 mM CaCl₂ 10 mM BME pH 7.5 and then eluted
with the same buffer containing 0.1 mM EGTA instead of CaCl₂ pH 7.5. Two
mL fractions were collected and their absorbance measured at 280 nm. Fractions
containing the protein were pooled and concentrated against 40% glycerol and
stored in aliquots at -20°C.

Purification of Bovine Brain and Heart Phosphodiesterase Using
Anti-Phosphodiesterase Monoclonal Affinity Chromatography

The above two enzymes were purified as previously described by Sharma
et al. (155). One kg of tissue was partially thawed, cut into pieces and then
homogenized. Homogenate was then centrifuged at 4000 rpm for 45 min and the
supernatant passed through glass. The supernatant fraction was applied
onto an anion exchange DEAE-cellulose column (9 x 12 cm) previously equilibrated
with buffer B containing 0.1 mM EGTA, 1 mM EDTA and 10 mM BME pH 7.5. The
column was washed with 2-3 bed volumes of the same buffer containing
0.05 M NaCl pH 7.5 and then eluted at a higher salt concentration (0.35 M NaCl
pH 7.5). Protease inhibitors (TLCK, TPCK, PMSF) to a concentration of 1 mg/
L each was added to the eluate and then applied onto anti-phosphodiesterase
Sepharose 4B. The column was washed with the equilibrating buffer containing
0.2 M NaCl and eluted with the same buffer containing 25 M MgCl₂ pH 7.5.
Fractions containing enzymatic activity were pooled, dialyzed and concentrated
with an Amicon ultrafiltration system using Diaflo ultrafiltration membrane (PM
10). The concentrated enzyme sample was then divided into aliquot containing
10% sucrose and stored at -20°C.
J. Purification of Bovine Brain, Heart and Placental Phosphodiesterase

Using ACC-1 and ACAP-1 Monoclonal Antibody Affinity Chromatography

Most of the steps are as previously described in section (H) except that the eluate obtained from Affi-Blue column chromatography was dialyzed and to which Ca$^{2+}$ and crude calmodulin were added to a final concentration of $10^{-3}$ M and $10^{-6}$ M respectively. In addition protease inhibitors were added to a final concentration of 1 mg/mL and applied onto ACC-1 or ACAP-1-Protein-A-Sepharose previously equilibrated with buffer B containing 0.1 M CaCl$_2$, 0.1 M NaCl and 10 mM BME pH 7.5. In the case of ACC-1 monoclonal antibody affinity chromatography, the column was washed with the same buffer and then eluted with buffer containing 1 mM EGTA. However, with the ACAP-1 monoclonal affinity chromatography, the column was washed with the same buffer containing 1 mM EGTA and then eluted with buffer containing 2 M MgCl$_2$ pH 7.5. As previously described by Hansen and Barac (6), the binding of ACC-1 to certain calmodulin-binding proteins and not others was found to be very useful in the purification of two calmodulin dependant cyclic nucleotide phosphodiesterase isozymes present in bovine brain. These two forms of the enzyme can be separated by subjecting the eluate of ACC-1 through ACAP-1 monoclonal affinity chromatography. The lower molecular weight form of the enzyme binds to the affinity column whereas the higher molecular weight is obtained in the breakthrough fraction. Fractions containing enzymatic activity eluted off ACAP-1 affinity chromatography column were pooled, dialyzed and then concentrated against 40% glycerol in buffer B pH 7.5. The breakthrough fraction from ACAP-1 affinity chromatography containing the lower Mr enzyme was concentrated against 40% glycerol in buffer and stored in aliquots at -20°C.
E. Separation of Phosphodiesterase from Calmodulin Using Organomercurial Sepharose Affinity Chromatography

The enzyme preparations employing the ACC-1 and ACAP-1 monoclonal affinity chromatography are co-purified with calmodulin. Calmodulin was removed by first dialyzing the sample to remove β-mercapto ethanol (BME) and then applied onto organomercurial Sepharose pre-equilibrated with buffer B containing 0.1 M EGTA, 0.1 M NaCl pH 7.5. The column was washed with the same buffer and the bound enzyme eluted with the same buffer containing 10 mM BME. Fractions having enzymatic activity were pooled and concentrated against buffer B containing 40% glycerol.

L. Assay of Cyclic Nucleotide Phosphodiesterase

Phosphodiesterases isolated by the various procedures were initially checked for their activities by an enzyme coupled assay. Following the hydrolysis of the substrate by the enzyme, and formation of P1 by 5'-nucleotidase present in the same mixture, ammonium molybdate and α-naphthol sulfonic acid were added to form the coloured chromogen. The absorbance was measured at 660 nm (155).

M. Gel Electrophoresis

SDS gel electrophoresis was performed following the protocol of Laemml (161) and 10% acrylamide gels were routinely used.

N. Protein Estimation

Protein was estimated according to the procedure of Bradford using bovine serum albumin as standard (162).
RESULTS

1. Characterization of the Fluorescent Cyclic Nucleotide Derivatives

All analogues (Ant and Mant derivatives of cAMP and cGMP) were checked for their purity by thin layer chromatography. Each analogue was chromatographically pure as indicated by a single fluorescent spot. Their Rf values are indicated in Table 4.

The absorption spectra of all the analogs at pH 8.0 are shown in Figures 6 and 7. The spectra of the Ant- and Mant-cAMP forms exhibited two maxima as indicated in Table 5. The spectra of the Ant and Mant-cGMP forms were also similar except that a distinct shoulder around 280 nm was also exhibited.

The fluorescent characteristics of equimolar concentration of Ant-5'- and Ant-3',5' as well as Mant-5' and Mant-3',5' cyclic nucleotides were investigated spectrofluorometrically. The relative fluorescence quantum yield ratio of Ant 5'-AMP/Ant 3',5'-cAMP and Mant-5'-AMP/Mant-3',5'-cAMP were found to be about 1.3 and 1.5 respectively (Figures 8 and 9). However, in relation to the Mant-5'-GMP/Mant 3',5'-cGMP a 2-fold decrease in the fluorescence quantum yield ratio was observed (Figure 9B). The Ant-5',GMP/Ant-3',5'-GMP (Figure 9A) appeared to have identical emission characteristics.

2. Development of a Direct Discontinuous Fluorescent Assay

Titration studies of a 1:1 mixture of Ant-AMP/Ant-cAMP with zirconyl chloride (8 mM) in sodium citrate (10 mM) pH 7, indicated an increase in the relative fluorescence quantum yield ratio of Ant-AMP to Ant-cAMP of about 1.7 (Figure 10). Under these conditions zirconyl chloride did not affect the fluorescent
Table 4. Rp Values of Fluorescent Cyclic Nucleotide Derivatives

<table>
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<td>ANT-cAMP</td>
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<td>MANT-GMP</td>
<td>0.63</td>
<td>MANT-GMP</td>
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a. TLC was performed with 1-propanol, NH$_4$OH, water (6:3:1 v/v/v) containing 0.5g/litre of EDTA on silica gel.
Figure 2  Absorption Spectra of cyclic-AMP and AMP derivatives.

A. Absorption Spectra of ANT-AMP (29 μM) and ANT-cAMP (28 μM) in 40 mM Tris-HCl at pH 7.5; ——— Ant-AMP; ——— ANT-cAMP.

B. Absorption Spectra of MANT-AMP (29 μM) and MANT-cAMP (28 μM) in 40 mM Tris-HCl at pH 7.5; ——— MANT-AMP; ——— MANT-cAMP.
Figure 7 Absorption spectra of cyclic-GMP and GMP derivatives.

A. Absorption spectra of ANT-GMP (32 μM) and ANT-cGMP (32 μM) in 40 mM Tris-HCl pH 7.5: ——— ANT-GMP; ——— ANT-cGMP.

B. Absorption spectra of MANT-GMP (32 μM) and MANT-cGMP (32 μM) in 40 mM Tris-HCl at pH 7.5: ——— MANT-GMP; ——— MANT-cGMP.
<table>
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<th>Compound</th>
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a Concentration of the various derivatives
Figure 9 Fluorescent emission spectra of cyclic-AMP and AMP derivatives.

A. Fluorescence emission spectra of ANT-AMP (28 μM) and ANT-cAMP (28 μM) in 40 mM Tris-HCl pH 7.5. Emission spectra were run with excitation at 300 nm: ——— ANT-AMP, ——— ANT-cAMP.

B. Fluorescence emission spectra of MANT-AMP (28 μM) and MANT-cAMP in 40 mM Tris-HCl at pH 7.5. Emission spectra were run with excitation at 350 nm: ——— MANT-AMP, ——— MANT-cAMP.
Figure 6 Fluorescent emission spectra of cyclic-GMP and GMP derivatives.

A. Fluorescent emission spectra of ANT-GMP (31 μM) and ANT-dGMP (31 μM) in 40 mM Tris-HCl pH 7.5. Emission spectra were run with excitation at 360 nm.

--- ANT-GMP. --- ANT-dGMP.

B. Fluorescent emission spectra of MANT-GMP (31 μM) and MANT-dGMP (31 μM) in 40 mM Tris-HCl pH 7.5. Emission spectra were run with excitation at 360 nm.

--- MANT-GMP. --- MANT-dGMP.
Figure 9

A

FLUORESCENCE INTENSITY

B
Figure 10 Titration curve of 1:1 mixture of ANT-AMP and ANT-cAMP with zirconyl chloride.

A 1:1 mixture of ANT-AMP and ANT-cAMP (0.8 nmol) was titrated against zirconyl chloride in sodium citrate buffer (10 mM, pH 7). Inset: fluorescence intensity of the derivatives plotted against concentration of zirconyl chloride (□) ANT-AMP, (□) ANT-cAMP.
Figure 10

[Graph showing the relationship between [ZrOCl₂] (M x 10⁻³) and the ANTI-AMP-AMP Fluorescence Intensity Ratio. The graph includes data points and a curve that illustrates the increase in fluorescence intensity with increasing [ZrOCl₂] concentration.]
intensity of ANT-cAMP. This suggested the selective interaction of zirconium with ANT-AMP. An apparent binding constant of ~2 mM of zirconyl chloride of ANT-AMP even estimated from Figure 10. (See insert.) The selective interaction of zirconyl chloride with ANT-AMP was further elucidated when ANT-AMP adsorbed onto DEAE-Sephadex in the presence of zirconyl chloride. The elution profile of the fluorescent derivatives of AMP and cAMP in the presence and absence of zirconyl chloride are shown in Figure 11. In the absence of zirconyl chloride neither ANT-AMP nor ANT-cAMP were adsorbed onto DEAE-Sephadex (Figure 11A, B, C) and no further fluorescence can be detected upon introduction of 2M NaCl (Figure 11). This suggested that neither of the derivatives bind to DEAE-Sephadex. However, in the presence of zirconyl chloride (10 mM) in 20 mM sodium citrate buffer pH 7, ANT-AMP was adsorbed onto DEAE-Sephadex (Figure 11D). As can be seen in Figure 11E ~5% of the applied ANT-AMP leaked from the column. This leakage would appear to be a result of overloading the column with ANT-AMP (~6 µmol applied). In the presence of zirconyl chloride ANT-cAMP did not bind to DEAE-Sephadex (Figure 11D). In a 1:1 mixture of ANT-AMP/ANT-cAMP (Figure 11F) following the wash of ANT-cAMP with about 20 mL of buffer (20 mM Sodium Citrate, 2 mM zirconyl chloride pH 7), ANT-AMP is eluted upon introduction of buffer 2 M NaCl pH 7. In the absence of citrate buffer ANT-AMP, ANT-cAMP, and their mixtures were adsorbed onto DEAE-Sephadex and selective wash or elution with NaCl was difficult to achieve. These results suggest that a citrate-Zr⁺⁺- ANT-AMP complex with a net anionic charge may be involved for the selective adsorption onto DEAE-Sephadex. The zirconium (Zr⁺⁺) is postulated to interact through the dianion of the monophosphate ester form of ANT-AMP and the citrate ion. A highly speculative structure is shown in Figure 12. This complex with a net anionic charge is then postulated to
Figure 11: Elution profile of ANT-AMP and ANT-cAMP (8 nmol) on DEAE-Sephadex.

(A,D) ANT-cAMP; (B,E) ANT-AMP; (C,F) 1:1 mixture ANT-AMP/ANT-cAMP (8 nmol). (O) Absence of zirconyl chloride; (●) Presence of zirconyl chloride. Arrow indicates point at which 0.1 M NaCl was introduced.
Figure 11

FLUORESCENCE INTENSITY (425 nm)

FRACTION NUMBER
Figure 12 Speculative structure of citrate - Zr - ANT-AMP complex with a net anionic charge.

A indicates base residue (Adenine or Guanine), Zr indicates zirconium, R indicates hydrogen atom or methyl group.
Figure 12
Figure 13 Standard curve of ANT-AMP obtained with 8:2 mixture of ANT-AMP.

Varying amounts of ANT-AMP (0.4-12.8 nmol) were made in 40 mM Tris-HCl pH 7.5. Zirconyl chloride (100 mM) was then added and applied into DEAE-Sephadex. Column was washed with sodium citrate (10 mM) buffer containing zirconyl chloride (2 mM) pH 7.5. The bound AMP was then eluted with sodium chloride (0.1M) in a total volume of 5 mL and then measured its fluorescence. Each data point represents the mean ± SD of four determinations with different columns.
Figure 13

[Graph showing fluorescence (425 nm) vs. [ANT-AMP] (nmol)]
in Figure 12. This complex with a net anionic charge is then postulated to
bind to DEAE-Sephadex. The results presented here would suggest that this
interaction does not occur with ANT-cAMP, as the cyclic nucleotide cannot act
as a bidentate oxygen ligand for Zn$^{2+}$ through its phosphate moiety. Furthermore,
identical elution profiles were obtained with AMP and cAMP, suggesting that
the anthraniloyl moiety does not play a role in the adsorption onto DEAE-
Sephadex in the presence of Zn$^{2+}$.

The selective separation of ANT-AMP from ANT-cAMP in this manner was
adapted to the assay of Ca$^{2+}$/calmodulin activatable cyclic nucleotide
phosphodiesterase from bovine brain. In performing the standard curve (Figure
12) ANT-cAMP to ANT-AMP ratio of 3:1 was maintained to mimic the enzymatic
reaction. Under these conditions over the whole range of concentrations studied
the AMP eluted was free of ANT-cAMP contaminants as determined by thin layer
chromatography. The standard curve was linear within the range of 0.4 to 4
nmol ANT-AMP. Some deviation from linearity was observed above the
concentration of 4 nmol.

To determine the amount of enzymatic ANT-cAMP hydrolysis, ANT-cAMP
(concentration range 4.2-524 μM) was incubated with phosphodiesterase from
bovine brain with saturating amounts of calmodulin in the presence and absence
of Ca$^{2+}$. Upon incubation for 4 min at 30°C, the reaction was quenched by
boiling and m-cresol chloride in citrate buffer was added to a final concentration
of 10 μM. The mixture was then applied to a column of DEAE-Sephadex and
washed with 20 mL of buffer to remove ANT-cAMP. ANT-AMP was then eluted
with 2M sodium chloride and quantitated. Under the above assay conditions, ANT-
AMP formed by Ca$^{2+}$/calmodulin dependent phosphodiesterase (PDE) did not exceed
4 nmol. Rates of ANT-AMP formation with respect to ANT-cAMP concentrations.
Figure 1. Rate of hydrolysis of ANT-cAMP by bovine brain cyclic nucleotide phosphodiesterase.

A. Substrate concentrations ranged from 4.25 to 524.8 μM. (a) Presence of Ca²⁺ (1 mM); (b) Presence of EGTA (0.1 mM). Each data point represents the mean ± SD of four determinations with different columns.

B. Plot of $V^{-1}$ against $S^{-1}$ of the rate of hydrolysis of ANT-cAMP in the presence of Ca²⁺ (1 mM). $K_m = 90 ± 10$ μM, $V_{max} = 1.43 ± 0.05$ nmol min⁻¹.
exhibited normal Michaelis-Menten kinetic behaviour (Figure 13A). A \( K_m \) of 90 ± 10 \( \mu \)M and \( V_{max} \) of 1.43 ± 0.5 nmol min\(^{-1}\) was obtained from the linear double reciprocal plot in the presence of Ca\(^{2+}\) (Figure 13A). The rate of hydrolysis of ANT-cAMP by brain PDE was determined to be about 17 fold less in comparison to that of cAMP. Under the present conditions employed, Ca\(^{2+}\)/calmodulin activated the enzyme-catalyzed hydrolysis of ANT-cAMP by approximately six-fold. The amount of activation was identical to that observed with cAMP as determined by the ANSA-molybdate assay. This direct discontinuous assay for phosphodiesterase was also attempted with a crude homogenate of the bovine brain. The standard curve obtained with a mixture of authentic ANT-AMP added to crude homogenate was indistinguishable from that illustrated in Figure 13, indicating that the cell components do not interfere with the assay. The enzymatic assay of phosphodiesterase in crude homogenate produced a \( K_m \) of about 125 ± 20 \( \mu \)M.

This assay was subsequently modified for the large scale, semi-automated kinetic analysis of PDEs. This was accomplished by the addition of a peristaltic pump and a flow cell to the fluorometer. In this manner, the product of the enzymic reaction (ANT-5; MANT-5 of AMP and GMP) was eluted off the mini-columns as a sharp peak. Furthering the interfacing of the fluorometer to a Commodore 64 enabled the automated integration of the elution peak by a computer programme for the purpose of rapid and accurate quantitation. With the modified assay, about 20-30 samples could be run per hour (See Appendix III). Figures 15 and 16 illustrate the standard curve of authentic ANT and MANT derivatives of AMP and GMP obtained in this manner. The standard curves were linear within the range of 0.4 nmol to ~ 5 nmol for the ANT and MANT-AMP
Figure 15: Standard curves of AMP derivatives obtained by the modified procedure.

Varying amounts of 8:2 mixture of (i) ANT-cAMP and ANT-AMP; and (ii) MANT-cAMP and MANT-AMP; were made in 40 mM Tris-HCl pH 7.5 respectively. Zirconyl chloride (100 mM) was then added to each sample and applied onto DEAE-Sephadex. Column was washed with sodium citrate (10 mM) containing zirconyl chloride (2 mM) at pH 7.5. The bound AMP-derivative was then eluted with sodium chloride (2M) and allowed to pass through a flow cell in the fluorometer via a peristaltic pump. ANT derivatives were excited at 330 nm with emission at 430 nm and MANT derivative were excited at 350 nm with emission at 445 nm.

( □ ) ANT-AMP; ( △ ) MANT-AMP
Figure 16 Standard curves of GMP derivatives obtained by the modified procedure.

Varying amounts of 8:2 mixture of (i) ANT-cGMP and ANT-GMP; and (ii) MANT-cGMP and MANT-GMP; were made in 40 mM Tris-HCl at pH 7.5 respectively. Zirconyl chloride (10 mM) was then added to each sample and applied onto DEAE-Sephadex. Column was washed with sodium citrate (10 mM) containing zirconyl chloride (2 mM) pH 7.5. The bound GMP derivative was then eluted with sodium chloride (2M) and allowed to pass through a flow cell in the fluorometer via a peristaltic pump. ANT derivatives were excited at 330 nm with emission at 427 nm. MANT-derivatives were excited at 380 nm with emission at 441 nm.

[ ] ANT-GMP; [ ] MANT-GMP.
derivatives, and within the range of 0.4 nmoI-10 nmoI for the ANr and MANT-
GMP derivatives. Preliminary investigations of the catalyzed hydrolysis of ANT-
cAMP exhibited valid kinetic data with the modified assay procedure. This direct
discontinuous assay was subsequently employed for a comprehensive kinetic
investigation on the rates of hydrolysis of ANr-cAMP, MANT-cAMP, ANr-cGMP
and MANT-cGMP for phosphodiesterases isolated from bovine brain, heart and
placental tissue.

3: Characterization and Kinetic Studies of the Bovine Brain, Heart and
Placenta Phosphodiesterases Purified Using Calmodulin Sepharose Affinity
Chromatography

The overall purification of bovine placenta phosphodiesterase by
calmodulin-Sepharose affinity chromatography is reported in Table 6. The enzyme
activities were measured colorimetrically by an enzyme coupled assay (155). In
the presence of saturating amounts of calmodulin, presence of Ca\textsuperscript{2+}, about 53
fold purification with a specific activity of 1.05 μMoles min\textsuperscript{-1}mg\textsuperscript{-1} was obtained
using native cAMP as substrate. The specific activities for all the three enzymes
are shown in Table 7. The heart and brain phosphodiesterase revealed about
four to five fold in activation whereas the placental phosphodiesterase exhibited
only about two fold in its activation by calmodulin. The enzyme preparations
when analyzed by SDS gel electrophoresis, were homogenous with apparent
molecular weights of 60-63 Kd for brain and 57-59 Kd for the heart and placenta
(Figure 17).

The initial rates of the enzymatic reaction v versus substrate
concentrations for the bovine brain phosphodiesterase were investigated with
MANT-cAMP and MANT-cGMP as substrates. Normal Michaelis-Menten kinetic
### Table 6. Purification of Bovine Placenta Phosphodiesterase

<table>
<thead>
<tr>
<th>Sample Fraction</th>
<th>Total Protein mg</th>
<th>Total Activity μmoles of Pi/min</th>
<th>Specific Activity μmoles of Pi/min/mg</th>
<th>Purification</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant 1200 rpm x 1 hr</td>
<td>3450</td>
<td>69</td>
<td>0.02</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>45% (NH₄)₂SO₄ precipitation</td>
<td>1232</td>
<td>50.4</td>
<td>0.04</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>Affi-Blue</td>
<td>242</td>
<td>34</td>
<td>0.14</td>
<td>2.5</td>
<td>48</td>
</tr>
<tr>
<td>Calmodulin-Sepharose</td>
<td>1.34</td>
<td>14.1</td>
<td>1.05</td>
<td>53</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 7. Calmodulin Activation of Calmodulin-Sepharose Affinity Purified Phosphodiesterases

<table>
<thead>
<tr>
<th>Protein (calmodulin)</th>
<th>Brain</th>
<th></th>
<th></th>
<th>Heart</th>
<th></th>
<th></th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp. Activity µMoles P_i min^{-1} mg^{-1}</td>
<td>0.524</td>
<td>0.288</td>
<td>0.444</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>2.744</td>
<td>2.498</td>
<td>1.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 17 SDS polyacrylamide gel electrophoresis of bovine cyclic nucleotide phosphodiesterases purified by calmodulin-Sepharose affinity chromatography.

Lanes: b, heart phosphodiesterase (15 μg); c, brain phosphodiesterase (12 μg); d, placenta phosphodiesterase (12 μg); e and f, molecular weight standards: bovine serum albumin, 66 Kd; ovalbumin, 45 Kd; glyceraldehyde-3-phosphate dehydrogenase, 35 Kd; carbonic anhydrase, 29 Kd; trypsinogen, 24 Kd; trypsin inhibitor, 20 Kd; α-Lactalbumin, 14 Kd.
Plots of enzyme velocity as a function of substrate concentration with bovine brain cyclic nucleotide phosphodiesterase purified by calmodulin-Sepharose affinity chromatography.

(A) MANT-cAMP; (B) MANT-cGMP

Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Mg$^{2+}$, 1 mM Ca$^{2+}$ and 4.13 µg of enzyme.

$K_m$ and $V_{\text{max}}$ values were obtained via simplex fit of the data $v$ versus $s$. The curves were drawn by determining the theoretical values of $v$ against $s$ by substituting the calculated values of $K_m$ and $V_{\text{max}}$ in the Michaelis-Menten equation.

The curve drawn without data points indicates the theoretical curve obtained in the absence of calmodulin.
Figure 18

A

B

$\nu \text{ (mmol/min/mg)} \times 10^4$

$[\text{MANT-CAMP}]$

$[\text{MANT-COMP}]$
Figure 19: Plots of $v^{-1}$ versus $s^{-1}$ of the rates of hydrolysis of MANT-cAMP and MANT-cGMP by bovine brain cyclic nucleotide phosphodiesterase.

(A) MANT-cAMP; (B) MANT-cGMP

Standard reaction mixture (0.2 mL), pH 7.5, contained 36 mM Tris-HCl, 1 mM Mg$^{2+}$, 1 mM Ca$^{2+}$, (10$^{-6}$ M) calmodulin and 4.13 µg of enzyme.

Data presented in the Lineweaver-Burk format for the different symbols (+, □, ◊, △) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.

The line drawn without data points indicates the theoretical curve obtained in the absence of calmodulin.
Figure 20 Plots of $v^{-1}$ versus $s^{-1}$ on the rate of hydrolysis of MANT-cAMP and MANT-cGMP by bovine heart cyclic nucleotide phosphodiesterase.

(A) MANT-cAMP; (B) MANT-cGMP

Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Mg$^{2+}$, 1 mM Ca$^{2+}$ (10$^{-6}$M) calmodulin and 6.28 µg of enzyme.

Data presented in the Lineweaver-Burk format for the different symbols (+, □, △, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.

The line drawn without data points indicates the theoretical curve obtained in the absence of calmodulin.
Figure 21: Plots of $v^{-1}$ versus $s^{-1}$ of the rates of hydrolysis of MANT-cAMP and MANT-cGMP by bovine placenta cyclic nucleotide phosphodiesterase.

(A) MANT-cAMP; (B) MANT-cGMP.

Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Mg$^{2+}$, 1 mM Ca$^{2+}$ (10$^{-6}$) M calmodulin and 4.5 µg of enzyme.

Data presented in the Lineweaver-Burk format for the different symbols (+, −, Δ, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.

The line drawn without data points indicates the theoretical curve obtained in the absence of calmodulin.
Figure 21

\[ \frac{1}{v} \text{(mg/ml/min)} \times 10 \]

vs.

\[ \frac{1}{[\text{NAD+}]} \text{(1/micromolar)} \]

\[ \frac{1}{v} \text{(mg/ml/min)} \times 10 \]

vs.

\[ \frac{1}{[\text{NAD+}]} \text{(1/micromolar)} \]
Table 6. Effect of Calmodulin on the Kinetic Parameters of Cyclic Nucleotide Phosphodiesterases Purified by CaM-Sepharose affinity chromatography.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Calmodulin (10^{-7} M)</th>
<th><em>K_m</em> (µM)</th>
<th><em>V_{max}</em> (nmol min^{-1}mg^{-1})</th>
<th><em>K_m</em> (µM)</th>
<th><em>V_{max}</em> (nmol min^{-1}mg^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>-</td>
<td>279.0±10</td>
<td>141.0±4</td>
<td>101.8±3</td>
<td>77.1±1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>169.0±4</td>
<td>434±12</td>
<td>167.5±3</td>
<td>212.4±2</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>167.0±4</td>
<td>44.8±2</td>
<td>200.0±5</td>
<td>50.0±4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>69.0±5</td>
<td>101.5±3.7</td>
<td>500.0±7</td>
<td>200.0±10</td>
</tr>
<tr>
<td>Placenta</td>
<td>-</td>
<td>166.0±10</td>
<td>2.3±3</td>
<td>136.0±4</td>
<td>11.0±2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>64.5±3</td>
<td>9.3±1</td>
<td>335.0±4</td>
<td>35.6±3</td>
</tr>
</tbody>
</table>
behaviour on the rates of hydrolysis of the two substrates were observed (Figure 18). Similar observations were also made for the bovine heart and placenta phosphodiesterases. Lineweaver-Burk plots (v$^{-1}$ versus s$^{-1}$) on the rates of hydrolysis of the two substrates for the bovine brain, heart, and placenta are represented in Figures 19-21. The only exception to this was an upward curvature observed in L-B plots with the heart PDE in the absence of calmodulin (CaM), a possible indication of substrate induced negative cooperativity. The $K_m$'s and $V_{max}$'s estimated by the fit of the combined data to the Michaelis-Menten equation via simplex fit are summarized in Table 8. In the presence of calmodulin, brain phosphodiesterase displayed a 1.7 fold decrease in $K_m$ with MANT-cAMP as substrate. A larger decrease, about 2.5 fold, was observed for the heart and placenta phosphodiesterase. On the other hand calmodulin increased the $K_m$ about 1.6 fold for the brain enzyme and even higher (2.5 fold) for the heart and placenta enzymes when MANT-cGMp was employed as substrate.


The enzymes purified by ACAP-1 affinity chromatography were analyzed by SDS gel electrophoresis. The enzymes preparation were found to be purified to apparent homogeneity, revealing a single protein band for the heart and placenta phosphodiesterase. On the other hand, the bovine brain phosphodiesterase, purified by ACC-1 affinity chromatography showed two bands with apparent molecular weights of 60 Kd and 63 Kd (Figure 22).
Figure 22 SDS polyacrylamide gel electrophoresis of bovine brain, heart, and placenta cyclic nucleotide phosphodiesterases, purified by ACC-1 and ACAP-1 monoclonal affinity chromatography lanes: a, ACC-1 purified brain phosphodiesterase (10 µg); d, ACAP-1 purified placenta phosphodiesterase (8 µg); c, molecular weight standards: bovine serum albumin, 66 Kd; ovalbumin, 45 Kd; glyceraldehyde-3-phosphate dehydrogenase, 35 Kd; carbonic anhydrase, 29 Kd; trypsinogen, 24 Kd; trypsin inhibitor, 20 Kd; α-lactalbumin 14 Kd.

B. SDS polyacrylamide gel electrophoresis of bovine heart (lane b) and placenta (lane c) phosphodiesterase purified by ACAP-1 monoclonal affinity chromatography. Lane a, molecular weight standards (as above).
Table 6. Specific Activities of ACAP-1, ACC-1 Monoclonal Affinity Column Purified Cyclic Nucleotide Phosphodiesterases

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Brain (μMoles Pi min⁻¹ mg⁻¹)</th>
<th>Heart (μMoles Pi min⁻¹ mg⁻¹)</th>
<th>Placenta (μMoles Pi min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC-1</td>
<td>50b</td>
<td>37-80</td>
<td>31.85</td>
</tr>
<tr>
<td>ACAP-1</td>
<td>45.7c</td>
<td>40.06</td>
<td>36.95</td>
</tr>
<tr>
<td></td>
<td>52.48d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Prior to purifying the enzyme using monoclonal affinity chromatography, the purification steps described in Table 6 were also performed, except that the CaM-Sepharose affinity purification step was omitted.

b Mixtures of 60 and 63 Kd.

c Eluate of ACAP-1.

d Breakthrough of ACAP-1 (Fraction that was not bound to ACAP-1.)
The brain 62 Kd and 60 Kd isoenzymes were separated by exploiting the differential affinity of the ACAP-1 and ACC-1 antibodies for these PDE forms. The enzyme fraction obtained from the ACC-1 antibody which reacts with both isoforms, was reapplied to a column of ACAP-1, which is specific for the lower MW isozyme. In this manner, the breakthrough of the ACAP-1 column contained the larger MW protein, while the MgCl₂ eluate contained the lower. The activities of the enzymes were measured using native cAMP as substrate. In Table 5 the specific activities of bovine brain, heart and placenta phosphodiesterase purified by using the above procedures are reported. After isolation and characterization of the monoclonal affinity purified enzymes they were compared for their action on the rates of hydrolysis for the fluorescent derivatives of cAMP and cGMP. In these studies the enzyme activities were measured in the presence of an excess of Ca²⁺ and calmodulin. The kinetic data exhibited normal Michaelis-Menten kinetic behaviour with all the enzymes. The double reciprocal plots on the rates of hydrolysis as a function of the concentration of the various derivatives employed are illustrated in Figures 23-30. With MANT-cAMP and MANT-cGMP as substrate, the brain (60-61) Kd and 63 Kd phosphodiesterase exhibited no significant change in its $K_m$. However, the heart and placenta enzymes displayed a lower $K_m$ with MANT-cGMP (16 μM and 17 μM) respectively (Table 10). All the enzymes displayed lower $K_m$'s with MANT-cGMP as compared to ANT-cGMP as substrate. In addition, the largest $V_{max}$ values were displayed by all the enzymes with ANT-cGMP as substrate. The largest variation in the $K_m$ values between the phosphodiesterases was obtained with ANT-cAMP, where $K_m$ values of 133 μM and 155 μM were obtained for the brain 60 Kd and the placenta enzymes respectively, whereas the heart and brain 63 Kd enzymes displayed $K_m$ that were approximately three fold lower.
Figure 23  Cyclic-AMP derivative substrates: double reciprocal plots of velocity as a function of substrate concentration with bovine brain (60–61 Kd cyclic nucleotide phosphodiesterase purified by monoclonal affinity chromatography.

(A) ANT-cAMP; (B) MANT-cAMP

The standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Mg$^{2+}$, 1 mM Ca$^{2+}$ (3 x 10$^{-5}$M) of calmodulin and (1.5 x 10$^{-8}$M) concentration of enzyme.

Data presented in the Lineweaver-Burk format for the different symbols (+ □ △ ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 23

A

\[ \frac{1}{v} \text{(mg/min/mmol)} \times 10 \]

\[ \frac{1}{[\text{ANT-AMP}]} \text{ (1/micromolar)} \]

B

\[ \frac{1}{v} \text{(mg/min/mmol)} \times 10 \]

\[ \frac{1}{[\text{MANT-AMP}]} \text{ (1/micromolar)} \]
Figure 24  Cyclic-GMP derivative substrates: double reciprocal plots of velocity as a function of substrate concentration with (60-61) 
Kd bovine brain cyclic nucleotide phosphodiesterase purified 
by monoclonal affinity column chromatography.

(A) ANT-cGMP  (B) MANT-cGMP

The standard reaction mixture in 0.2 mL, pH 7.5, contained
36 mM Tris-HCl, 1 mM Mg^{2+}, 1 mM Ca^{2+} (3 \times 10^{-6} M) of calmodulin 
and (1.6 \times 10^{-9} M) concentration of enzyme.

Data presented in the Lineweaver-Burk plot for the different 
symbols (+, □, △, ◇) represent the data obtained from different 
kinetic runs. The solid line is the theoretical best fit of the 
data to the Michaelis-Menten equation via simplex fit.
Figure 24

A

B
Figure 25  Cyclic-AMP derivative substrates: double reciprocal plots of velocity as a function of substrate concentration with 63 Kd bovine brain cyclic nucleotide phosphodiesterase purified by monoclonal affinity chromatography.

(A) ANT-cAMP; (B) MANT-cAMP.

Standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Ca\(^{2+}\), 1 mM Mg\(^{2+}\) (3 x 10\(^{-6}\)M) of calmodulin and (1.96 x 10\(^{-8}\) M) concentration of enzyme.

Data presented in the Lineweaver-Burk plot for the different symbols (+, Δ, □, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 25

A

B

\( \frac{1}{v} \) (mg/min/mmol) \times \text{10}^{-6} \n
\frac{1}{[\text{ANT-CAMP}]} \text{ (1/MICROMOLAR)}

\( \frac{1}{v} \) (mg/min/mmol) \times \text{10}^{-6} \n
\frac{1}{[\text{MANT-CAMP}]} \text{ (1/MICROMOLAR)}
Figure 26: Cyclic-GMP derivative substrates: double reciprocal plots of velocity as a function of substrate concentration with bovine brain 63 Kd cyclic nucleotide phosphodiesterase purified by monoclonal affinity chromatography.

(A) ANT-cGMP; (B) MANT-cGMP.

Standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Ca^{2+}, 1 mM Mg^{2+}, (3 \times 10^{-6})M) of calmodulin and (1.96 \times 10^{-8}M) concentration of enzyme.

Data presented in the Lineweaver-Burk plot for the different symbols (+, □, △, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 26

A

B

$\frac{1}{V}$ (mg/min/mmol) x 10

$\frac{1}{[\text{ANT-cGMP}]}$ (1/MICROMOLAR)

$\frac{1}{V}$ (mg/min/mmol) x 10

$\frac{1}{[\text{MANT-cGMP}]}$ (1/MICROMOLAR)
Figure 27  Cyclic-AMP derivatives as substrates: double reciprocal plots of velocity as a function of substrate concentration with bovine heart cyclic nucleotide phosphodiesterase purified by monoclonal affinity chromatography.

(A) ANT-cAMP; (B) MANT-cAMP.

Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Ca\(^{2+}\), 1 mM Mg\(^{2+}\) (9 x 10\(^{-6}\)M) of calmodulin and (1.75 x 10\(^{-8}\)M) concentration of enzyme.

Data presented in the Lineweaver-Burk plot for the different symbols (+, □, Δ, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 27

A

B
Figure 28 Cyclic-GMP derivatives as substrates: double reciprocal plots of the velocity as a function of substrate concentration with bovine heart cyclic nucleotide phosphodiesterase purified by monoclonal affinity chromatography.

(A) ANT-cGMP; (B) MANT-cGMP.

Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Ca$^{2+}$, 1 mM Mg$^{2+}$ (3 x 10^{-6}) of calmodulin and (1.75 x 10^{-6}M) concentration of enzyme.

Data presented in the Lineweaver-Burk plot for the different symbols (+, □, △, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 28

A

B

\[ \frac{1}{V} \text{ (mM/min/nmol) x 10} \]

\[ \frac{1}{[\text{ANT-cGMP}]} \text{ (1/MICROMOLAR)} \]
Figure 29 Cyclic-AMP derivatives as substrates: double reciprocal plots of the velocity as a function of substrate concentration with the bovine placenta cyclic nucleotide phosphodiesterase purified by the monoclonal affinity chromatography.

(A) ANT-3AMP; (B) MANT-cAMP.

Standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Mg\(^{2+}\), 1 mM Ca\(^{2+}\), (3 \times 10^{-6}M) of calmodulin and (1.6 \times 10^{-6}M) of enzyme.

Data presented in the Lineweaver-Burk plot for the different symbols (+, □, △, ◊) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fix.
Figure 30  Cyclic-GMP derivatives as substrates: double-reciprocal plots of the velocity as a function of substrate concentration with the bovine placenta cyclic nucleotide phosphodiesterase purified by monoclonal affinity chromatography.

(A) ANT-cGMP; (B) MANT-cGMP.

Standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Mg\(^{2+}\), 1 mM Ca\(^{2+}\), (3 \times 10^{-6} M) of calmodulin, and (1.6 \times 10^{-9} M) of enzyme.

Data presented in the Lineweaver-Burk plot for the different symbols (+, ●, △, ○) represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 30

A

B
Table 19. Kinetic Constants of Bovine Brain, Heart and Placenta
Phosphodiesterase Purified by Monoclonal Antibody Chromatography^a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ANT-cAMP</th>
<th>MANT-cAMP</th>
<th>ANT-cGMP</th>
<th>MANT-cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>Brain</td>
<td>36.8±3</td>
<td>1.04</td>
<td>45.5±6</td>
<td>.80</td>
</tr>
<tr>
<td>Heart</td>
<td>133±5</td>
<td>.945</td>
<td>29.9±5</td>
<td>0.46</td>
</tr>
<tr>
<td>Placenta</td>
<td>153.7±6.0</td>
<td>2.2</td>
<td>52.8±3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

a Enzymatic reactions were carried out in the presence of 1 mM Ca^{2+} and
(3 x 10^{-6}M) of calmodulin.

b $K_m$ (μM).

c $V_{max}$ (μMoles min^{-1} mg^{-1}) and the uncertainties were within 5% of
experimental error.
5. Investigations of the Kinetic Effects of Reduced Recombinant Oncomodulin (r-ONC) Oxidized Recombinant Oncomodulin (r-ONC-d) on Heart and Placenta Cyclic Nucleotide Phosphodiesterase Catalyzed Hydrolysis of ANT-cGMP

The Ca^{2+}/calmodulin dependent cyclic nucleotide phosphodiesterases from the heart and placenta tissues were isolated using calmodulin-Sepharose affinity chromatography. The kinetic parameters of these enzymes were investigated with respect to the effect of calmodulin and recombinant oncomodulin. The r-ONC and r-ONC-d were checked electrophoretically before investigating their effects on the enzymes. The reduced and the oxidized protein revealed single peaks with apparent molecular weights of approximately 11,800 and 23,000 daltons respectively (Figure 31). The effect of calmodulin, and reduced and oxidized oncomodulin were investigated on the rates of hydrolysis of ANT-cGMP. Activity was measured at pH 7.5 in the presence of excess Ca^{2+} (1 mM) and saturating amounts of either calmodulin, or reduced or oxidized oncomodulin. The plots of the rate of hydrolysis as a function of ANT-cGMP concentration displayed normal Michaelis-Menten kinetic behaviour (Figures 32A-37B). Linear patterns on the Lineweaver-Burk plot were observed on the rates of hydrolysis of ANT-cGMP for the heart and placenta PDE (Figures 32B-37B). With calmodulin, an approximately 2.4 and 5 fold increase in the \( K_m \) were observed for the heart and placenta phosphodiesterases (Table 11). The \( K_m \) values for the heart enzyme increased only about 1.6 fold when investigated, in the presence of either, the reduced or oxidized form of r-ONC. However, for the placenta enzyme, about a 1.6 fold increase in \( K_m \) was only seen with the oxidized form of r-ONC. The \( K_m \) value obtained with the reduced form of r-ONC (\( K_m \); 245.5 \( \mu \)M) was similar to that observed with calmodulin (\( K_m \); 285 \( \mu \)M). The similar \( K_m \) values observed
**Figure 31** SDS polyacrylamide gel electrophoresis of reduced and oxidized recombinant oncomodulin.

Lanes: a. recombinant oncomodulin (10 μg) at time t=0; b. recombinant oncomodulin at time t=2 hrs; c. recombinant oncomodulin at time t=16 hrs (completely oxidized); d. recombinant oncomodulin (reduced form) in the presence of β-mercaptoethanol; e. Molecular weight standards: bovine serum albumin 66 Kd; ovalbumin 45 Kd; glyceraldehyde-3-phosphate dehydrogenase 35 Kd; carbonic anhydrase 29 Kd; trypsinogen 24 Kd; trypsin inhibitor 20 Kd; α-Lactalbumin 14 Kd.
Figure 31
Figure 32  The effect of calmodulin on bovine heart cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

A. Rates of hydrolysis of ANT-cGMP by bovine heart cyclic nucleotide phosphodiesterase. (●) Presence of calmodulin (10⁻⁶ M). Standard reaction in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, Ca²⁺ and 9.75 µg of enzyme. (●) Absence of calmodulin. Theoretical curve obtained from the data is the fit of the data to the Michaelis-Menten equation via simplex fit.

B. Plot of ν⁻¹ versus s⁻¹ of the rates of hydrolysis of ANT-cGMP in the presence of calmodulin. Inset represents the same plot in the absence of the protein. Data presented in Lineweaver-Burk format for the different symbols represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 32

A

$V$ (NMOL/MIN/MG)

[ANT-CGMP] (MICROMOLAR)

B

$1/V$ (nmol/min/mg)

$1/[ANT-cGMP]$ (1/MICROMOLAR)
Figure 33. The effect of reduced recombinant oncomodulin on bovine heart cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate:

A. Rate of hydrolysis of ANT-cGMP by bovine heart cyclic nucleotide phosphodiesterase. (*) Presence of reduced recombinant oncomodulin (10^{-6} M); (+) Absence of reduced recombinant oncomodulin.

Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Ca^{2+} and 9.75 μg of enzyme.

B. Plot of $v^{-1}$ versus $s^{-1}$ of the rates of hydrolysis of ANT-cGMP in the presence of reduced recombinant oncomodulin. Inset represents the data obtained in the absence of the activator protein. Data presented in the Lineweaver-Burk format for the different symbols represent data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 34 The effect of oxidized recombinant oncomodulin on bovine heart cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

A. Rate of hydrolysis of ANT-cGMP by bovine heart cyclic nucleotide phosphodiesterase. (*) Presence of oxidized recombinant oncomodulin (10^{-5}M); (×) Absence of oxidized recombinant oncomodulin. Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Ca^{2+} and 9.75 μg of enzyme.

B. Plot of $v^{-1}$ versus $s^{-1}$ of the rate of hydrolysis of ANT-cAMP in the presence of oxidized recombinant oncomodulin. Inset represents the data obtained in the absence of the activator protein. Data presented in the Lineweaver-Burk format for the different symbols represent data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 34

A

$V$ (NMOL/MS/MG) vs [ANT-CGMP] (MICROMOLAR)

B

$1/v$ (mol/min/mg) vs $1/([ANT-CGMP]$ (1/MICROMOLAR)
Figure 35  The effect of calmodulin on bovine placenta cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate.

A. Rate of hydrolysis of ANT-cGMP by bovine placenta cyclic nucleotide phosphodiesterase. (*) Presence of calmodulin (10^{-6} M); (•) Absence of the activator protein. Standard reaction mixture in 0.2 mL, pH 7.5 contained 36 mM Tris-HCl, 1 mM Ca^{2+} and 7.24 µg of enzyme.

B. Plot of v^{-1} versus s^{-1} of the rate of hydrolysis of ANT-cAMP in the presence of calmodulin. Data presented in the Lineweaver-Burk format for the different symbols represent data obtained from different kinetic runs. The solid line is the theoretical, best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 35

A

$V$ (nmoles/min/mg) vs. [ANT-CGMP] (micromolar)

B

$\frac{1}{v}$ (mmol/min/mg) vs. $\frac{1}{[\text{ANT-CGMP}]}$ (1/micromolar)
Figure 36 The effect of reduced recombinant oncomodulin bovine placenta cyclic nucleotide phosphodiesterase with ANT-cGMP as substrate

A. Rate of hydrolysis of ANT-cGMP by bovine placenta phosphodiesterase. (*) Presence of reduced recombinant oncomodulin (10^-6 M); (+) Absence of the activator protein. Standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Ca^{2+} and 7.24 μg of enzyme.

B. Plot of v^{-1} versus s^{-1} of the rate of hydrolysis of ANT-cGMP in the presence of the protein. Inset represents the same plot in the absence of the protein. Data presented in the Lineweaver-Burk format for the different symbols represent data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 36

A

\[ V \text{ (NMOLES/MIN/HR)} \]

\[ [\text{ANT-CGMP}] \text{ (MICROMOLAR)} \]

B

\[ \frac{1}{V} \text{ (mmol/min/mg)} \]

\[ \frac{1}{[\text{ANT-cGMP}]} \text{ (1/MICROMOLAR)} \]
Figure 37  The effect of oxidized recombinant oncomodulin on bovine placenta cyclic nucleotide phosphodiesterase activity with ANT-cGMP as substrate

A. Rate of hydrolysis of ANT-cGMP by bovine placenta phosphodiesterase (++) Presence of oxidized recombinant oncomodulin; (++) Absence of the activator protein. Standard reaction mixture in 0.2 mL, pH 7.5, contained 36 mM Tris-HCl, 1 mM Ca^{2+} and 7.24 μg of enzyme.

B. Plot of 1/v versus 1/s of the rate of hydrolysis of ANT-cGMP in the presence of the protein. Inset represents the same plot in the absence of the protein. Data presented in the Lineweaver-Burk format for the different symbols represent the data obtained from different kinetic runs. The solid line is the theoretical best fit of the data to the Michaelis-Menten equation via simplex fit.
Figure 37

A

B

\[ \frac{1}{v} \text{ (mol/min/mg)} \text{ vs. } \frac{1}{[\text{ANT-cGMP}]} \text{ (1/micromolar)} \]
Table 11. Kinetic constants of the effect of calmodulin/oncomodulin on Heart and Placenta Phosphodiesterase

<table>
<thead>
<tr>
<th>Effectors(^b)</th>
<th>Heart</th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) ((\mu)M)</td>
<td>(V_{max}) (nmoles min(^{-1})mg(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>90.7±6.2</td>
<td>92.4±6.1</td>
</tr>
<tr>
<td>Calmodulin(^b)</td>
<td>214±10.1</td>
<td>327±10.0</td>
</tr>
<tr>
<td>Reduced oncomodulin</td>
<td>150±5.6</td>
<td>154±7.4</td>
</tr>
<tr>
<td>Oxidized oncomodulin</td>
<td>152.7±2.7</td>
<td>216.7±6.7</td>
</tr>
</tbody>
</table>

\(b\) ± indicates absence or presence of the effector

\(a\) ANT-cGMP was used as substrate. Reactions were carried out in the absence of imidazole, saturating amounts of Ca\(^{2+}\) (1 mM) and the protein (10\(^{-6}\)M).
here for the placenta enzyme appears to indicate that both these forms of protein affect the enzyme allosterically to the same extent thus having similar affinity towards ANT-cGMP as substrate. However, this speculation was not supported by the observed \( V_{\text{max}} \)'s with reduced r-ONC (\( V_{\text{max}} \): 166.3 nmoles min\(^{-1}\)mg\(^{-1}\)) and calmodulin (\( V_{\text{max}} \): 247.5 nmoles min\(^{-1}\)mg\(^{-1}\)). An approximately 5.4 fold and 3.6 fold increase in the \( V_{\text{max}} \) was observed for the calmodulin and reduced r-ONC as compared to their absence. No such increases in the \( V_{\text{max}} \) were observed with heart phosphodiesterase.

6. The Dose Dependent Activation of Heart and Placenta Phosphodiesterases by Recombinant Oncomodulin (ONC) and Calmodulin (CaM)

The enzymes purified by calmodulin Sepharose affinity chromatography were further investigated with respect to effect of varying amounts of recombinant oncomodulin. Both the reduced recombinant oncomodulin (r-ONC) and the oxidized recombinant oncomodulin (r-ONC-d) stimulated the heart PDE approximately 50% and 85% respectively of maximal calmodulin activation. The concentration of (r-ONC) and (r-ONC-d) required for half maximal activation was estimated to be about 1.7 \( \times \) 10\(^{-7}\)M and 3 \( \times \) 10\(^{-8}\)M respectively (Figure 38). Only the effect of the reduced form of r-ONC was investigated with the placenta phosphodiesterase and the half maximal activation was observed at about 9 \( \times \) 10\(^{-8}\)M (Figure 39). The enzyme was stimulated about 25% maximal activation of calmodulin. These stimulatory effects were further investigated with PDE's purified from these tissues by monoclonal affinity chromatography (ACAP-1 and ACC-1).
Figure 38 Differential stimulation of calmodulin-Sepharose affinity
purified bovine heart phosphodiesterase by reduced and
oxidized recombinant oncomodulin. (+) oxidized form; (*) reduced
form. Enzyme activity was determined as a percentage of the
maximal activation obtained with calmodulin. Protein
concentration of the reduced and oxidized form of recombinant
oncomodulin was varied from $10^{-10}$M to about $10^{-5}$M.
Figure 39 The effect of reduced concentrations of recombinant oncomodulin with Calmodulin-Sepharose affinity purified bovine placenta phosphodiesterase. Enzyme activity was determined as a percentage of the maximal activation obtained with calmodulin. Protein concentration of the reduced form of recombinant oncomodulin was varied from \((10^{-10})\text{M}\) to about \((10^{-5})\).
Figure 39
The calmodulin-Sepharose affinity purified preparation of PDE, with added Ca$^{2+}$/calmodulin, were passed through ACAP-1 and ACC-1 monoclonal affinity columns separately. The calmodulin-dependent phosphodiesterase was eluted and subsequently passed through organomercurial Sepharose affinity resin to remove calmodulin. In Table 12 the enzyme activation of the different fractions are summarized. The enzyme preparation free of calmodulin contamination was titrated with the reduced and oxidized form of r-ONC. No effect of the two forms of oncomodulin on the heart and placenta phosphodiesterase could be observed. However the enzymes could be stimulated in their activity by calmodulin exhibiting half-maximal activation at a concentration of 3.16 x 10^{-9} M. When the heart enzyme activity and protein content in the breakthrough fraction of ACAP-1 monoclonal affinity chromatography were investigated, about 70% in enzyme activity and 85% in protein content of that of CaM-Sepharose affinity purified enzyme were observed (Table 12). Similarly, for the placenta enzyme, in the breakthrough fraction of ACAP-1 monoclonal affinity chromatography about 48% in enzyme activity and 30% in protein content of that of CaM-Sepharose affinity purified enzyme were observed. These observations indicate that the enzyme activations observed by the two forms of recombinant oncomodulin on the CaM-Sepharose affinity purified enzymes do not bind to ACAP-1 monoclonal affinity column and are thus present in the breakthrough fraction. This was, therefore, the reason why the ACAP-1 monoclonal affinity purified Ca$^{2+}$/CaM dependant enzymes could not be stimulated by the two forms of recombinant oncomodulin.
Table 12. Heart and Placenta Phosphodiesterase Activities of the CaM-Sepharose Fraction, ACAP-1 eluate and ACAP-1 breakthrough fraction.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction</th>
<th>Total Activity µMoles/Pi/min</th>
<th>Protein content mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>Calmodulin-Sepharose affinity</td>
<td>0.600</td>
<td>0.258</td>
</tr>
<tr>
<td></td>
<td>ACAP-1 affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eluate</td>
<td>1.550</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Breakthrough</td>
<td>0.43</td>
<td>0.218</td>
</tr>
<tr>
<td>Placenta</td>
<td>Calmodulin-Sepharose Affinity</td>
<td>0.312</td>
<td>0.275</td>
</tr>
<tr>
<td></td>
<td>ACAP-1 affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eluate</td>
<td>1.665</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Breakthrough</td>
<td>0.152</td>
<td>0.08</td>
</tr>
</tbody>
</table>
The effect of calmodulin concentration on the stimulation of heart and placenta phosphodiesterase purified by monoclonal (ACAP-1) affinity column chromatography. Enzyme activity was determined as a percentage of the maximal activation obtained with calmodulin. Protein concentration of calmodulin was varied from $10^{-10}$M to about $10^{-7}$M.
7. Differential Stimulation of Brain and Heart Cyclic-AMP Phosphodiesterase by Oncomodulin

The cyclic nucleotide phosphodiesterase from bovine heart and brain were purified using anti-phosphodiesterase (C1 antibody) (160). The enzyme preparations were homogenous as revealed by SDS gel electrophoresis, showing one band at an apparent molecular weight of 59 Kd for the heart and 61 Kd for the brain as previously described (160) (Figure 41).

The primary initial concern was to have the oncomodulin used for the present investigation to be absolutely free of calmodulin contamination. Figure 42A illustrates that by HPLC it was possible to separate, with baseline resolution, oncomodulin from calmodulin or parvalbumin. The purified oncomodulin was ensured to be free of calmodulin contamination as shown in Figure 42B. In addition, further assurance of purity was achieved by taking advantage of the Cys-16 of oncomodulin in the use of organomercurial affinity chromatography (Figure 43A). Mammalian calmodulin lacking this Cys residue was also passed through the same affinity column and found not to be retained. To further assure the purity of calmodulin, anti-calmodulin IgG was added to separate reaction mixtures containing either calmodulin or oncomodulin. In the reaction mixture containing calmodulin, stimulation of enzymatic activity was reduced, however, no reduction in activity was observed when anti-calmodulin IgG was added to the oncomodulin containing reaction mixture (Table I3). The oncomodulin purified by HPLC, and the organomercurial Sepharose affinity resin were used to demonstrate the effect of the protein on brain and heart cyclic nucleotide phosphodiesterase.
**Figure 41**, SDS polyacrylamide gel electrophoresis of bovine brain and heart cyclic nucleotide phosphodiesterases purified by C1 monoclonal affinity chromatography.

Lanes: a, molecular weight standards: bovine serum albumin, 66 Kd; ovalbumin, 45 Kd; glyceraldehyde-3-phosphate dehydrogenase, 35 Kd; carbonic anhydrase, 29 Kd; trypsinogen, 24 Kd; trypsin inhibitor, 20 Kd; α-Lactalbumin, 14 Kd; b, heart phosphodiesterase (3 µg); c, brain phosphodiesterase (6 µg).
Figure 42 The reverse-phase HPLC purification of oncomodulin free of calmodulin contamination. A: Demonstration of base-line resolution in separation of calmodulin, oncomodulin and parvalbumin (20 μg of each protein). B: Preparative purification of oncomodulin (1 mg) showing no evidence for presence of calmodulin. (Courtesy of Dr. J.P. MacManus Animal/Cell Physiology Group, Division of Biological Sciences, Ottawa, Canada.)
Figure 43 Use of sulphydryl affinity chromatography to obtain oncomodulin free of possible contamination. A: Calmodulin was not retained and B: The thiol-reduced fraction of HPLC pure oncomodulin (Figure 42) was retained by the organomercurial resin. The arrow indicates the start of elution with β-mercaptoethanol.
Figure 43

A. Calmodulin

B. Oncomodulin

PROTEIN (μg/mL)

FRACTION (mL)
Oncomodulin was found to stimulate the heart phosphodiesterase approximately five fold higher than the brain phosphodiesterase (Figure 44). The concentration of oncomodulin required for half maximal activation of bovine heart enzyme was estimated to be about (2 x 10^{-7}M). A comparison of the stimulation of heart and brain phosphodiesterase by both calmodulin and oncomodulin is presented in Figure 45. Both calcium-binding proteins stimulated the heart enzyme activity to the same extent (Figure 45A). However, the brain and heart enzymes had different apparent relative affinities for calmodulin, which was even more obvious with oncomodulin. The concentration of calmodulin required for half-maximal activation of heart phosphodiesterase (5 x 10^{-10}M) was about four fold lower than for the brain enzyme (2 x 10^{-9}M). These differences were more dramatic for oncomodulin, where the heart enzyme had an apparent affinity (2 x 10^{-7}M) twenty-five fold lower than that estimated for the brain enzyme (Figure 45C).

These differences observed between the brain and heart phosphodiesterase indicate that the two enzymes are different. The activation by oncomodulin was seen with the heart PDE purified by Cl monoclonal antibody affinity chromatography and not observed with the ACAP-1 or ACC-1 monoclonal antibody purified enzyme, further indicating, that the enzyme purified by the Cl and the ACAP or ACC-1 were of different forms of PDE.
<table>
<thead>
<tr>
<th>Condition</th>
<th>-IgG</th>
<th>+IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO STIMULATION</td>
<td>4.8 (±15)</td>
<td></td>
</tr>
<tr>
<td>Calmodulin (5.5 x 10^-9 M)</td>
<td>15.4 (±36)</td>
<td>10.3 (±.50)</td>
</tr>
<tr>
<td>Oncomodulin (5.0 x 10^-7 M)</td>
<td>11.2 (±.50)</td>
<td>11.0 (±.60)</td>
</tr>
</tbody>
</table>
Figure 44 Similar stimulation of bovine heart phosphodiesterase by oncomodulin purified by reverse phase HPLC ( □ ) and organomercurial resin ( ○ ). Enzyme activities were measured by the enzyme coupled assay.
Figure 44
Figure 45  Differential stimulation of brain (A) and heart (B) phosphodiesterase by oncomodulin (□ or ○), and calmodulin (□ or ○). Enzyme activities were measured by the enzyme coupled assay. Values given are mean ± SEM of at least three separate determinations. C: Replot of A & B to demonstrate the greater sensitivity to oncomodulin of phosphodiesterase from heart compared to brain.
Figure 45

(A) Brain

(B) Heart

(C) MODULATOR (M)

nMOL P₁ / MINUTE
DISCUSSION

Since the existence of multiple molecular forms of phosphodiesterase was initially reported by Thompson and Appleman (135), other laboratories have also shown that multiple forms of phosphodiesterase are present in a variety of tissues and cells (30,33). Many reports have appeared regarding the number of phosphodiesterases present in these different tissue/cell types, as well as the kinetic characteristics of each (139) which may be related to the different methods by which the enzymes were isolated.

It was a goal of the present study to evaluate different molecular forms of Ca\(^{2+}\)/calmodulin dependent phosphodiesterase from bovine brain, heart and placental tissues purified by identical procedures and then to compare their kinetic characteristics with respect to their steady state kinetic parameters, \(K_m\) and \(V_{\text{max}}\), for the hydrolysis of ANT-cAMP, MANT-cAMP, ANT-cGMP and MANT-cGMP.

Substrates utilized here were first reported by Hiratsuka (135). The direct discontinuous kinetic assay presently employed was developed in this laboratory. A disadvantage of using the substrate analogs is that the kinetic constants obtained cannot be directly compared to previous studies where native substrates have been employed. For example, with ANT-cAMP, the \(K_m\) and \(V_{\text{max}}\) that was obtained with the brain PDE was 4.5 fold higher and 50-fold lower, respectively, in comparison to the native substrate. Although a direct comparison is not possible a comparison of the ratios of the kinetic constants with cAMP and cGMP to those obtained for their derivatives is a logical alternative.

The enzymes from the three bovine tissues were purified by two procedures with a common initial purification step: in both cases the soluble tissue
homogenates were precipitated by (NH₄)₂SO₄ (45% saturation). In the CaM-
Sepharose procedure, the fraction precipitated, subsequent to solubilization and
dialysis was subjected to chromatography on affi-blue Sepharose; the protein
fraction eluted by high salt was dialysed and finally subjected to CaM-Sepharose
chromatography. All the latter purification steps were carried out prior to
application onto a column of anti-PDE protein-A-Sepharose. The enzymes from
the various tissues were purified ~50 fold by the CaM-Sepharose and 150-200
fold by the monoclonal procedure. The specific activities of the enzymes fall
well in the range of those previously reported (Table 14).

The enzymes purified by CaM-Sepharose affinity chromatography displayed
catalytic properties of the "high $K_m$" form with both substrates employed (Table
8). The brain PDE was found to be kinetically distinct from the heart and placenta
enzymes as evident by the display of different kinetic parameters. The effect
of calmodulin on the kinetic parameters were substrate dependent. The calmodulin
activated brain PDE had similar $K_m$ values for the cAMP and cGMP analogues,
whereas the $K_m$s with MANT-cGMP for the CaM-activated placenta and heart
enzyme was approximately 5- and 7-fold larger, respectively, in comparison to
MANT-cAMP (Figure 46). The $K_m$ values observed here with MANT-cAMP as
substrate for the calmodulin-dependent PDEs are in agreement with that reported
by other investigators (55,58,59). However, the 5-7 fold higher $K_m$s reported here
for the cGMP-derivative are very unusual in that previous reports indicate that
the $K_m$ ratios of cAMP/cGMP for the enzymes purified by CaM-Sepharose range
between 10 and 23. If the ratio of $V_{max}/K_m$ is used as a measure of substrate
specificity, then the enzyme from brain appears to be specific for both substrates
<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ units/mg</th>
<th>Specific Activity (Units/mg)$^0$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(basal)</td>
<td>310±70</td>
<td>.26</td>
<td>30</td>
<td>55</td>
</tr>
<tr>
<td>(activated)</td>
<td>100±20</td>
<td>1.56</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(basal)</td>
<td>150±50</td>
<td>25$^b$</td>
<td>167</td>
<td>51</td>
</tr>
<tr>
<td>(activated)</td>
<td>150±50</td>
<td>50$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>(activated)</td>
<td>n.d.$^b$</td>
<td>364</td>
<td>58</td>
</tr>
<tr>
<td>cGMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(activated)</td>
<td>200±40</td>
<td>280</td>
<td>96</td>
<td>167</td>
</tr>
<tr>
<td>cGMP</td>
<td>(basal)</td>
<td>3.0±5</td>
<td>n.d.</td>
<td>166</td>
</tr>
<tr>
<td>(activated)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3.4-10</td>
<td>166</td>
</tr>
<tr>
<td>cGMP</td>
<td>(activated)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>300</td>
</tr>
<tr>
<td>cGMP</td>
<td>(activated)</td>
<td>n.d.</td>
<td>2.3</td>
<td>169</td>
</tr>
<tr>
<td>cAMP</td>
<td>(basal)</td>
<td>13.3±5.2</td>
<td>0.12</td>
<td>n.d.</td>
</tr>
<tr>
<td>(activated)</td>
<td>28.9±8.3</td>
<td>0.89</td>
<td>n.d.</td>
<td>164</td>
</tr>
<tr>
<td>cGMP</td>
<td>(basal)</td>
<td>4.76±5.5</td>
<td>1.09</td>
<td>n.d.</td>
</tr>
<tr>
<td>(activated)</td>
<td>2.68±3</td>
<td>13.51</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>60 kDa</td>
<td>66-70</td>
<td>6</td>
<td>n.d.</td>
</tr>
<tr>
<td>(basal)</td>
<td>10-12.5</td>
<td>166</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>(activated)</td>
<td>66-70</td>
<td>1.7</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Molecular Weight (kDa)</td>
<td>Specific Activity (U/mg)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
<td>--------------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>cAMP</td>
<td>(activated)</td>
<td>26-35.5</td>
<td>10</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(basal)</td>
<td>6.0-7.0</td>
<td>3.0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(activated)</td>
<td>1.1-1.2</td>
<td>30.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>aAMP</td>
<td>(activated)</td>
<td>16-18</td>
<td>17</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(basal)</td>
<td>2.5-2.6</td>
<td>53</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a determined at constant substrate concentration

b not determined

32-320 rat brain
24-400 bovine brain
Figure 46 Bar graphs comparing the kinetic constants of CaM-Sepharose affinity purified bovine phosphodiesterases.

Kinetic constants, \( K_m \) (top row) and \( V_{\text{max}} \) (bottom row) with respect to the substrate analogs MANT-cAMP and MANT-cGMP.

(■) Absence of calmodulin; (■■) Presence of calmodulin.
whereas that of the heart and placenta appear to be more cAMP specific. In the presence of calmodulin, with MANT-cGMP as substrate, a decrease in the 
$K_m$ and $V_{max}$ was observed as compared to an increase in the $K_m$ and $V_{max}$ for the heart and placenta. A calmodulin-sensitive cyclic GMP phosphodiesterase Mr 74,000 from bovine brain have been reported to have the opposite effect in that the Ca$^{2+}$/CaM increases the $K_m$ and $V_{max}$ for cAMP and decreases the $K_m$ and increases the $V_{max}$ for cGMP hydrolysis (163).

The data presented here suggest that the brain placenta and heart enzymes purified by CaM-Sepharose display different kinetic properties than previously reported and are therefore distinct PDE forms. However, it must be stressed that the observed kinetic parameters could arise from the denaturation of the enzymes, proteolytic or otherwise during the lengthening purification procedures. In addition CaM-Sepharose purification procedure is not 100% specific for PDE as evidenced by the presence of minor contaminant bands on SDS PAGE. The presence of cNMP binding proteins as contaminants would potentially result in higher $K_m$ values.

The conformation specific ACC-1 and ACAP-1 (2) enabled the purification of brain low and high Mr PDE forms as well as Mr ~60,000 species from the heart and the placenta. The kinetic parameters obtained with ANT- and MANT- derivatives of both cNMPs clearly indicate that the four PDE forms are clearly distinct from each other and from those purified by the CaM-Sepharose procedure. With the MANT- derivatives the cAMP/cGMP $K_m$ ratios were near unity for the brain isozymes; ratios of 4.2 and 3.3 were obtained with the heart and the placenta enzymes respectively. Thus in terms of substrate specificity (approximately by $V_{max}/K_m$) the brain enzymes are specific to both cNMPs whereas the heart and the placenta enzymes appear to be cGMP specific. Although the brain PDEs
obtained by either purification procedure appear to be similar with respect to substrate specificity, the $K_m$s displayed by the monoclonal-purified enzymes were 4-7 fold less.

A model for the catalytic sites of cGMP-dependent PDEs from rat liver and the slime mold has been proposed (133). In this model, the cNMPs are bound to the enzyme by $H$-bonding and ionic interactions at the base and at the ribosyl phosphate residue (Figure 48a). These authors conclude that the cGMP analogues are more strongly bound and therefore display lower $K_m$s primarily because of greater $H$-bonding/polarizability potential with guanine in comparison to adenine. The sugar moiety containing the cyclic phosphate is thought to be immobilized with a primary interaction between cationic side chain on the enzyme and the delocalized negative charge on the cyclic phosphate. A weaker interaction at the 2'-OH has also been proposed. As can be seen from Figure 5, ANT and MANT derivatives employed in this study differ only with respect to N-methyl substitution. Yet this slight structural variation between the derivatives can give rise to such large differences in the kinetic parameters. The results obtained here are thought to be related to formation of an intramolecular $H$-bond between the ANT or MANT nitrogen and the delocalized negative charge on the 3',5'-cyclic phosphate oxygen (Figure 48b). The formation of such a hydrogen bond is less likely with the MANT-derivatives because of the steric hindrance introduced by the N-methyl group. This intramolecular $H$-bond, when formed (ANT-derivative), would effectively reduce the negative charge on the cyclic phosphate oxygen, thus decrease the magnitude of binding interactions between the cationic active site of protein side chain and the pseudo
Figure 47 Bar graph comparing the kinetic constants of monoclonal affinity purified bovine phosphodiesterases.

Kinetic constants $K_m$ (top row) and $V_{\text{max}}$ (bottom row) with respect to the substrate analogs ANT-cAMP, MANT-cAMP, ANT-cGMP and MANT-cGMP.
Figure 47
Figure 48a: Model for the interactions of cGMP with the A* site of the cyclic nucleotide phosphodiesterase. Part of an amino acid (A) functioning as a hydrogen donor; part of an amino acid (B) functioning as a hydrogen acceptor; x: positively charged amino acid side-chain (133).

Figure 48b: Model for the possible intramolecular H-bonding ANT-cAMP between the nitrogen and the delocalized negative charge of cyclic phosphate.
substrate. The effect of this on the kinetic parameter would be an increase in $K_m$ and $V_{\text{max}}$. This is precisely what is observed. MANT-derivatives of cNMPs displayed lower $K_m$'s and higher $V_{\text{max}}$ in comparison to the ANT-derivatives. However, if the active-site cationic protein side chain is closer than the amino hydrogen to the cyclic phosphate negative charge, then the intramolecular hydrogen bonding effect would be minimized thus making the ANT and MANT derivatives kinetically indistinguishable. This is thought to be the case with the 63,000 Mr brain and the heart enzyme where the $K_m$'s for ANT and MANT-cAMP were comparable within experimental error. On the other hand, the proposed cationic active-site side chain would appear to be more distant from the cyclic phosphate negative change in the placenta and the lower Mr brain enzymes. This conclusion is reached since the ANT-cAMP derivatives with H-bonding potential displayed ~3-fold higher $K_m$'s in comparison to the MANT derivatives.

The low and high Mr brain enzymes purified in the present study were kinetically distinct from those reported by Sharma et al (161): both Sharma enzymes gave cAMP/cGMP $K_m$ ratios ~10, whereas in the present study both isoforms displayed cAMP/cGMP ratios of near unit (MANT-derivatives). In addition, the lower Mr consistently displayed lower $V_{\text{max}}$ with all the pseudosubstrates. The $V_{\text{max}}$ ratios cAMP/cGMP for the lower Mr enzyme was .25 and .4 for both derivatives, whereas the ratio for the higher Mr PDE were .167 and .68 with the ANT- and MANT-derivatives respectively. In contrast, Sharma et al reported cAMP/cGMP $V_{\text{max}}$ ratios for the lower Mr and higher Mr enzymes of 1.8 and .33 respectively. One potential reason for the observed differences could be related to the degree of phosphorylation of the enzymes in the present study. However, this is unlikely since, phosphorylation of the lower Mr enzymes while decreasing the CaM affinity by 20-fold (5nM to 10nM) appears not to affect $K_m$ nor $V_{\text{max}}$. 
(161). Furthermore, artifactual change in the kinetic parameters as a result of lowered CaM saturation in response to lowered CaM affinity can also be ruled out as the present studies were performed in the presence of excess CaM (1E-7M).

Oncomodulin is a tumor protein which has been detected in approximately 85% of all tumor cell lines and tumors from mouse, rat and man (120). Recently, oncomodulin was found to be a normal constituent of human and rodent placenta. It has been shown that this protein belongs to the calmodulin multigene family of calcium binding proteins which can undergo a calcium dependent conformational change (128,130) and mimic calmodulin in stimulation of rat heart cyclic nucleotide phosphodiesterase (126).

Sharma and Wang (161) have purified bovine brain PDEs using CI monoclonal affinity chromatography and reported the enzyme to have higher $V_{\text{max}}$ towards cAMP hydrolysis. In the present study employing the same monoclonal affinity column, brain and heart PDEs were purified and compared with respect to their effect with calmodulin and oncomodulin. Both calcium binding proteins stimulated the heart enzyme activity to the same extent. However, the heart and brain enzymes had different relative affinities for calmodulin, which was even more obvious with oncomodulin. The apparent calmodulin binding affinity for the heart enzyme was estimated to be about four fold lower than that observed for the brain enzyme. With oncomodulin the relative apparent affinities were even more dramatic as the heart enzyme had an apparent affinity twenty-five fold lower than that estimated for the brain enzymes. These observations of the differential stimulation of the brain and heart enzyme cAMP hydrolysis by oncomodulin and calmodulin indicate the two enzymes to be different which agrees with the electrophoretic differences observed here and previously reported (65).
An attempt was made to make similar investigation with the PDEs isolated using ACC-1 and ACAP-1 monoclonal affinity column. It was mentioned earlier, that the PDEs purified by the ACC-1 or ACAP-1 monoclonal affinity could be distinct from those purified by the C1 or A6 monoclonal affinity column. The dimerization of oncomodulin via the Cys-16 amino acid residue has been demonstrated electrophoretically in this laboratory. The disulphide linked form of ONC appears to have properties of calmodulin (165). Hence, the second goal of this study was to investigate the apparent binding affinities of the PDE towards these Ca$^{2+}$-binding proteins CaM, ONC and ONC-d. To this end bovine heart and placenta phosphodiesterase purified by ACC-1 and ACAP-1 monoclonal affinity were investigated as to the rates of ANT-cGMP hydrolysis. The ACAP-1 or ACC-1 purified enzymes were first separated from calmodulin using organomercurial Sepharose resin before investigating their effect on the calcium binding proteins (r-ONC, r-ONC-d and CaM). Surprisingly, neither the heart nor placenta PDEs were stimulated by r-ONC and r-ONC-d. However, they were stimulated by calmodulin with apparent affinities (concentration at half maximal activation) of 3.16 x 10$^{-9}$ M and 1.7 x 10$^{-9}$ M for the heart and placenta PDEs, respectively. Therefore these results suggest that there exists a class of Ca$^{2+}$/calmodulin dependent phosphodiesterase in the heart and placental tissue that can be activated by reduced or oxidized r-ONC. This form of the enzyme cannot be purified with the ACAP-1 or ACC-1 antibodies, however, can be purified using C1 monoclonal affinity columns. Thus, it can be speculated that the two types of monoclonal affinities employed here, purify different forms of Ca$^{2+}$/calmodulin dependent enzymes and only the C1 monoclonal affinity purified appears to be sensitive to oncomodulin.
Owing to the inavailability of C1 monoclonals, the kinetic parameters of CaM-Sepharose purified heart and placenta enzyme were investigated instead on the rates of hydrolysis of ANT-cGMP. With ONC-d the heart enzyme had an apparent affinity (3 x 10^{-6} M) about 10-fold lower than that estimated with r-ONC (1.7 x 10^{-7} M) (Figure 34). This indicated that ONC-d behaves more like calmodulin. The effect of only the reduced form of ONC could not be investigated with placenta because of the lack of tissue availability. An apparent affinity of about (2.5 x 10^{-7} M) was displayed by the placenta PDE towards ONC. The kinetic parameters determined on the rates of substrate hydrolysis by heart and placenta PDEs were more informative. In the presence of these Ca^{2+} binding proteins, both the heart and placenta enzymes displayed an increase in the $K_m$ as well as the $V_{max}$. With calmodulin, heart and placenta $K_m$s for ANT-cGMP increased about 2.5 fold and 4 fold respectively with a corresponding increase $\sim 3.6$ and $\sim 5.36$ fold in the $V_{max}$. However, in the presence of reduced ONC the placenta exhibited similar $K_m$ value with about 3.5 fold increase in the $V_{max}$. These types of observations were not seen with the heart enzyme (Table 11). This distinct kinetic difference for the two enzymes was further examined in the presence of r-ONC-d. Both the enzymes display similar $K_m$ (150-160 µM), however, only about a 2 fold increase in the $V_{max}$ was observed for the placenta with a larger increase in the $V_{max}$ for the heart (Table 11). Although the two enzymes appear to display similar kinetic constants in the presence of calmodulin, the values differ with r-ONC and r-ONC-d, which can be used as a means to distinguish the heart and placenta PDE. Furthermore, since higher $V_{max}$ values have been observed in the presence of r-ONC and r-ONC-d as compared to calmodulin, it indicates that these calcium binding proteins may be regulatory in nature like calmodulin. The inability of oncomodulin
to the same extent as calmodulin may be attributed to the source of oncomodulin, which has been isolated from rat hepatoma. The exact role of oncomodulin is not known but from the results presented here it appears to have a regulatory effect on PDEs which in turn controls the levels of cyclic nucleotide concentrations especially in tissues where it has been reportedly found.

The present comparative kinetic study apart from detecting two CaM-dependent PDE forms from the placenta, have added to the list of kinetically distinct CaM-dependent PDEs from the brain and the heart. It should be pointed out that these distinct kinetic PDE forms we have isolated could be denatured, proteolytically or chemically altered or that the distinct kinetic parameters we have observed could be caused by proteins that have been co-purified. On the other hand if the results presented here, are a true indication of the in vivo situation, what would be the need for so many different PDE forms? It would be tempting to speculate that by having many CaM-dependent PDEs each with a particular substrate specificity and affinity in addition to differing affinities for CaM, the cNMP concentrations would decrease in the form of a non-linear gradient. The advantage to the cell of a non-linear decrease in [cNMP] would be sequential turning of processes with specific cNMP affinities in response to the gradient. Theoretically, the CaM-dependent decrease subsequent to cessation of external stimuli could be simulated once the kinetic parameters for all the CaM-dependent PDE forms have been determined. Furthermore, comparison of the simulated cNMP gradients from normal and transformed tissues could be potentially useful in implicating such gradients in growth and proliferation.

In conclusion, the original aspects of the present study were:
1. The successful development of a direct discontinuous fluorescent assay for determining the rates of substrate hydrolysis by bovine PDEs.

2. Bovine placenta Ca\(^{2+}\)/calmodulin sensitive phosphodiesterase has been isolated and characterized for the first time in this laboratory.

3. Catalytic properties of the brain PDEs were differentiated from the heart and placenta PDEs.

4. Four different forms of calmodulin dependent PDEs were isolated employing monoclonal affinity columns and characterized kinetically.

5. The effect of monomer and the dimer form of recombinant oncomodulin was demonstrated with respect to its stimulatory effect on bovine brain heart and placenta PDE.
APPENDIX I

Steady State Kinetics of Enzyme Catalyzed Reactions

Michaelis-Menten theory assumes that the enzyme E first combines with the substrate to form the enzyme substrate complex ES. The latter then breaks down in a second step to form the free enzyme and product P

$$E + S \xrightleftharpoons[k_1^{-1}]{k_2} ES \xrightarrow{k_2} E + P$$

The initial velocity of an enzyme-catalyzed reaction is equal to the rate of breakdown of the enzyme substrate complex. Therefore the first order rate equation can be represented by:

$$v_0 = k_2 [ES]$$

Within seconds of mixing enzyme and substrate, the concentration of enzyme substrate complex reaches a steady state. Since the concentration of the complex is constant, its rate of change is zero. That is

$$\frac{d[ES]}{dt} = 0$$

The formation of [ES] can only occur one way, whereas the disappearance of the complex can occur two ways, either the conversion to reactants or the conversion to product. Thus,

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] - k_4[ES]$$

The total enzyme concentration at any instant of the reaction can be represented by

$$E_T = [E] + [ES]$$

where E is the concentration of free enzyme and [ES] is the concentration of enzyme substrate complex.
\[ [ES] = [E]_T - [E] \]  

(5)

Substituting 3 in 5 and rearranging

\[ [ES] = \frac{[E]_T [S]}{k_{-1} + k_2/k_{+1}} + [S] \]  

(6)

Substituting equation 6 in the first order rate equation

\[ V_0 = \frac{k_2 [E]_T [S]}{k_{-1} + k_2/k_{+1}} + [S] \]  

(7)

the composite team \( k_{-1} + k_2/k_1 \) is referred to as the Michaelis constant \( K_m \)

\[ K_m = k_{-1} + k_2/k_1 \]  

(8)

Therefore, equation 7 can be rewritten as follows,

\[ V_0 = \frac{k_2 [E]_T [S]}{K_m + [S]} \]  

(9)

For any given enzyme reaction \([E]_T\), \( k_2 \), and \( K_m \) are constants. Considering two limiting cases, first for low substrate concentrations \([S] \ll K_m \) equation 9 reduces to

\[ V_0 = \frac{k_2 [E]_T}{K_m} [S] \]  

(10)

Since the terms in parentheses in equation 10 are all constants, the rate law can be written as

\[ V_0 \approx k_2 [E]_T [S] \]  

(11)

and the reaction approaches first order. Second, at high substrate concentrations \([S] \gg K_m \) equation 10 reduces to

\[ V_0 = k_2 [E]_T \]  

(12)

Both \( k_2 \) and \([E]_T\) are constant. We define the maximum rate of reaction \( V \) as

\[ V = k_2 [E]_T \]  

(13)
Since the maximum rate of reaction is independent of substrate concentration, the reaction is zero order.

Equation (9) can be rewritten as

$$V_o = \frac{V[S]}{K_m + [S]}$$

Equation 14 is the Michaelis-Menten rate law. When the substrate concentration equals $K_m$, equation 14 becomes

$$V_o = \frac{1}{2} V$$

The value of $K_m$ then is that substrate concentration which gives half maximal reaction velocity. The enzyme is half-saturated when $[S] = K_m$. The value of $K_m$ characterizes the interaction of an enzyme with a given substrate. The allosteric effectors of regulatory enzymes can alter the rate of reaction by changing the value of $K_m$ for a substrate. Most allosteric regulatory enzymes have altered $K_m$ values in the presence of allosteric effectors.
APPENDIX II

Definition of $R_f$ value.

$$R_f = \frac{\text{Distance moved by the solute}}{\text{Distance moved by the solvent front}}$$
APPENDIX III

Diagrammatic representation for the large scale, semi-automated kinetic analysis of PDEs.
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EDUCATION

1981-1987 Ph.D. Biochemistry
Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada

Department of Chemistry, University of Windsor, Windsor, Ontario, Canada

1977-1979 M.Sc. Radiation Biophysics
Department of Medical Biophysics, University of Dundee, Scotland, United Kingdom

1975-1976 B.Sc. (Hons) in Physics
Department of Biophysics, Punjab University, Chandigarh, India

AWARDS

1986-87 University of Windsor Scholarship
1997 Summer Research Assistantship

AFFILIATIONS

Member of the American Association of Clinical Chemists

PUBLICATIONS
