Purification and characterization of bovine calmodulin-dependent phosphodiesterases and non-enzymatic glucosylation of calmodulin.

Jaswinder Grewal
University of Windsor
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PURIFICATION AND CHARACTERIZATION OF BOVINE CALMODULIN-DEPENDENT
PHOSPHODIESTERASES

AND

NON-ENZYMATIC GLUCOSYLATION OF CALMODULIN

by

Jaswinder Grewal

A Dissertation
Submitted to the Faculty of Graduate Studies and Research 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

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1991
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   Year: 1980
   Chapter: Calmodulin
   Authors: C. B. Klee, T. H. Crouch and P. G. Richman
   Page: 496
   Figure to be used: Primary structure of calmodulin.

2) Journal: Annual Review of Pharmacology and toxicology
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   Year: 1985
   Title of the Article: The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation
   Authors: L. J. Ignarro and P. J. Kadowitz
   Page: 186
ABSTRACT

PURIFICATION AND CHARACTERIZATION OF BOVINE CALMODULIN-DEPENDENT
PHOSPHODIESTERASES

AND

NON-ENZYMATIC GLUCOSYLATION OF CALMODULIN

In the present study, three calmodulin-dependent phosphodiesterases, $M_r$ 60,000 (low) and $M_r$ 63,000 (high) from the bovine brain as well as $M_r$ 59,000 species from heart, have been compared with respect to their steady-state kinetic parameters for the hydrolysis of cAMP and cGMP and their 2'-O-anthraniol- and 2'-O-(N-methylanthraniloyl)-derivatives. Kinetic studies with the native substrates indicate that the high molecular weight brain phosphodiesterase is cGMP specific whereas, the heart and low molecular weight brain enzymes are equally specific to cAMP and cGMP hydrolysis. These enzymes appear to be kinetically distinct from those previously isolated from the bovine brain and heart (Sharma et al. (1988) Mol. Aspects. Cell. Reg. 5, 265-295). With exception of some differences in $V_{\text{max}}$, the isoforms were indistinguishable with respect to their relative affinities for cAMP and cGMP. Using pseudosubstrates, a distinction could be made between the three enzymes. The major effect of the substitutions, at the 2'-O-position of the cyclic nucleotides, was on the maximal velocity where, the $V_{\text{max}}$ values were 1-11% of those observed with the native substrates. These results indicate that the substitutions have little effect on the relative affinities of the enzymes for the substrates but, lower their catalytic efficiency. The high $M_r$ brain enzyme displayed higher kinetic parameters with the anthraniloyl derivatives. The low $M_r$ brain enzyme exhibited such a trend with cGMP derivatives and not cAMP derivatives. The heart phosphodiesterase was insensitive to the structural differences of the 2'-O- substituents. The higher kinetic parameters observed with the brain enzyme are thought to be related to the formation of an intramolecular hydrogen bond. The formation of such a bond would effectively interfere in the interactions between the active-site
residues and the pseudosubstrates. The insensitivity of the heart enzyme to the derivatives could arise if the active site of this enzyme differs in such a way as to not allow the formation of the intramolecular H-bond.

A calmodulin-dependent phosphodiesterase from uterine smooth muscle has been partially purified and characterized. The enzyme was purified about 100-fold by DEAE ion-exchange, blue-Sepharose and calmodulin-Agarose affinity chromatography. The uterine isoform is stimulated 2-3 fold by calmodulin with an apparent $K_{activity}$ of approximately 10 nM. Furthermore, unlike most of the other calmodulin-dependent phosphodiesterases, the uterine enzyme hydrolyzes cAMP and cGMP with nearly equal affinity ($K_m \approx 30 \mu M$). Comparison of inhibition profiles of the uterine enzyme with the aortic enzyme, with various drugs, indicates that both the enzymes had identical drug sensitivities. Selective inhibitors of calmodulin-dependent phosphodiesterases, vinpocetine and 8-methoxymethyl isobutylxanthine, inhibited both the isoforms with same potency ($IC_{50}$s for vinpocetine and 8-methoxymethyl isobutyl xanthine being about 30 $\mu M$ and 12 $\mu M$, respectively. It has been previously demonstrated that the calmodulin-dependent phosphodiesterases from vascular smooth muscles, exhibit different kinetic properties but are characterized by having similar drug sensitivities. In view of the above observation plus the fact that vascular tissues are of limited supply and contain low amounts of these phosphodiesterases, employing the uterine enzyme for screening new drugs would be useful.

In vitro non-enzymatic glucosylation of calmodulin has been demonstrated. A new method has also been developed for quantitating the extent of glucosylation of proteins. This assay is based on the periodate oxidation of the glucosylated protein releasing formaldehyde. The latter is complexed with the reagent 3-methyl-2-benzothiazolinone hydrazone to produce a chromogen with an absorption maxima at 630 nm. This assay is more sensitive than most of the methods currently employed for quantitating glucosylated proteins. Using this assay it has been shown that in the presence of calcium, approximately 2 mols of glucose are incorporated per mol of calmodulin. The effect of the modification was studied on three enzymes, heart
phosphodiesterase, calcineurin and (Ca^{2+}+Mg^{2+})-ATPase. It was observed that glucosylation of CaM did not drastically alter its ability to stimulate the three target enzymes.
Dedicated to my mother and father

Mohinder Kaur and Balwant Singh Grewal
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<td>advanced glucosylation end-products</td>
</tr>
<tr>
<td>β-ME</td>
<td>beta-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Ca²⁺</td>
<td>calcium</td>
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<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaN</td>
<td>calcineurin</td>
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<td>CGI-PDE</td>
<td>cGMP-inhibited phosphodiesterase</td>
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<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>EGTA</td>
<td>ethylene glycol bis (beta-aminoethyl ether) N, N', N'-tetraacetate</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
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<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methyl-xanthine</td>
</tr>
<tr>
<td>IC50</td>
<td>inhibitor concentration which brings about a loss of 50% of enzyme activity</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>Lys</td>
<td>lysine</td>
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<td>MBTH</td>
<td>3-Methyl-2-benzothiazolinone hydrazone</td>
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<tr>
<td>Mg²⁺</td>
<td>magnesium</td>
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<td>MLCK</td>
<td>myosin light chain kinase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Mr</td>
<td>relative molecular weight</td>
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<tr>
<td>NBT</td>
<td>nitrobluetetrazolium</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PDE</td>
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<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>TPM</td>
<td>tetraazapentamethine dye</td>
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<td>Ul-PDE</td>
<td>uterine phosphodiesterase</td>
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CHAPTER 1-Introduction

1. OVERVIEW

Ca\textsuperscript{2+} plays a key role in the regulation of various biological processes. It acts as a messenger transmitting the signal received by the cell to many intracellular biochemical processes. The external signal is delivered to the cell by molecules collectively termed as \textit{first messengers}. Some examples of the first messengers are noradrenaline, serotonin, histamine, and lectins. Stimulation of the cell by these first messengers triggers the release of \textit{second messengers} which in turn communicate the message to the enzymes and other proteins present in the cell. Ca\textsuperscript{2+} functions as a second messenger. Cyclic nucleotides (cAMP and cGMP), the calcium-binding protein (CaM) and phosphatidylinositol 4,5-bisphosphate are some other examples of second messengers.

Stimulation of the cell results in an influx of calcium and the intracellular Ca\textsuperscript{2+} ion concentration increases from 10\textsuperscript{-7} M to 10\textsuperscript{-3} M. At high cytosolic Ca\textsuperscript{2+} levels, the ion binds to CaM and induces a conformational change in the protein. In this active conformation, CaM can associate with its target proteins and regulate their activity. At the end of this external stimulation, the Ca\textsuperscript{2+} levels of the cell drop down to 10\textsuperscript{-7} M, the Ca\textsuperscript{2+}-CaM complex dissociates and CaM returns to its inactive conformation and can no longer regulate its target enzymes. Ca\textsuperscript{2+} can also directly stimulate enzymes like the membrane bound adenylate cyclase which catalyzes the synthesis of other second messengers (cAMP from ATP).

The present study describes two projects, purification and characterization of calmodulin-dependent phosphodiesterases and \textit{in vitro} glucosylation of calmodulin. The introduction is divided into four sections and includes a review on the calcium binding protein, calmodulin, followed by description on the general features of the enzyme, phosphodiesterase. The latter section also includes a detailed discussion on Ca\textsuperscript{2+}/CaM-dependent PDEs. Finally, the process of non-enzymatic glucosylation is discussed followed by the goal of the present study.
1.1 CALMODULIN

W.Y. Cheung first detected CaM in 1970 as a protein factor which stimulates the enzyme cyclic nucleotide phosphodiesterase (PDE) from bovine brain (Cheung, 1970). Kakiuchi and Yamazaki (1970 a,b) made similar observations and were first to demonstrate that PDE stimulation by CaM is Ca\(^{2+}\)-dependent. CaM is present, in varying concentrations, in all mammalian tissues (Appleman et al., 1973; Wells and Hardman, 1977). Highest concentrations of the protein are found in the brain and the testis, 500 mg/kg and 450 mg/kg, respectively (Klee and Vanaman, 1982). The molecular weight of CaM, based upon its amino acid sequence is 16,790 daltons. It is a heat stable protein and can withstand temperatures as high as 80\(^\circ\). (Klee et al., 1980). The primary structure of CaM is shown in Figure 1. The amino acid composition of CaM is characterized by the presence of single residues of histidine and trimethyllysine (Watterson et al., 1980). The N-terminal residue of all calmodulins isolated to date is blocked by acetylation. Acetyllanine is the N-terminal amino acid of bovine brain CaM (Klee and Vanaman, 1982). The presence of 27 glutamate and 23 aspartate residues result in the highly acidic nature of the protein (pl 4.0-4.3). CaM primary structure is devoid of tryptophan and cysteine residues, an exception being spinach CaM which shows a single cysteine residue (Lukas et al., 1984). CaM also has a high phenylalanine to tyrosine ratio. The latter gives the protein a characteristic UV absorption spectrum where the fine structure of the absorption band of phenylalanine residues is readily detectable. CaM has an extinction coefficient of 1.8-2.0 M\(^{-1}\) cm\(^{-1}\) at 276 nm (Klee et al., 1980)

1.2 CALMODULIN-DEPENDENT CELLULAR PROCESSES

CaM is a major intracellular Ca\(^{2+}\)-binding protein in eukaryotic cells. It regulates various cellular processes (summarized in Figure 2) for example, regulation of cyclic nucleotide levels, regulation of cell motility, regulation of metabolic pathways, and the regulation of Ca\(^{2+}\) pumps
Figure 1: Primary structure of bovine brain calmodulin. The amino acids are represented by one letter codes: 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 Ca\(^{2+}\)-binding domains are designated as I-IV and the darker circles indicate amino acids forming the \(\alpha\)-helices of the Ca\(^{2+}\)-binding domains. (Klee, 1980; Reproduced with permission, from the Annual Review of Biochemistry, Vol. 49, \(\textcopyright\)1980 by Annual Reviews Inc.).
Figure 2: Regulation of cellular processes and enzymes by Ca\(^{2+}\)/CaM. Abbreviations: PDE, cyclic nucleotide phosphodiesterase; AC, adenylate cyclase; Phos. b kinase, phosphorylase b kinase; CaM, calmodulin; ATPase, Mg\(^{2+}\)-activated, Ca\(^{2+}\)-dependent ATPase, MLCK, myosin light chain kinase (Kincaid and Vaughan, 1986; reproduced with permission).
(Klee and Vanaman, 1982).

**Regulation of cyclic nucleotide levels:** Cyclic nucleotides are synthesized by the enzymes adenylate cyclase and guanylate cyclase. Only adenylate cyclase is regulated by CaM. The degradation of cyclic nucleotides is brought about by the enzyme cyclic nucleotide phosphodiesterase.

CaM regulated adenylate cyclase has been demonstrated in the brain (Brostrom et al., 1975), C6 astrocytoma cells (Brostrom et al., 1976) and pancreatic islets (Valverde et al., 1979). CaM stimulation of adenylate cyclase is dependent on the presence of a guanine nucleotide binding protein factor, G/F (Brostrom et al., 1976). It was later demonstrated that the stimulation of adenylate cyclase by CaM requires the presence of both, G/F and a GTP analog, 5'-guanylylimidodiphosphate (GppNHP) (Toscano et al., 1979). CaM stimulation of adenylate cyclase increases the maximum velocity of the enzyme without any affect on the $K_m$.

To date, 7 major families of the enzyme phosphodiesterase have been identified. Of these, only one type of PDE is regulated by CaM. Within these CaM-dependent PDEs, several isoforms have been identified (Beavo, 1988). It has been postulated that interaction of CaM-dependent PDEs with CaM requires four Ca$^{2+}$ ions bound to the calcium binding domains of CaM. The $V_{\text{max}}$ calculated for PDE in the presence of CaM is about 10-15 fold higher than in the absence of CaM (Sharma et al., 1988).

**Regulation of cell motility:** The two major proteins responsible for cell motility are actin and myosin (Clarke and Spudich, 1977). For muscle contraction, the actin-myosin (acto-myosin) complex formation is essential. This complex is formed only if myosin is phosphorylated. Myosin kinase catalyzes the phosphorylation of myosin and is composed of two subunits, a large subunit, the apoenzyme and a small regulatory subunit which binds Ca$^{2+}$ (Pires and Perry, 1977). The regulatory subunit was identified to be CaM (Yagi et al., 1978; Drabikowski et al., 1978).

CaM is also involved in the maintenance of cell ultrastructure. Microtubules are essential components of the cytoskeleton (Klee and Vanaman, 1982) and are associated with the various aspects of cell movement, like flagellar and ciliary motility and also chromosome movement.
during mitosis and meiosis (Walsh et al., 1978). Proper functioning of the cytoskeletal processes requires polymerization of microtubules. The polymerization in turn occurs only if the cytoskeletal proteins are phosphorylated. The latter process is catalyzed by a kinase which in turn is regulated by CaM (Klee and Vanaman, 1982).

**Regulation of Ca$^{2+}$ fluxes:** The various processes regulated by Ca$^{2+}$ are dependent on the transient increase in the intracellular concentrations of the ion. Ca$^{2+}$ transport across the plasma membrane is controlled by two processes: non-energy linked Na$^{+}$/Ca$^{2+}$ exchange and the energy requiring Ca$^{2+}$ transport catalysed by the membrane bound enzyme Ca$^{2+}$-ATPase which is CaM-dependent (Bond and Clough, 1973).

Cytosolic Ca$^{2+}$ levels in muscles are also regulated by a Ca$^{2+}$-dependent ATPase located in the sarcoplasmic reticulum (SR) membrane system. The activity of this enzyme is coupled with transport of Ca$^{2+}$ from the cytosol into the SR lumen. The activity of this enzyme is regulated by cAMP- and Ca$^{2+}$-dependent phosphorylation. The Ca$^{2+}$-dependent modification of Ca$^{2+}$-ATPase is mediated by the protein phospholamban which in turn has to be phosphorylated to be active (Tada et al., 1975, 1979). The phosphorylation of phospholamban is brought about by a kinase which is stimulated by CaM (Katz and Remtulla, 1978). Thus CaM not only interacts with Ca$^{2+}$ but also regulates the concentrations of the ion.

The other processes regulated by CaM include lipid metabolism, glycogen metabolism, egg fertilization, cell proliferation, chemotaxis and secretion processes.

1.3 **CALCIUM-BINDING TO CALMODULIN**

CaM is a member of the Troponin C superfamily which includes the proteins, troponin C, parvalbumins, S-100 protein, oncomodulin and the vitamin D-dependent protein. These proteins are characterized by the presence of Ca$^{2+}$-binding domains having a helix-loop-helix structure (Kretsinger, 1976). CaM has four calcium binding domains numbered I, II, III and IV starting from the amino terminus, Figure 1. Domains II and III are connected by a long central helix.
The tertiary structure of CaM, with four Ca$^{2+}$ ions bound to it, is dumb-bell shaped (Babu et al., 1985). The two lobes at each end are connected by a α-helix and each lobe consists of a set of calcium binding sites (Babu et al., 1985).

X-ray crystallographic and $^{113}$Cd-NMR studies on various calcium-binding proteins indicate that the oxygen ligands which participate in Ca$^{2+}$ binding are contributed by the following: β-carboxyl group of aspartic acid, carboxylate oxygens of glutamic acid, carbonyl oxygens of asparagine and glutamine and the hydroxyl oxygens of threonine and serine. X-ray crystallographic studies have further elucidated that the Ca$^{2+}$ ion bound to the domain exhibits an octahedral structure (Kretsinger and Nockolds, 1973; Szebenyi et al., 1981; Babu et al., 1985).

The binding of Ca$^{2+}$ to CaM exhibits positive cooperativity and causes a stepwise change in the conformation of the protein. The cooperativity is observed only in the presence of Mg$^{2+}$ (Klee et al., 1988). The authors fit the data obtained from Ca$^{2+}$ binding studies of CaM to a model which assumed the presence of two independent sets of sites; each set consists of two highly cooperative Ca$^{2+}$ binding domains, one having high affinity for Ca$^{2+}$ and the other set having low affinity for Ca$^{2+}$. In the absence of Mg$^{2+}$, the affinities of the Ca$^{2+}$ binding sites of the two sets are very similar and show no positive cooperativity. From these results, it was postulated that in the presence of Mg$^{2+}$, the binding of Ca$^{2+}$ to the high affinity sites is increased with a simultaneous decrease in the binding of Ca$^{2+}$ to the low affinity sites. Hence, binding of the first two Ca$^{2+}$ ions takes place at the high affinity sites (Klee et al., 1988). This effect of Mg$^{2+}$ was observed only in the intact protein and not in CaM fragments. It is postulated that the central α-helix probably contains a Mg$^{2+}$-binding site (Klee et al., 1988). The dissociation constants of CaM for Mg$^{2+}$ are in the range of 7 mM to 30 mM (Iida and Potter, 1988).

The binding of Ca$^{2+}$ to CaM results in conformational changes which occur in two steps (Evans et al., 1988). First, binding of two molecules of Ca$^{2+}$ causes a large change in the environment of the carboxyl-terminal region of CaM. This region has the III and the IV Ca$^{2+}$-binding domains. It was also shown that the two high affinity sites are located in the carboxy-terminal half of the protein. In view of the above observations, it was postulated that the first two
mols of Ca$^{2+}$ bind at III and the IV Ca$^{2+}$-binding domains. Binding of two additional Ca$^{2+}$ ions occurs at the low affinity sites (I and II) and results in further, though less drastic, conformational changes (Cox et al., 1984; Tsalkova and Privalov, 1985; Evans et al., 1988).

1.4 CALMODULIN-TARGET PROTEIN INTERACTIONS

As stated earlier, CaM stimulates various enzymes in a Ca$^{2+}$-dependent manner. CaM also interacts with various naturally occurring and synthetic peptides as well as hydrophobic drugs.

Calmodulin-Drug Interactions: Several hydrophobic drugs have been shown to inhibit CaM activity. These drugs gained much pharmacological interest as they could potentially be used as specific CaM inhibitors (Hidaka et al., 1981; Prozialeck, 1984; Van Belle, 1984). However, it was observed that most of these drugs inhibit not only CaM activity but also other non-calmodulin dependent processes (MacNeil et al., 1988). Despite, the non-specificity of these agents, they are a useful means of studying the mechanism of action of CaM.

Binding studies of CaM with phenothiazine antipsychotics indicate that there are 1-3 drug binding sites per CaM molecule (Weiss et al., 1982). Studies (LaPorte et al., 1980) have indicated that hydrophobic regions of CaM are involved in CaM-drug interactions. The greater the hydrophobicity of the drug, the tighter the CaM-drug interaction (La Porte et al., 1980). However, certain less hydrophobic drugs, for example quinacrine, are also potent inhibitors of CaM. This indicated that hydrophobicity is probably one of the many properties which dictates the binding characteristics of the drugs. Some of the other factors which contribute towards the potency of a particular drug are the geometric structure of the drug and its ionic characteristics. The latter was confirmed by the observation (La Porte et al., 1980) that slight modifications in the chemical structure greatly altered the ability of the compound to bind to CaM. Furthermore, pH titrations of trifluoperazine, a positively charged phenothiazine, binding to CaM suggested that ionic forces play an important role in CaM-drug interaction. In the above titrations, maximum binding was
observed in the pH range 4-8. Trifluoperazine, a phenothiazine, has a pKₐ of 8.1 and is positively charged in pH range 4-8. The isoelectric point of CaM being 4.0, the protein has an overall negative charge in the above pH range 4-8. Therefore, trifluoperazine binding to CaM is mediated by attraction of oppositely charged species (Weiss et al., 1982). Studies have also shown that the mechanisms of action of the various drugs are dissimilar (Orosz et al., 1990). The variability in the mechanism is related to the specificity of the drug for CaM. Orosz et al. (1990) studied the mechanism of action of the drugs trifluoperazine (TFP) and KHL-8430 on the enzymes phosphofructokinase (PFK) and phosphodiesterase (PDE). The enzyme PFK is inhibited by CaM. It was found that KHL-8430 has a greater affinity for CaM when CaM is in association with the enzymes whereas trifluoperazine binds to CaM first and then brings about the inhibition of the two enzymes. 

Calmodulin-Peptide Interactions: CaM can bind to a large number of peptide hormones and other endogenous peptides (DeGrado, 1988; Anderson and Malencik, 1986). The interaction of the peptides with CaM, is related to the presence of basic amphiphilic helices in the peptide. It was experimentally shown that these basic amphiphilic helices are important in binding to CaM (DeGrado et al., 1985; Cox et al., 1985). This was demonstrated by binding studies of CaM with a series of peptides comprising of amino acid residues leucine, lysine and tryptophan (DeGrado et al., 1985). The affinity of these peptides for CaM is strong with dissociation constants in the range of 10⁻⁹ to 10⁻¹⁰ M. Both hydrophobic and electrostatic interactions contribute towards the strong affinity of these peptides for CaM. CaM-binding domains of many proteins have been sequenced and mapped. The domains are characterized by the presence of a large number of positively charged and hydrophobic residues. The domains lack negatively charged residues (O'Neill and DeGrado, 1990). The CaM binding domains of many proteins have structures similar to the positively charged amphiphilic α-helices and hence it was postulated that this structure could be a common feature of CaM-target protein interactions. However, further studies with different peptides showed that CaM interaction with these peptides was unique to the peptide and
hence, it is unlikely that every CaM target protein would have a similar CaM binding domains (O'Neil and DeGrado, 1990).

As mentioned before, crystallographic studies indicate that CaM has two Ca\textsuperscript{2+}-binding lobes separated by a central helix and that there is little interaction between the two lobes. These Ca\textsuperscript{2+}-binding lobes are characterized by the presence of hydrophobic amino acids which can effectively interact with two positively charged amphiphilic \(\alpha\)-helices. Contrary to what was expected, only one peptide binds to a CaM molecule. It was later shown, that the two Ca\textsuperscript{2+}-binding lobes are closer than predicted in the crystal structure. Furthermore, it has been postulated that the central helix acts as a flexible arm allowing the two lobes to interact with each other forming a binding site which then binds the peptide (O'Neil and DeGrado, 1990).

The flexible arm hypothesis is also supported by photoaffinity labeling studies (Kauer et al., 1986; O'Neil et al., 1989; O'Neil and DeGrado, 1989). Two CaM-binding peptides were synthesized where one peptide had the photolabel benzophenylalanine (Bpa) at position 3 and the other at position 13 (Figure 3). Fluorescent studies of peptide binding to CaM show that these positions (3 and 13) are in contact with the CaM surface (O'Neil et al., 1987). With the photolabel at position 3 (P\textsubscript{3}), the methionine 144 located in the C-terminal lobe of CaM was labelled. The peptide having the photolabel at position 13 (P\textsubscript{13}), labelled methionine 177 of the N-terminal lobe of CaM. These results suggest that both the lobes of CaM are capable of interacting with the peptide (O'Neil and DeGrado, 1990). An alternative explanation for the above result is that interaction of the peptide causes a conformational change in the CaM molecule where the two lobes are close together only for a very short period of time but that this conformation is arrested due to the formation of covalent bonds between the photolabelled peptide and CaM. To check this possibility, a model peptide was synthesized with the photolabel at position 13 and a tryptophan residue at position 3 (P\textsubscript{13}, Figure 3). The peptide was then crosslinked to CaM and the fluorescence properties of the tryptophan residue were examined. As a control, similar experiments were performed with CaM and a control peptide (P\textsubscript{C}) having a tryptophan residue at position 3 but no photolabel, Figure 3. Fluorescent studies showed that the
SEQUENCE OF THE PEPTIDES USED FOR PHOTOLABELLING STUDIES

$P_1$: Ac Leu Lys $Bpa$ Lys Leu Leu Lys Leu Leu Lys Leu Lys Leu Gly.


Figure 3: Primary structure of the peptides used for photolabelling studies. $Bpa$, photolabel benzophenylalanine. (adapted from O'Neill and Degrado, 1990)
the interaction of the covalent complex of CaM-photolabeled peptide was identical to the non-
covalent complex of CaM with the control peptide (Pc). These results clearly indicate that the
two lobes of CaM together take part in the formation of a target binding site.

**Calmodulin-Protein Interactions:** CaM interacts with proteins and enzymes in much the same
way as it interacts with drugs and various peptides. Both the interaction and activation by CaM
are Ca²⁺-dependent. The removal of Ca²⁺ results in decreased affinity of CaM for its target
enzymes.

Many models have been proposed to explain the mechanism of Ca²⁺/CaM-dependent
stimulation of enzymes by CaM (Haiech et al., 1981; Delville et al., 1985). In the case of CaM-
dependent phosphodiesterase, it was shown that a large increase in the affinity of CaM for the
enzyme was due to stepwise binding of the Ca²⁺ to CaM (Huang and King, 1985; Gregori et al.,
1985). The Ca₄⁺⁺⁻⁻⁻⁻CaM complex increases the affinity of PDE for CaM by about 10,000 fold as
compared to Ca²⁺ free CaM. Huang and King estimated the t₁/₂ for the dissociation of CaM
from Ca₄⁺⁺⁻⁻⁻⁻CaM-PDE complex to be approximately 12 minutes. The dissociation of Ca²⁺ from
Ca₄⁺⁺⁻⁻⁻⁻CaM-PDE complex was much faster, less than a tenth of a second. This indicates that
the level of control is probably associated with Ca²⁺-binding to CaM-PDE complex rather than
the dissociation of CaM from Ca₄⁺⁺⁻⁻⁻⁻CaM-PDE complex (Sharma et al., 1988). The affinity of
Ca²⁺ for the set of high-affinity sites is enhanced only moderately (2-10 fold), whereas for the low
affinity sites the affinity is enhanced by several orders of magnitude. In view of the above
observations, it was postulated that at resting Ca²⁺ concentration, the affinity is just sufficient to
stabilize the CaM-PDE complex though the complex is inactive. With a slight increase in Ca²⁺
concentrations there is a rapid and large increase in the affinity of PDE for CaM and the complex
is now activated. In operational terms, this means that PDE does not have to compete with other
CaM-binding proteins for CaM.

CaM interacts with the various target enzymes with varying affinities. The binding
constants for CaM-enzyme interactions calculated so far are in the range of 10⁻⁵ to 10⁻¹⁰ M
(Sharma et al., 1988). CaM-binding domains of many enzymes have been identified. For
example, the CaM-binding domain of rabbit skeletal muscle enzyme was identified by; subjecting
the protein to CNBr fragmentation followed by assaying the fragments for CaM activity
(Blumenthal and Krebs, 1988). The assay for measuring CaM activity was based on the ability of
the fragment to inhibit CaM-dependent MLCK activity. The CaM-dependent fragments were
further cleaved by CNBr and analysed for their ability to bind CaM. Only one peptide was able to
interact with CaM. The affinity of this peptide for CaM was as strong as the affinity of intact
MLCK for CaM. The peptide corresponded to the C-terminal region of MLCK. These results
indicated that the CaM-binding domain of MLCK resides in the C-terminal region of the protein
(Blumenthal and Krebs, 1988). Furthermore, studies with MLCK CaM-binding domain peptides
indicate that the peptides interact with both the N-terminal and C-terminal lobes of CaM (Klevit
and Blumenthal, 1987).

Another approach towards understanding CaM-target protein interactions involves the
chemical modification of CaM and studying the effect of the modification on CaM function.
Carbamoylation of CaM lysine residues leads to 50% decrease in the ability of CaM to stimulate
phosphodiesterase whereas, in the case of Ca^{2+}-ATPase, the modification has no effect (Guerini
et al., 1987). Modification of the arginine residues had an opposite effect where
phosphodiesterase is not affected whereas stimulation of Ca^{2+}-ATPase by CaM is lowered by
40% (Guerini et al., 1987). The above observations indicate that the basic residues of CaM play
an important role in CaM-target protein interaction. The above observations further prove that
interactions of CaM with its target proteins are unique.

In summary, CaM is a ubiquitously occuring Ca^{2+}-binding protein. CaM has four Ca^{2+}-
binding domains, two exhibiting high affinity for Ca^{2+} and the other two exhibiting low affinity for
the ion. The Ca^{2+}-CaM structure is dumb-bell shaped with two lobes connected by the central
helix. Each lobe contains a set of Ca^{2+}-binding sites. Binding of Ca^{2+} to CaM induces a
conformational change in the protein structure making it more hydrophobic. This conformational
change allows CaM to associate with the various target proteins and regulate their activity.
Binding studies of CaM with drugs indicate that the mechanism of action of this protein involves
both hydrophobic and electrostatic interactions. Experiments with synthetic peptides indicate that CaM-binding domains of target enzymes may contain a positively charged helical region which associates with CaM. Peptide binding studies also indicated that the target proteins are capable of interacting with both lobes of CaM. The central helix acts as a flexible tether and brings the two lobes close together. The studies of CaM with the various drugs, peptides and target proteins collectively indicate that the mechanisms of action of CaM may share some common features but are specific for each target protein.
2. PHOSPHODIESTERASES

Cyclic nucleotide monophosphates (cNMP) act as intracellular second messengers and, like calcium, undergo rapid concentration changes in response to the stimulation received by the cell. Cyclic nucleotide metabolism is controlled by the enzymes adenylyl/guanylyl cyclases and cyclic nucleotide phosphodiesterase. PDE catalyzes the hydrolysis of the cyclic 3',5'-phosphate bond resulting in the formation of the corresponding 5'-nucleotide (Figure 4) (Tremblay, et al., 1988).

As many isoforms of PDE have been identified, attempts have been made to develop a nomenclature system which will account for not only the known isoforms but also isoforms yet to be characterized (Sharma et al., 1988; Beavo, 1988). On the basis of substrate specificity, molecular size, kinetic properties, regulation by intracellular factors and inhibition by selective drugs, Beavo (1988) classified the various PDEs into seven groups. These are as follows:

CaM-dependent PDEs: Ca$^{2+}$/CaM-dependent PDE activity has been observed in most tissues (Sharma et al., 1988; Beavo, 1988; Murray, 1990). Kinetic studies with these isoforms have exhibited $K_m$ for cAMP in the range of 10 μM-200 μM (Beavo, 1988; Sharma et al., 1988). The Ca$^{2+}$/CaM-dependent PDEs have also been referred to as the high $K_m$ PDEs. The native molecular weight of Ca$^{2+}$/CaM PDEs isolated from bovine brain and heart is about 120 kDa. SDS-PAGE experiments show the isoforms exist as homodimers with each subunit having a molecular weight of ~60 kDa (Sharma et al., 1988). An exception is the Ca$^{2+}$/CaM-dependent isoform isolated from bovine brain which reportedly has a subunit molecular weight of 75 kDa (Shenolikar et al., 1985).

cGMP-stimulated PDE (CGS-PDE): cGMP concentrations as low as 0.1-5.0 μM stimulate the activity of this isoform about 50-fold. Only one isoform of this particular PDE has been purified. CGS-PDE has been demonstrated in the bovine heart (Beavo et al., 1982; Martins et al., 1982) rat liver (Beavo et al., 1971), platelets (Hidaka and Asano, 1976), adipose tissue (Klotz and Stock, 1972) and adrenal medulla (Egrie and Siegel, 1977). The stimulatory effect of cGMP is
Figure 4: Hydrolysis of cyclic nucleotides by the enzyme phosphodiesterase, resulting in the formation of the corresponding 5'-nucleotide. (Adapted from Kincaid and Vaughan, 1986).
rapid and short lived as it is itself hydrolysed. Studies with cGMP derivatives and various inhibitors indicate that the enzyme has two cGMP binding sites, a allosteric site and a catalytic site. The allosteric site exhibits higher affinity for cGMP and regulates the action of the catalytic site.

**cGMP-specific PDEs:** Two major isoforms of cGMP-specific PDEs have been isolated, one from the lung (Francis et al., 1980) and the platelets (Coquil et al., 1980) and the other from photoreceptors. cGMP-specific PDE exhibits at least 50-fold greater affinity for cGMP as compared with cAMP. Experiments utilizing cGMP derivatives indicated that the lung and the platelet enzymes have a cGMP binding site which is distinct from the catalytic site (Coquil et al., 1980; Francis et al., 1980). Molecular weights of the cGMP-specific isoforms vary in the range from 150-180 kDa (Beavo, 1988).

**Low Km PDEs:** This family consists of isoforms which have 50-fold greater affinity for cAMP as a substrate. The two major isoforms purified are the cGMP-inhibited PDE (CGI-PDE) and the noninhibited isoform. CGI-PDEs are inhibited by cGMP concentrations as low as 1-2 μM. Isoforms of this type have been demonstrated in rat adipose tissue, platelets and bovine heart (Beavo, 1988). CGI-PDE from bovine cardiac tissue has a molecular weight of 110 kDa. The platelet CGI-PDE is phosphorylated by cAMP-dependent protein kinase (Macphee et al., 1987). Noninhibited Low Km PDEs have also been identified in many tissues and as the term indicates these isoforms are not inhibited by cAMP or cGMP. Although, this isoform is selectively inhibited by low concentration of the drug, RO 20-1724, and hence has also been termed as RO 20-1724 sensitive low Km PDE (Davis et al., 1984).

**Nonspecific intestinal PDE:** This isoform will catalyze the hydrolysis of cyclic nucleotides comprised of both purine and pyrimidine bases (Helfman et al., 1981). The isoform exhibits much higher affinity towards cAMP as compared to other cyclic nucleotides (Kelly and Butler, 1977).

The two other families of PDEs are the non-mammalian PDEs and "Other" Mammalian PDEs. The latter includes isoforms which have been partially characterized. Non-
mammalian PDEs include isoforms from lower species, e.g., low \( K_m \) PDE from Drosophila (Chen et al., 1988) and two isoforms, low and high \( K_m \), from yeast (Sass et al., 1986).

### 2.1 \( \text{Ca}^{2+}/\text{CaM-Dependent Phosphodiesterases} \)

PDEs belonging to this family have been found in all mammalian tissues. The richest source of the CaM-dependent isoform is the bovine brain (5-10 mg/kg). CaM-dependent PDEs have also been demonstrated in non-mammalian tissues, i.e., chicken gizzards, frog ovaries and a fungus, *Neurospora crassa* (Sharma et al., 1988).

\( \text{Ca}^{2+}/\text{CaM} \)-dependent PDEs have been purified by ion exchange (Sharma et al., 1980), gel filtration (Kincaid et al., 1981), blue dextran (Morrill et al., 1979) and CaM-Sepharose affinity chromatography (Klee and Krinks, 1978). Sharma et al. (1984) identified the presence of two isoforms in bovine brain with the aid of monoclonal antibodies. These isoforms are the 60 kDa (low \( M_r \) weight) and the 63 kDa (high \( M_r \) weight) isoforms. The authors developed three types of monoclonal antibodies (A6, C1 and A2) to the brain isoforms. C1 and A2 antibodies were specific for the 60 kDa species whereas the A6 antibody could react with both the low and high \( M_r \) enzymes. The two enzymes were separated by passing the partially purified isoforms over the A6-Sepharose affinity column, followed by passing the eluate from A6-Sepharose over an A2 Sepharose column. In this way the low \( M_r \) isoform could be separated from the high \( M_r \) isoform.

Hansen and Beavo (1982) developed an anti-heart phosphodiesterase monoclonal antibody (ACAP-1) which recognizes the \( \text{Ca}^{2+}/\text{CaM} \) induced PDE conformation. CaM or PDE alone do not bind to the antibody. Later, the authors developed another conformation specific monoclonal antibody (ACC-1) which could bind both the low \( M_r \) and high \( M_r \) isoforms from the brain (Hansen and Beavo, 1985). Like ACAP-1, monoclonal antibody ACC-1 recognizes \( \text{Ca}^{2+}/\text{CaM} \) and \( \text{Ca}^{2+}/\text{CaM-PDE} \) complexes and not PDE or \( \text{Ca}^{2+} \)-free CaM. The authors subjected the partially purified PDE to ACC-1 sepharose chromatography. The eluate from the latter was then applied
over ACAP-1 column which binds only the low Mr enzyme. By employing the above procedure, the authors were successful in separating the two isoforms.

CaM-dependent PDEs associate with CaM in the presence of calcium. Sharma et al. (1980) determined the molecular weight of the PDE-CaM complex to be 159 kDa, indicating that 2 moles of CaM are bound per mole of the enzyme. The PDE-CaM complex has a subunit structure of \( \alpha_2\beta_2 \), where \( \alpha \) is a PDE subunit and \( \beta \) a CaM subunit. Interaction of \( \text{Ca}^{2+} \), CaM and PDE is shown in Figure 5 (Mutus et al., 1984). Chau et al. (1982) determined the dissociation constants of CaM for \( \text{Ca}^{2+} \), \( K_1, K_2, K_3 \) and \( K_4 \) to be 7.5 \( \mu \text{M} \), 2.7 \( \mu \text{M} \), 31 \( \mu \text{M} \) and 30 \( \mu \text{M} \), respectively. Dissociation constants CaM-PDE complex and \( \text{Ca}^{2+}/\text{CaM-PDE} \) complex have been determined to be \( 10^{-5} \text{ M} \) and \( 10^{-10} \text{ M} \), respectively (Huang et al., 1981; Chau et al., 1982; Mutus et al., 1984). The \( \text{Ca}^{2+} \) dissociation constants from the CaM-PDE complex have yet to be determined. The large amounts of PDE required limit the determination of \( \text{Ca}^{2+} \) dissociation constants from CaM-PDE complex. Modification of PDE with a sulphydryl fluorescent probe, S-mercuric-N-dansyl-cysteine (SMNDC) for studying the \( \text{Ca}^{2+} \), CaM and PDE interactions has been demonstrated (Mutus et al., 1984). Experiments with modified PDE showed that the fluorescence properties of the CaM-PDE complex in the presence and absence of \( \text{Ca}^{2+} \) were different and that one could monitor the binding of \( \text{Ca}^{2+} \) to CaM-PDE complex.

### 2.2 KINETIC PROPERTIES OF CALMODULIN-STIMULATED PHOSPHODIESTERASES

Kinetic analysis of the various CaM-stimulated PDEs show that the kinetic properties of these enzymes vary. This is expected if each isoform performs a specific function and is regulated by different factors. The literature cAMP \( K_M \) values of \( \text{Ca}^{2+}/\text{CaM} \)-dependent PDE isolated from the bovine brain, range between 10 \( \mu \text{M} \)-200 \( \mu \text{M} \) (Sharma and Wang, 1986; Shenolikar et al., 1985; Morrill et al., 1979; Klee et al., 1979; Tucker et al., 1981). The different kinetic properties observed for this enzyme could arise as a result of the following: (1) lack of homogenous enzyme preparation (2) different assay conditions (3), presence of multiple isoforms
INTERACTION OF CALMODULIN WITH Ca^{2+} AND PDE

![Diagram showing interaction of calmodulin with Ca^{2+} and PDE with reaction constants and equilibrium constants.]

Figure 5: Energy coupling between Ca^{2+} binding of calmodulin and the association of CaM with PDE. $K_1$-$K_4$, dissociation constants of Ca^{2+} and CaM; $K'_1$-$K'_4$, dissociation constants of protein complexes and Ca^{2+}; $K_a$-$K_g$, dissociation of constants of PDE and liganded CaM. (Sharma et al., 1988; reproduced with permission).
of this enzyme. To date, three Ca\textsuperscript{2+}/CaM-dependent PDEs have been purified from the bovine brain (Smoake et al., 1981; Sharma et al., 1984).

A common feature of the Ca\textsuperscript{2+}/CaM-PDEs is the higher affinity of these enzymes for cGMP as a substrate (Beavo, 1988). For the four well characterized PDEs, heart, low $M_r$ and high $M_r$ brain and lung enzymes, the $K_m$s obtained for cGMP are at least 10 fold lower than that observed for cAMP (Sharma et al., 1988) indicating that these PDEs have greater affinity for cGMP. The two exceptions are: the isoform from mouse testis (Rossi et al., 1988) and the isoform from human myometrium (Leroy et al., 1987). The testis enzyme exhibited nonlinear kinetics with low and high $K_m$s of 2 $\mu$M and 20 $\mu$M for both cAMP and cGMP, respectively. The substrates acted as non-competitive inhibitors to each other which indicated that the enzyme has two distinct catalytic sites. The human myometrial PDE has equal affinity for cAMP and cGMP.

2.3 REGULATION OF CALMODULIN-DEPENDENT PHOSPHODIESTERASES

Each PDE isoform contributes to the overall regulation of cyclic nucleotide levels. The mechanism of action of each isoform is in turn regulated by various factors. Some of the factors regulating the activity of CaM-dependent PDEs are phosphorylation, differential CaM affinity and Ca\textsuperscript{2+} sensitivity of the enzyme.

CaM affinity of PDEs: The CaM-dependent PDEs exhibit varying affinities for CaM (Sharma et al., 1988; Beavo, 1986). The primary structure, kinetics and the immunological properties of the low $M_r$ isoform from the brain and the 59 kDa isoform from the heart are very similar (Beavo, 1988). However, the affinity of these enzymes for CaM is different; the heart enzyme has 5 fold higher affinity for CaM as compared to the brain isoform (Mutus et al., 1985; Sharma, et al., 1988). It has been postulated that the difference in CaM affinity is related to the estimated concentration of CaM in the two tissues. The CaM content of the brain is about 10 times higher than that of the heart (Sharma and Wirch, 1980). The lung enzyme reportedly has CaM as a subunit (Sharma and Wang, 1986). It has been postulated that by having CaM as a subunit the
enzyme does not have to compete with the other CaM-binding proteins for CaM.

**Phosphorylation:** Protein kinases bring about the phosphorylation of PDEs rendering them inactive (Sharma et al., 1988). Phosphorylation is discussed here with respect to the low and high $M_r$ from brain PDEs. The low $M_r$ (60 kDa) isoform is a substrate for a cAMP-dependent protein kinase (Sharma et al., 1980). Two moles of phosphate are incorporated per mole of the enzyme subunit. The high $M_r$ (63 kDa) enzyme also undergoes phosphorylation and is a substrate for Ca$^{2+}$/CaM-dependent protein kinase. Like the low $M_r$ enzyme, 2 moles of phosphate are incorporated per mole of the high $M_r$ PDE (Sharma et al., 1980). In both cases, the phosphorylated enzyme can be dephosphorylated by a CaM-dependent phosphatase, calcineurin.

In response to the stimulation received by the cell, the intracellular concentration of the second messengers, Ca$^{2+}$ and cNMPs are elevated for a short duration of time. How do these second messengers regulate phosphorylation and in turn PDE activity? In an attempt to answer the question, Sharma et al. (1988) developed a working hypothesis as outlined in Figure 6, A and B.

The low $M_r$ enzyme exhibits high $K_m$ for cAMP and is dependent on Ca$^{2+}$ for maximal activation. When the cell is activated, the enzyme adenylate cyclase (AC) is stimulated and there is an increase in the cAMP levels. AC catalyzes the conversion of ATP to cAMP. The increased levels of cAMP activate the cAMP-dependent protein kinase which phosphorylates the low $M_r$ isoform. The latter is now inactive and cannot degrade cAMP. As a result of AC stimulation and low $M_r$ PDE inhibition, there is a sharp increase in cAMP levels which in turn triggers other cAMP-dependent biochemical processes. In the later stages of cell stimulation, there is an influx of Ca$^{2+}$ ions. At high concentrations of Ca$^{2+}$, a Ca$^{2+}$-CaM complex is formed which activates the phophatase, calcineurin (CaN). The latter dephosphorylates the low $M_r$ enzyme rendering it active. The isoform can now bind Ca$^{2+}$-CaM complex and bring about the degradation of the cNMPs. The cAMP levels decrease to normal levels. The process is represented in Figure 6, A.

The postulated mechanism of regulation of the high $M_r$ isoform by phosphorylation is
Figure 6: Regulation of (A) low M₉ (60 kDa) isoform and (B) high M₉ (63 kDa) isoform from brain by phosphorylation and dephosphorylation. (Sharma et al., 1988; reproduced with permission).
slightly different (Figure 6, B). Upon cell stimulation, the increase in Ca^{2+} concentration results in the formation of the Ca^{2+}-CaM complex which activates the CaM-dependent protein kinase. The latter phosphorylates the high M_r isoform rendering it inactive. The enzyme adenylate cyclase is also activated by Ca^{2+}-CaM complex followed by a rapid increase in the cAMP concentrations. At later stages of cell activation, the Ca^{2+} concentrations increase further and the enzyme calcineurin is activated. The latter dephosphorylates the high M_r isoform which then degrades cAMP thereby decreasing the concentration of cAMP to normal levels. For the above hypothesis to work, the adenylate and CaM-dependent protein kinase should be activated by lower concentrations of Ca^{2+} as compared to the high M_r PDE and the phosphatase, CaN. Piascik et al. (1980) has demonstrated that adenylate cyclase is in fact activated by lower Ca^{2+} concentration as compared to the PDE. The Ca^{2+} sensitivity of the protein kinase has yet to be determined.

**Ca^{2+} sensitivity of PDE:** As described in the previous section, phosphorylation of PDE changes the affinity of the enzyme for CaM. Sharma and Wang (1985) demonstrated that the activity of the phosphorylated and non-phosphorylated PDEs is similar at CaM concentrations \( \geq 10 \text{ nM} \). In the brain, the CaM levels are in the range of 10-100 \( \mu \text{M} \) and hence in vivo activity of CaM-dependent PDEs would not be effected by phosphorylation. Huang et al. (1981) had earlier shown that the activation of PDE depends on the presence of both Ca^{2+} and CaM and that Ca^{2+} binding to CaM exhibits positive cooperativity. Titrations of PDE at varying CaM concentrations with Ca^{2+}, indicated that increasing Ca^{2+} concentration results in increased sensitivity of the PDE for CaM. It has been postulated that phosphorylation alters the Ca^{2+} sensitivity of the enzyme and not the affinity of the enzyme for CaM.

### 2.4 PHOSPHODIESTERASE: PHARMACOLOGICAL SIGNIFICANCE

Cyclic nucleotides play a key role in smooth muscle relaxation. It has been shown that high levels of cAMP and cGMP are associated with the process of muscle relaxation (Ignarro and
in Figure 7. A transient increase in the cAMP or cGMP levels, activates protein kinases which bring about phosphorylation of myosin light chain kinase (MLCK). cAMP- and cGMP-dependent protein kinases have been demonstrated in vascular smooth muscle (Ignarro and Kadowitz, 1985; Lincoln, 1989; Murray, 1990). The modified MLCK is inactive and cannot associate with the Ca\(^{2+}\)-CaM complex. The function of MLCK is to phosphorylate myosin which can then bind to actin forming the actomyosin complex. For the muscle to contract the association of actin and myosin is required. If MLCK is phosphorylated it cannot modify myosin, the actomyosin complex is not formed and the muscle stays in a relaxed state. cAMP and cGMP also aid in decreasing cytosolic Ca\(^{2+}\) levels in vascular smooth muscle cells which are also involved in the process of vasodilation (Murray, 1990; Lincoln, 1989).

PDE is the sole means of degradation of cyclic nucleotides and therefore is a crucial point for the regulation of cNMP levels. Many pharmacological agents/drugs have been developed which have been shown to inhibit PDE activity and result in vasodilation (Kauffman et al., 1987). Ohoka et al. (1990) demonstrated a cAMP-specific PDE inhibitor, E-1020, which increased cAMP levels but decreased Ca\(^{2+}\) levels in cultured vascular smooth muscle cells. Thus, E-1020 could be a potential vasodilating agent. Kauffman et al. (1987) independently demonstrated \textit{in vitro} relaxation of vascular smooth muscle which is accompanied by a decrease in the PDE activity of the cells.

To date, various types of PDE have been isolated from vascular tissues and are being used to screen new drugs as potential vasodilative/cardiotoxic agents. These fall into three types; the Ca\(^{2+}\)/CaM-dependent PDEs, low K\(_{m}\) PDEs (CGI-PDE and RO 20-1724-sensitive PDE) and the cGMP-specific PDEs (Nicholson et al., 1991). Several drugs have been developed which bring about selective inhibition of the above enzymes (Beavo, 1983; Nicholson et al., 1991). 8-methoxymethyl IBMX (isobutylmethylxanthine) and vinpocetine are specific for Ca\(^{2+}\)/CaM-dependent PDEs. The CGI-PDE is selectively inhibited by agents like cilostamide, finoximone and milrinone. The other low K\(_{m}\) PDE is inhibited by drugs, rolipram and RO 20-1724, and has also been referred to as RO 20-1724-sensitive PDE. cGMP-specific PDE is
Figure 7: Illustration of the physiological role of PDE in vascular smooth muscle (Ignarro and Kadowitz, 1985; reproduced with permission, from the Annual Review of Pharmacology and Toxicology, Vol. 25, ©1985 by Annual Reviews Inc.).
specifically inhibited by drugs, M&B 22,948, dipyridamole and MY-5445. The potency of the above mentioned inhibitors towards the various isoforms is relative to the degree of purification of the isoform. Most of the inhibition studies have been performed on crude DEAE-extracts which probably contain a mixture of PDEs and therefore the potency of a particular drug represents an average. This is also of significance in vivo since the drug might inhibit more than one isoform. IBMX and papaverine are two non-specific inhibitors of phosphodiesterase enzymes.

Selective PDE inhibitors have also been shown to relax non-vascular smooth muscle i.e human myometrium, where the drugs were used in the treatment of premature labor. In the human myometrium two PDE isoforms have been identified, the Ca\(^{2+}\)/CaM-PDE and cAMP-specific PDE (Leroy et al., 1989). As in vascular tissue, IBMX and papaverine were non-specific inhibitors of the two PDEs isolated from the myometrium. M&B 22,948, a cGMP-specific inhibitor was found to be a potent inhibitor of the myometrial Ca\(^{2+}\)/CaM-dependent PDE. Such a result was also obtained with the Ca\(^{2+}\)/CaM-PDEs isolated from aorta, heart and platelets (Leroy et al., 1989). cAMP-specific PDE from human myometrium was found to be selectively inhibited by rolipram. The inhibition of this PDE was accompanied by in vitro relaxation of the myometrial smooth muscle. Various PDE inhibitors are also being evaluated as potential bronchodilators and anti-platelet aggregatory agents.

Cyclic nucleotide phosphodiesterases catalyse the hydrolysis of cyclic nucleotides to the corresponding 5'-nucleotides. Many isoforms of PDEs have been isolated and characterized. Ca\(^{2+}\)/CaM-PDEs have been extensively studied and have been shown to be present to some extent in most tissues (Beavo et al., 1988; Sharma et al., 1988). The kinetic properties of the various isoforms of this type of PDE different. These isoforms can also be regulated by phosphorylation. PDEs have gained much clinical interest as they can be specifically inhibited. This inhibition results in a rise in the cyclic nucleotide levels. Increased levels of cAMP and cGMP are associated with vascular relaxation. Therefore, PDEs are excellent targets of cardiotonic drugs.
3. **NON-ENZYMATIC GLUCOSYLATION**

The normal glucose levels in the human body are in the range of 3.6-8.1 mM. These levels are maintained by the biochemical processes regulated by two major hormones, insulin and glucagon (Wilson and Foster, 1985). Insulin stimulates the processes involved in utilizing glucose and glucagon stimulates processes which synthesize glucose. Therefore, insulin helps in decreasing blood glucose levels whereas glucagon aids in elevating blood glucose levels. Other hormones like cortisol and epinephrine also regulate the concentrations of glucose in the body (Wilson and Foster, 1985).

The glucose levels in aging tissues and in diseased states like diabetes are increased considerably (Bunn, 1981). Hyperglycemic conditions in these states have been associated with long-term chronic complications (Brownlee et al., 1984). Some of the complications involve the development of cataracts (Stevens et al., 1978), altered platelet function (Winocour et al., 1985) and increased risk of heart disease, stroke and kidney failure (Kennedy and Baynes, 1984). The tissues which are freely permeable to glucose are damaged most. It is thought that these long-term chronic complications are a result of two processes (Kennedy and Baynes, 1984). In the first process, the glucose enters the cell where it is converted to sorbitol by the action of the enzyme, aldose reductase. Sorbitol in turn is converted to fructose by the enzyme, sorbitol dehydrogenase. The cell is not freely permeable to sorbitol and fructose and therefore, these sugars accumulate inside the cell. The influx of glucose continues as its concentration inside the cell is not drastically altered. The accumulation of the sugars causes an osmotic imbalance and the cell imbibes water. The latter causes swelling and damages the cell (Suarez, 1989).

The other process where hyperglycemic conditions contribute to long-term complications is the attachment of sugar residues to the protein amino groups by non-enzymatic means. Such a protein modification can alter the protein function and/or structure. For example, glycosylation of aldose reductase stimulates the enzyme activity. Aldose reductase catalyzes the conversion of glucose to sorbitol (Srivastava, 1989). The formation of sorbitol is accompanied by conversion
of one mole of NADPH to NADP⁺. When the enzyme is stimulated, more of glucose is converted
to sorbitol and NADPH consumption increases. This hinders other NADPH requiring processes.

Non-enzymatic glucosylation of proteins has shown to be prevalent in aging tissues and
in the diseased state like diabetes. The sugar residue reacts with the protein amino groups to
form a labile-Schiff base, aldimine. The latter undergoes Amadori rearrangement to form the
stable ketoamine, Figure 8 (Armbruster, 1987; Furth, 1988). The reaction is also known as the
Maillard reaction named after the scientist who in 1912 discovered that amino acids can react
with glucose (Monnier, 1989). Until recently, formation of the Amadori product was considered
as an irreversible reaction though it has now been shown that the Amadori product can undergo a
series of reactions, i.e., dehydration, condensation and other rearrangement reactions. These
reactions result in the formation of compounds which have been collectively referred to as the
advanced glucosylation end-products (AGE) (Brownlee et al., 1988). The reaction resulting in the
formation of AGEs is very slow and irreversible. AGEs form covalent bonds between two
proteins resulting in protein crosslinking. For example, these end-products cause crosslinking of
collagen making it insoluble and resistant to digestion which in turn contribute to the long term
complications seen in diabetics like, cardiovascular disease, nephropathy, indurated skin and
joint stiffness (Brownlee et al., 1988).

3.1 NOMENCLATURE

The term glucosylation was used as most of the processes studied involved modification
by glucose. In general, the process is also referred to as non-enzymatic glycosylation and
depending on the sugar, the term has been modified to include the name of the sugar, i.e.
attachment of fructose to protein is referred to as non-enzymatic fructosylation. IUPAC-IUB Joint
Commission on Biochemical Nomenclature has recommended the use of the term "glycation" for
the reaction of any sugar with a protein (Armbruster, 1987). The terms glycation and
glycosylation have been used interchangeably.
Figure 8: Reaction of glucose with protein amino group to form 1-amino-1-deoxyfructosamine. (adapted from Armbruster, 1987)
3.2 FACTORS AFFECTING NON-ENZYMATIC GLUCOSYLATION

The chemical groups most susceptible to glucosylation are the amino groups of lysine residues and the N-terminus amino acid of proteins. Although, arginine and histidine residues can also react with glucose (Monnier, 1989). Non-enzymatic glucosylation is regulated by various factors. Some of these factors being pH, temperature, protein concentration, pK\textsubscript{a} of the amino group on the protein, concentration of sugar, type of sugar, time of exposure of the protein to the sugar and the half-life of the protein (Monnier, 1989). Non-enzymatic glucosylation of protein increases with the increase in pH, the type of sugar, concentration of both the sugar and the protein. The lower the pK\textsubscript{a} of the amino group, greater the reactivity towards the sugar (Baynes, 1989). Further, the environment of the amino group can affect its pK\textsubscript{a} (Baynes, 1989). For example, in hemoglobin the lysine residues located adjacent to acidic amino acids are more susceptible to undergo glucosylation (Bunn et al., 1979). The rate of glucosylation can also be altered by different buffering ions. Anionic buffers like phosphate, arsenate and bicarbonate can enhance the rate of glucosylation of a protein (Baynes, 1989). Watkins and coworkers demonstrated that phosphate binds to the basic regions of the protein. This changes the local environment of nearby lysine residues in a way to lower the pK\textsubscript{a} of the amino group of the residue making it more susceptible to glucosylation (Watkins et al., 1987).

The imidazole group of the histidine residue can also enhance the rate of glucosylation by catalyzing the Amadori rearrangement (Watkins et al., 1985; Shilton and Walton, 1990). The mechanism of action is outlined in Figure-9. The protonated form of the imidazole group (BH) of the protein functions as a acid catalyst in the formation of the Schiff base (II) from the protein-sugar adduct (I). The unprotonated form of the imidazole group (B) acts as a base catalyst where it removes a proton from C-2 of the schiff base resulting in the formation of the enol (III). The latter undergoes tautomerization resulting in the formation of the stable ketoamine (IV). Such an effect has been demonstrated in vivo with glucosylated horse liver dehydrogenase.
EFFECT OF IMIDAZOLE GROUP ON THE RATE OF GLUCOSYLATION

Figure 9: Effect of imidazole on the rate of glucosylation. Structure I represents a glycosamine formed by reaction of an amino group of a protein, R, with glucose. B represents the imidazole group.
where, 75% of glucosylation had occurred at lysine 231 and glucosylation of other lysine residues was 10% or less (Shilton and Walton, 1991). With X-ray crystallographic models of the enzyme it was shown that the lysine 231 was in close vicinity to the imidazole group of histidine 348 residue.

Sugars like fructose, mannose, galactose, xylose, arabinose, and ribose can also react with protein amino groups (Monnier, 1989) and it has been reported that the rate of glycosylation with these sugars is greater than that obtained with glucose.

### 3.3 PROCESSES AFFECTED BY NON-ENZYMATIC GLUCOSYLATION

Non-enzymatic glucosylation can alter processes like enzyme activity, binding of metabolites to protein, proteolytic degradation of the protein, nucleic acid function and protein crosslinking (Brownlee et al., 1984). Glucosylation of an essential lysine residue could result in altered catalytic properties of the enzyme, for example glucosylation of Lys 41 of ribonuclease A causes a total loss of enzyme activity (Eble et al., 1983). Incubation of cathepsin B, a sulfhydryl protease, with glucose for 2 weeks results in complete inactivation of the enzyme (Coradello et al., 1981). In contrast, Walton et al., (1988) have purified in vivo glucosylated alcohol dehydrogenase from horse liver, where the activity of the enzyme was stimulated by glucosylation.

Modification of the protein structure, which interferes with its binding ability will result in an altered biochemical process. 2,3-diphosphoglycerate binds reversibly to hemoglobin (Hb) and decreases the affinity of Hb for oxygen (Perutz, 1979). The binding of oxygen to Hb in turn decreases the affinity of the protein towards 2,3-diphosphoglycerate. The reversibility of 2,3-diphosphoglycerate and oxygen binding to Hb, maintains the equilibrium between deoxy- and oxy-Hb levels (Perutz, 1979). Non-enzymatic glucosylation of Hb decreases its affinity for oxygen, disturbing the equilibrium between deoxy- and oxy-Hb. Another example where glucosylation interferes with protein-receptor interactions is glucosylation of the peptide hormone,
human prolactin where the modified hormone has decreased affinity for its receptor (Haro et al., 1990).

Nucleic acids can also undergo the process of non-enzymatic glucosylation. For example, glucosylation of DNA of bacteriophage f1 alters its ability to transflect E.Coli (Brownlee et al., 1984). It has been reported that the rate of reaction of DNA with glucose can be enhanced by the presence of amino acids like lysine. First the lysine residue reacts with the sugar and the sugar-lysine intermediate can then bind to DNA (Brownlee et al., 1984). It has been postulated that such amino acid-DNA complexes may be responsible for the increased amounts of protein crosslinking to the nucleic acids observed with aging (Bojanovic et al., 1970).

The best example of protein crosslinking due to glucosylation is that of lens proteins observed in cataracts (Kennedy and Baynes, 1984; Abraham et al., 1989). The lens proteins have a slow turnover rate and it has been postulated that glucosylation causes a change in the conformation of the protein. The conformational change exposes the protein sulphhydryl groups. Crosslinking of the lens proteins occurs by formation of intermolecular disulfide bonds (Abraham et al., 1989).

3.4 METHODS FOR DETERMINING THE EXTENT OF GLUCOSYLATION OF PROTEINS

The end product of Amadori rearrangement is 1-amino-1-deoxyfructosamine, commonly referred to as fructosamine (Armbruster, 1987). The product is structurally analogous to fructose and hence the latter has been used as a standard in many assays for quantitating non-enzymatically glucosylated proteins. It has been shown that prolonged elevation in blood glucose is correlated to increased levels of glucosylated proteins and measuring their levels provides an measure of average blood glucose levels (Bunn, 1981). The two proteins used extensively for measuring blood glucose levels over a period of time are hemoglobin (Hb) and serum albumin (Bunn, 1981). The life time of serum albumin is shorter than Hb therefore, measurement of glycated serum albumin provides a means of monitoring blood glucose levels over a shorter
duration of time. The methods used so far for determination of the extent of glucosylation in proteins can be grouped into three main categories: chromatographic, chemical and electrophoretic methods (Furth, 1988).

**Chromatographic methods:**

Non-enzymatic glucosylation of the protein can alter the net charge on the protein thus, facilitating its separation from non-glucosylated protein by ion-exchange chromatography. The cation exchanger, carboxymethyl-cellulose has been used to separate glucosylated fraction from non-glucosylated fraction of Hb (Day et al., 1979). The protein is applied on the column at neutral pH and is eluted with a low pH buffer (pH 6.2).

Affinity chromatography has also been used to separate the glucosylated protein from non-glucosylated protein (Furth et al., 1988). 3-aminophenylboronate agarose binds to the cis-diol group of the sugar residue on the protein (Gould et al., 1984). The bound protein can be eluted with sorbitol or by low pH (Gould et al., 1984; Mallia et al., 1981). This affinity chromatography method has been employed as a routine method for measuring percent glucosylated Hb in clinical settings. A major disadvantage of the chromatographic methods is that they detect the percent of glucosylated protein thus, the stoichiometry of the number of fructosamines per mole of the protein cannot be calculated.

**Chemical methods:**

(a) Thiobarbituric acid (TBA) method: This is one of the most commonly used assay for determining glucosylated proteins. The assay involves acidic hydrolysis of the sugar residue to hydroxymethyl furfural (HMF) (Murtlashaw et al., 1983). The sugar free protein is removed by a precipitation step and the HMF produced is quantitated by reacting with TBA. The chromophore produced has an absorption maxima at 435 nm and an extinction coefficient of 32, 000 M\(^{-1}\) cm\(^{-1}\). The major disadvantage of the TBA assay is that the HMF produced is itself hydrolysed by acid which results in under estimation of glucosylated protein. Furthermore, any sugar with six or more carbons will also produce the furfural and has to be removed before quantitating the
glucosylated protein (Furth, 1988). The procedure is also tedious to perform. By this assay one can detect HMF levels in range of 7 nmols-25 nmols (Murtiashaw et al., 1983).

(b) Nitroblue tetrazolium method (NBT) method: This method exploits the reducing ability of the fructosamine (Johnson et al., 1982). At alkaline pH (10.8-11), the fructosamine forms an enediol which reduces the reagent nitroblue tetrazolium to form a chromophore with absorption maxima at 530 nm (Furth, 1988; Johnson et al., 1982). The assay is easy to perform, precise and cheap. The disadvantages of the assay are: low sensitivity and interferences from substances like glucose, glutathione and uric acid which contribute to color production. These compounds can be easily removed by gel filtration or dialysis. Another disadvantage is that the assay has no fixed endpoint and reduction continues to give blue-black color (Furth, 1988). 1-deoxy-1-morpholinofructose (DMF), a synthetic Amadori product, has been used as a standard and this assay can accurately quantitate concentrations of DMF in the range of 1 μmol-8 μmol (Johnson et al., 1982).

(c) Periodate oxidation based methods: These methods involve periodate oxidation of the fructosamine on the protein releasing formaldehyde (Furth, 1988). One mole of formaldehyde is produced per mole of fructosamine. The formaldehyde (HCHO) released is from the C-1 of the fructosamine and under the reaction conditions employed, the second molecule of HCHO (from C-6) is not released (Gallop et al., 1981). The reduction of fructosamine with sodium borohydride results in production of 2 moles of HCHO per mole fructosamine. Fructose is used as a standard in the periodate based methods as it is structurally analogous to the fructosamine. The HCHO released can be detected fluorometrically by complexing with acetyl acetone and ammonia to produce the fluorophore, diacetyldihydrolutidine (DDL). The fluorescence is linear in the range 10 nmols-40 nmols and 2-4 mg of the glucosylated protein is required (Gallop et al., 1981).

A spectrophotometric method has also been employed for detection of DDL (Ahmed and Furth, 1991). DDL has an absorption maxima at 405 nm and an extinction coefficient of 7780 M⁻¹ cm⁻¹. By this method fructose (standard) concentrations in the range of 5 nmols-40 nmols can be accurately detected. The periodate based methods are relatively easy to perform,
inexpensive and senstitive means for detecting glucosylated proteins. The major disadvantage of these methods is the interference from glucose and other sugars present in the glucosylated protein sample. However, the sugars could be removed by a simple step of dialysis. Enzymatically glucosylated proteins will also interfere with these methods.

(d) Borohydride reduction method: This assay involves the reduction of the fructosamine with radiolabeled borohydride (Furth, 1988). This method is probably one of the most sensitive methods of detecting glucosylated protein. The major drawback of this assay is the significant amount of peptide bond reduction which occurs during sodium borohydride reduction of the protein (Paz et al., 1970). This was demonstrated in the protein collagen which contains covalently bound carbonyl compounds which in turn form intra- and intermolecular cross-links. One method employed for the characterization of these carbonyl compounds is reduction with tritiated sodium borohydride (Paz et al., 1970). It was observed that use of sodium borohydride also resulted in the reduction of the peptide bonds (Paz et al., 1970).

(e) Assays on protein hydrolysates: (1) Furosine method: In this method the glucosylated protein is subjected to acid hydrolysis to produce furosine which is chromatographed on HPLC. Furosine is detected by measuring the absorbance at 280 nm (Schleicher and Wieland, 1981). The method is very sensitive (can detect pmol of glucosylated protein) and is very specific. The major disadvantage of this assay is the 18-hour hydrolysis step and the expensive means of detection i.e HPLC.

(2) Amino acid analysis: This method detects the modified amino acid. Fructosamine is first subjected to reduction by borohydride, followed by acid hydrolysis of the reduced fructosamine (Walton and McPherson, 1987). The product, N-(1-deoxyhexitol-1-yl) amino acid, can then be detected by high pressure liquid chromatography.

Electrophoretic methods:

Non-glucosylated Hb has been separated from glucosylated Hb by electrophoresis on agar gel, pH 6.3 (Mortensen and Christopherson, 1983). The non glucosylated Hb is retarded by negatively charged groups and the bands are quantified by scanning densitometry. Isoelectric
focussing on thin-layer polyacrylamide gel slabs, can also separate the glucosylated fraction from the non-glucosylated fraction (Mortensen and Christopherson, 1982). Microgram-milligram quantities of glucosylated protein is required and the method provides an estimate of percent of glucosylated protein and not the specific number of glucose molecules per mole of the protein.

3.5 NON-ENZYMATIC GLUCOSYLATION OF CALMODULIN

Kowluru et al. (1989) demonstrated in vitro non-enzymatic glucosylation of CaM. The calcium binding protein was incubated in phosphate buffer, pH 7.4 with 28 mM glucose in the presence of Ca\(^{2+}\) and EGTA for 9 days. The extent of glucosylation was greater in the presence of Ca\(^{2+}\) (2.5 mole of glucose/mole of CaM) as compared to EGTA (1.5 mol of glucose/mol of CaM). The degree of glucosylation reached a maximum by about 6 days and the extent of modification was same with glucose concentration in the range of 20-100 mM (Kowluru et al., 1989).

In the above study, the effect of glucosylation of CaM was studied on the following enzymes: brain phosphodiesterase, adenylyl cyclase and protein kinase. The apparent affinity of phosphodiesterase for glucosylated CaM was about 2-fold less as compared to the unmodified CaM whereas for adenylyl cyclase and protein kinase the apparent affinity for glucosylated CaM was increased by approximately 2.6-fold and 1.25-fold, respectively. At half-saturating concentrations, the extent of activation of PDE, adenylyl cyclase and protein kinase by glucosylated CaM was decreased by 70%, 40% and 30%, respectively. Furthermore, the extent of inhibition of the enzymes was greater when CaM was glucosylated in the presence of Ca\(^{2+}\) as opposed to in absence of Ca\(^{2+}\).

Kowluru et al. (1989) also studied the effect of glucosylation on Ca\(^{2+}\)-binding. The affinity of the glucosylated CaM for Ca\(^{2+}\) was decreased by 50%. The decrease in Ca\(^{2+}\) affinity, had no effect on the Ca\(^{2+}\) induced conformation of CaM. This indicates that the conformational
changes brought about by Ca$^{2+}$-binding do not require all the four Ca$^{2+}$-binding sites to be occupied (Kowluru et al., 1989).

There are eight lysine residues in calmodulin. Lysine 115 is trimethylated and the N-terminus of CaM is acetylated and therefore the amino groups at these positions cannot be glucosylated. CNBr cleavage of glucosylated CaM produced eight peptides. Four of the eight peptides had one or more lysine residues in their primary structure. Furthermore, these four peptides also showed the presence of [6-3H] glucose. The fragment corresponding to the amino acids 1-36, had highest extent of glycation (1.4 mole of glucose/mole of CaM). Tit;o amino acids residues contributing to the four Ca$^{2+}$-binding sites of CaM are: site I-residues 12-39, site II-residues 48-75, site III-residues 85-112 and site IV-residues 121-148. Ca$^{2+}$-binding sites, II and IV each have one lysine residue at the their C-terminal ends. Therefore it was postulated that glucosylation of the lysine residues in sites I and IV would not result in such a marked decrease in Ca$^{2+}$ binding at these sites. Ca$^{2+}$-binding site III has one lysine residue which is located in the center of the Ca$^{2+}$-binding domain III and can possibly cause steric hindrance in Ca$^{2+}$-binding. Domain I has three lysine residues and it was shown that the extent of glucosylation was highest in this region of CaM molecule therefore, it is quite possible that the Ca$^{2+}$-binding to this domain is also hindered.

3.6 CALMODULIN FUNCTION IN PLATELETS: RELEVANCE TO DIABETES

Platelets are non-nucleated, disc-shaped cells (Evatt et al., 1988). There are approximately 150,000-400,000 platelets per mL of blood. The life-span of platelets is about 10 days. CaM was first purified from platelets in 1974 by Smoake et al. (1974). The Intracellular concentration of CaM in platelets is about 30 μM (Jones, 1985) and the amount of CaM is ~0.56-1.2 pg per platelet.

Platelets play an important role in hemostasis by arresting bleeding of damaged blood vessels and by maintaining the endothelial surface of the vessels (Evatt et al., 1988). Platelets
are capable of aggregating with each other and on the damaged blood vessel area. Adhesion of platelets to the blood vessel, initiates a release reaction where substances are secreted from granules located inside the platelets. The substances released cause the platelets to aggregate. The sequence of events is Adhesion → Release reaction → Platelet aggregation (Weiss, 1975). Platelet aggregation can also be induced by exogenous substances and the process is termed as first-phase aggregation. When substances, secreted from intracellular platelet granules, cause aggregation the process is termed as second-phase aggregation (Weiss, 1975).

CaM regulates platelet aggregation by mediating the release reaction (Nishikawa, 1980). The latter is initiated by the force generated during contraction of the actomyosin complex. This complex is formed by the proteins actin and myosin. For contraction and actomyosin complex formation, the protein myosin has to be phosphorylated. Myosin light chain kinase (MLCK) catalyzes the phosphorylation of myosin and is regulated by calmodulin (Weiss et al., 1975).

In diabetics, there is enhanced platelet aggregation which contributes to the vascular complications observed in these patients (Nishikawa et al., 1980; Evatt, 1988). CaM levels in insulin-dependent diabetes mellitus patients were found to increased ~2-fold (Paoliisso, 1986). The authors suggested that monitoring CaM levels in diabetics could provide a useful indicator of altered platelet function. Furthermore, CaM antagonists trifluoperazine (TFP) and N-(6-amino-hexyl)-5-chloro-1-naphthalene sulphonamide (W7), inhibit the release reaction and second-phase aggregation (Nishikawa et al., 1980). Monitoring the extent of phosphate incorporated in myosin, at varying concentrations of W7 showed that the drug inhibits phosphorylation of myosin (Nishikawa et al., 1980). The above results indicate that CaM plays an important role in platelet aggregation by regulating the phosphorylation of the proteins.

In summary, non-enzymatic glucosylation of proteins can hinder protein structure and function. Many methods have been developed to quantitate non-enzymatic glucosylation of proteins. These methods can be broadly classified into three classes: chromatographic, chemical and electrophoretic methods. The calcium binding regulator protein, calmodulin can undergo in
**vitro** non-enzymatic glucosylation. This modification alters the ability of CaM to stimulate its target proteins. The Ca$^{2+}$-binding affinity of glucosylated CaM was decreased by 50% as compared to native CaM. Diabetic patients exhibit higher levels of CaM. In diabetic platelets, the CaM levels were increased by about 2-fold. Diabetics also exhibit increased platelet aggregability and it has been shown that CaM regulates of platelet aggregation. Therefore, monitoring platelet CaM levels in diabetic patients could provide a useful indicator of altered platelet aggregation.

Glucose is transported through platelet membrane by an active transport and by simple diffusion (Yardimci, 1980). In diseased states like diabetes mellitus and atherosclerosis, the platelet-active glucose transport is impaired and the sugar is transported inside the platelets by simple diffusion (Yardimci, 1980; Yardimci and Ulutin, 1986). In view of the above observation plus the fact that CaM is glucosylated *in vitro*, it is possible that in CaM undergoes non-enzymatic glucosylation *in vivo*. The consequence of such a modification would be to lower the effective concentrations of CaM which in turn could possibly result in impaired platelet function. The higher levels of CaM observed in diabetics could then result from overexpression of the protein in response to signals of impaired biochemical process.

### 3.7 AIM OF THE PRESENT STUDY

Two major Ca$^{2+}$/CaM-dependent PDEs have been isolated from bovine brain, the low $M_r$ (60 kDa) and high $M_r$ (63 kDa) species (Sharma et al., 1988; Beavo, 1988). Kinetic studies of the two brain PDEs and the bovine heart PDE (59 KDa) showed that all the three isoforms exhibit specificity towards cGMP as compared to cAMP (Sharma et al., 1988). These isoforms were isolated by use of monoclonal antibodies targeted against the PDEs.

Previously in our laboratory, the three isoforms were purified with aid of monoclonal antibodies targeted against the PDE-CaM complex (Karupiah, 1987). Kinetic studies were performed using cNMP analogs, 2'-O-anthraniloyl (ANT)- and 2'-O-methylanthraniloyl (MANT)- derivatives (Karupiah, 1987). These studies indicated that the two brain isoforms had equal
affinity for both substrates as judged from the $V_{\text{max}}/K_m$ ratios. The heart enzyme appeared to be cAMP specific (Karuppiyah, 1987). Thus it was interpreted that the isoforms isolated using PDE-CaM conformational monoclonal antibodies are distinct from the ones isolated by Sharma et al. (1988).

The PDEs purified by Karuppiyah could not be compared with the isoforms isolated by Sharma et al. (1988) as the kinetic studies were not performed with native substrates. It is possible that the difference in the kinetic parameters observed in Karuppiyah's study was a result of using cAMP and cGMP analogs.

The aim of this project was to isolate the three PDE isoforms with PDE-CaM conformational monoclonal antibodies and to study the catalytic properties of PDEs using native substrates. Furthermore, an attempt was made to elucidate the differences in the catalytic sites of these PDEs by taking into account the kinetic parameters obtained with cNMP analogs (Karuppiyah, 1987) in conjunction with the postulated structures of the analogs, predicted with the aid of energy minimization calculations using the computer program PC Model.

In the past few years PDE has gained much pharmacological interest. Increased levels of cNMPs are associated with smooth muscle relaxation (Ignarro and Kadowitz, 1985). Thus inhibition of PDE activity provides a point of control for regulating cNMPs levels. Various inhibitors have been synthesized which bring about inhibition of PDE activity and relaxation of smooth muscle (Kaufman et al., 1987). Many PDE isoforms have been isolated from smooth muscles (like vascular and bronchial tissues) and are being screened with drugs for analysing the ability of the drug to act as potential vasodilative/cardiotonic agents (Nicholson et al., 1991).

The major drawback in the use of PDEs for screening drugs is that relatively low amounts of these isoforms are present in these tissues, and that there is usually a limited supply of these tissues.

The second part of the phosphodiesterase project dealt with the isolation and characterization of bovine uterine phosphodiesterase. The aim of this study was: first, to purify a Ca$^{2+}$/CaM-dependent PDE from bovine uterus and to compare its drug sensitivity with
Ca²⁺/CaM-dependent PDE from bovine aorta. This would indicate whether the uterine isoform can be used as a substitute for screening new drugs. Secondly, an attempt has been made to characterize the bovine uterine PDE.

Chemical modification of CaM has been associated with altered function of the protein. CaM undergoes two posttranslational modifications both involving amino groups: Lys-115 is trimethylated and the amino terminus alanyl residue is acetylated. The second project of the present study was to perform \textit{in vitro} non-enzymatic glucosylation of CaM and study the effect of such a modification on the ability of CaM to stimulate its target enzymes. Furthermore, a method has been developed to quantitate the extent of glucosylation of CaM and other non-enzymatically glucosylated proteins. This method was a modification of the method previously used by Massamini \textit{et al.} (1979) for quantitating sialic-acid residues.

The platelet CaM levels of diabetic patients are shown to be about 2-fold higher as compared to normal individuals. CaM plays a key role in the regulation of platelet aggregation by the way of regulating the activity of the enzyme, myosin light chain kinase. As mentioned before, CaM is a dumbbell shaped molecule with two lobes connected by a \( \alpha \)-helix. Each lobe consists of two Ca²⁺-binding domains. Studies with MLCK calmodulin-binding domain peptides indicate that both lobes of CaM can interact with MLCK (Klevit and Blumenthal, 1987). Residues, Phe-16, Thr-26, Asp-64 and Phe-65, of the N-terminal region of CaM are directly involved in MLCK-CaM interactions. The three dimensional structure of CaM with four Ca²⁺ bound to it shows that Asp-64 and Thr-26 are close to the Lys-13 and Lys-30, respectively. Thus any modification of these lysine residues would interfere with the interaction of MLCK at the N-terminal region of CaM. Kowliuru \textit{et al.} (1989), postulated that Lys 13, 20 and 30 of CaM are the most likely sites of glucosylation. Hence it would be interesting to investigate whether CaM undergoes \textit{in vivo} non-enzymatic glucosylation. To this end, attempts were made to purify CaM from platelets of diabetic patients and to check whether the protein is glucosylated.
CHAPTER II Materials

Acetone
Acrylamide
Affi-gel 601
Affi-gel Heparin
1-Amino-2-Naphthol-4-Sulphonic Acid
Ammonium Acetate
Ammonium Molybdate
Ammonium persulfate
Anti-BSA
β-mercaptoethanol
Biorad protein Reagent
Bovine Brains/Heart
Bovine Serum Albumin
Bovine Uterus

Butanol
Calcium Chloride
Calmodulin-Agarose

Cyanogen Bromide
Cyclic Adenosine Monophosphate
Cyclic Guanosine Monophosphate
Diethylaminoethyl Cellulose
Ethanol

BDH
Sigma
BioRad
BioRad
Sigma
Sigma
BDH
Sigma
Sigma
Sigma
Biorad
CDN meat Packers, Leamington.
Sigma
Wolverine, Detroit /Peltreez, Arkansas
BDH
Sigma
Sigma
Company
Sigma
Sigma
Sigma
BDH
Ethylene Glycol-bis(β-aminooethyl ether) N,N,N',N'-. Sigma
Ethylenediaminetetraacetic Acid BDH
Ferric Chloride BDH
Formaldehyde Sigma
Fructose BDH
Glacial Acetic Acid Sigma
HEPES Sigma
Imidazole Sigma
Lauryl Sulphate BioRad
m-aminophenylboronate Agarose Sigma
Magnesium Acetate Sigma
Magnesium Chloride Sigma
Methanol BDH
3-Methyl-2-benzothiazolinone Aldrich Chemical Company
Hydrazone(MBTH) hydrochloride Sigma
N,N'-Methylene bis Acrylamide Sigma
N,N,N,N'-Tetramethylethylene diamine Sigma
5'-Nucleotidase (from Crotalus atrox) Sigma
Phenyl-Sepharose Sigma
PMSF Aldrich Chemical
Potassium Chloride Aldrich Chemical
Procion Blue HB Sigma
Protein-A BioRad, Sigma
SDS-PAGE Molecular weight Standards Pharmacia
Sephadex G-25 Pharmacia
Sepharose 4B BDH
Sodium Azide
Sodium Bisuphite
Sodium Chloride
Sodium Hydroxide
Sodium Periodate
Sodium Sulphite
Sorbitol
Tetraacetic Acid
TLCK
TPCK
Trichloroacetic Acid
Trizma Base
Trypsin
Trypsin Inhibitor
Urea
Zinc Sulphate
ACC-1 and ACAP-1 monoclonal antibodies

INSTRUMENTATION

Low Pressure chromatography Econo System
UV-160 Shimadzu Spectrophotometer
Electrophoresis Apparatus
Model J-6B Centrifuge
CHAPTER-III-Methods

1.0 PREPARATION OF SEPHAROSE ACC-1 AND ACAP-1 MONOCLONAL AFFINITY COLUMNS

Lyophilized ascites fluid containing the monoclonal antibodies (2 mg) were dissolved in buffer A (40 mM tris-HCl, 1.0 mM Mg(CH$_3$COO)$_2$, pH 7.4) containing 0.1 mM CaCl$_2$ and 0.1 M NaCl. 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$^\circ$C for about 1 hour. The stirred mixture was then packed in an Econo column (0.7 X 1.2 cm) and washed with the same buffer to remove the unbound protein. The monoclonal antibody affinity column was then stored in phosphate buffered saline at 4$^\circ$C until further use.

1.1 PURIFICATION OF BOVINE PDEs USING MONOCLONAL ANTIBODIES

Partially thawed tissues (1kg) were homogenized with 2 L of buffer A (pH 7.4) containing 0.1 mM EGTA, 1.0 mM EDTA, 10 mM β-mercaptoethanol and protease inhibitors (PMSF, TPCK, TLCK 1mg/L of each). The homogenates were centrifuged at 3500 X g for 45 minutes and the supernatant was filtered through glass wool. Solid (NH$_4$)$_2$SO$_4$ was added to the filtrate to give 45% saturation and the sample was incubated for 30 minutes. The pellet was collected by centrifugation, dissolved in buffer A and dialyzed overnight against the same buffer (2 X 12 L). The dialyzed sample was applied to blue-Sepharose column (4 X 10 cm), the column was washed with buffer A containing 0.15 M NaCl and 0.1 mM EGTA and the PDE was eluted with same buffer containing 1 M NaCl. The eluate was dialyzed overnight against buffer A (2 X 12 L) containing 1mM CaCl$_2$. CaM was added to the dialysate to a final concentration of 10 mM prior to its application onto either the ACC-1 or ACAP-1 matrix. The ACC-1 monoclonal antibody is reactive to both low and high M$_r$ brain enzymes, which were eluted with buffer A containing 1 mM
EGTA. ACAP-1 only binds the low M₉ isoform and was eluted with buffer A containing 2 M 
MgCl₂, pH 7.5. Thus, the two forms of the enzyme can be separated by subjecting the eluate of 
ACC-1 to ACAP-1. The purification was carried out at 4°.

1.2 PURIFICATION OF CALMODULIN

Bovine brain calmodulin was purified by a modified procedure of Sharma and Wang  
(1979). 1 kg of bovine brain was homogenized, using a Waring blender, in 4 L of 40 mM Iris-HCl;  
pH 7.4. 1.0 mM Mg(CH₃COO)₂ (buffer A) containing 40 μg/mL PMSF and 1.0 mM EGTA. The  
homogenates were then centrifuged at 3500 X g for one hour. The supernatant was then passed  
through glass wool and applied over DEAE-cellulose (9.5 X 10 cm) and subjected to two washes  
of, 0.05 M NaCl and 0.25 M NaCl in buffer A containing 0.1 mM EGTA. CaM was eluted with  
buffer A containing 0.5 M NaCl and 0.1 mM EGTA. The eluate from DEAE-cellulose was  
dialed overnight against water (2 X 16 L). The dialysate was made 5.0 mM CaCl₂ (final  
concentration) and applied to phenyl Sepharose (5 X 9 cm). The column was washed with buffer  
A containing 0.2 M NaCl and 0.1 mM CaCl₂ and CaM was eluted with buffer A plus 0.2 M NaCl  
and 1.0 mM EGTA. The purification except for the phenyl-Sepharose chromatography was  
carried out at 4°. The phenyl-Sepharose affinity chromatography was performed at room  
temperature. CaM was extensively dialyzed against water, lyophilized and stored at -20°.

1.3 PREPARATION OF BLUE-SEPHAROSE

Blue-Sepharose was prepared according to the method of Dean and Watson (1979).  
100 g (moist weight) of Sepharose 6B is suspended in 350 mL water and 100 mL of the 1%  
procion blue. The mixture was stirred for 10 minutes. 50 mL of 10% NaCl solution was added  
and the mixture stirred for one half hour at room temperature. 2.5 mL of 5 M NaOH is added to  
elevate the pH to 10, followed by incubation of the mixture for 3 days after which the Sepharose
is extensively washed with deionized distilled water, 1 M NaCl, 4-8 M Urea and water. Before using the affi-blue Sepharose the gel was equilibrated with buffer A.

1.4 PREPARATION OF CALMODULIN-AGAROSE

CaM-agarose was either bought from Sigma or prepared as described in the following section. Affi-gel 15 agarose (BioRad) was used as the solid phase in the preparation of CaM-agarose. This solid phase is an N-hydroxysuccinimide ester of derivatized crosslinked agarose. 30-40 mgs of bovine brain CaM was dissolved in 40 mM Heps buffer, pH 7.0. The CaM solution was combined with 10 mL of gel slurry which had previously been washed with deionized distilled water. The mixture was gently stirred for 4 hours at 4°C. The gel was then washed with 7 M urea containing 1.0 mM EGTA followed by a wash with deionized distilled water and 40 mM Heps buffer, pH 7.0. Using the above method 98% of CaM bound to affi-gel 15 agarose. The gel was stored at 4°C until further use.

1.5 PURIFICATION OF CaM-DEPENDENT UTERINE PHOSPHODIESTERASE

CaM-dependent uterine phosphodiesterase was purified by a modified procedure of Sharma et al. (1980). 1 kg of the tissue was homogenized with 4 L of buffer B (40 mM tris-HCl, pH 7.4; 2.0 mM Mg(CH$_3$COO)$_2$ and 10.0 mM β-ME) containing 1.0 mM EGTA, 0.2 mM PMSF and 15 mM benzamidine-HCl. The protease inhibitors (PMSF and benzamidine) were included in all the buffers used in the purification. The homogenate was centrifuged at 3500 X g for 1 hour at 4°C. The supernatant was passed through glass wool and applied to DEAE-cellulose (9.5 X 11 cm) previously equilibrated with buffer A. The column was washed with 2 L of buffer A containing 0.05 M NaCl and PDE was eluted with 1 L of buffer A containing 0.25 M NaCl. The DEAE eluate was applied to blue-Sepharose column (5.5 X 9 cm) which was then washed with 1 L of buffer B containing 0.1 M NaCl. The PDE was eluted from blue-Sepharose with 1 L of the same buffer.
containing 1.0 M NaCl. The blue-Sepharose eluate was dialyzed overnight against buffer B (2 X 16 L) containing 1.0 mM CaCl₂. The dialysate was subjected to CaM-agarose (2 x 3 cm) previously equilibrated with buffer B. The gel was washed with same buffer containing 0.1 mM CaCl₂ followed by the elution of uterine PDE with 1.0 mM EGTA. The purification was carried out at 4°C. The CaM-agarose eluate was then concentrated with 40% glycerol in buffer B and stored at -80°C until further use.

1.6 PREPARATION OF ANTI-BSA-PROTEIN-A SEPHAROSE

4 mg of anti-BSA (polyclonal antibody to bovine serum albumin) was dissolved in 0.1 M sodium phosphate buffer, pH 7.0. The antibody solution was mixed with 500 μL of protein-A Sepharose which had previously been equilibrated with the same buffer. The mixture was made 0.2% glutaraldehyde and gently stirred for 2 hours at 4°C. The anti-BSA-protein-A Sepharose was then extensively washed with 0.5 M acetic acid (pH 2.0) containing 0.1 M NaCl and equilibrated with 20 mM tris-HCl, pH 7.3. The column was stored at 4°C until further use.

1.7 IMMUNOADSORPTION OF UTERINE PDE AND BSA ON ANTI-BSA PROTEIN A SEPHAROSE

10 μg of uterine CaM-dependent PDE and BSA were separately mixed with 150 μL of anti-BSA-protein-A suspension. As control, the proteins (10 μg each of uterine PDE and BSA) were mixed with 150 μL protein-A suspension alone (no antibody). The samples were incubated for 1 hour at 4°C. The total suspension volume was adjusted to 350 μL with 20 mM tris-HCl, pH 7.3. The samples were then centrifuged at 15,000 X g. The supernatant was removed and the protein was precipitated with 0.1% TCA. The TCA treated samples were subjected to 10% SDS-PAGE after adjusting the pH of the sample to about 7.3 with 1.0 M tris-HCl, pH (7.3).
1.8 IMMUNOADSORPTION OF UTERINE PDE ON ACC-1-PROTEIN-A SEPHAROSE

ACC-1-protein-A Sepharose was prepared as described in section 1.0 of this chapter. 5 μg of uterine PDE plus 2 μg of CaM was incubated with 100 μL of ACC-1-protein-A suspension in 20 mM tris-HCl, pH 7.2 containing 1.0 mM CaCl₂. As control, the same amount of uterine PDE and CaM were incubated 100 μL protein-A suspension alone (no antibody). The total suspension volume was 150 μL. The samples were incubated at 4° for 1 hour followed by centrifugation at 15,000 X g for 5 min. The supernatants were removed and the protein precipitated with 0.1% TCA. The pH of the TCA treated samples was adjusted to about 7.3 and the samples were then subjected to 10% SDS-PAGE.

1.9 ELECTROPHORESIS

Electrophoresis was performed with 10 % SDS-PAGE gels using the method of Laemmli (1970). The gels were sandwiched between two layers of Biogel wrap and dried at room temperature.

2.0 PHOSPHODIESTERASE ASSAY

PDE activity was determined by a coupled assay of Sharma and Wang (1979). The assay involves hydrolysis of the cyclic nucleotide to the 5'-nucleotide. The latter is then subjected hydrolysis by the enzyme 5'-nucleotidase resulting in the release of phosphate and the corresponding nucleoside. The phosphate is then quantitated as phosphomolybdate complex which has an absorption maxima at 660 nm. The assay mixture contained PDE, saturating amounts of CaM and Ca²⁺, 5'-nucleotidase, 36.0 mM tris-HCl (pH 7.3) and 4.5 mM Mg(CH₃COO)₂. The volume was adjusted to 800 μL with deionized distilled water. The reaction was started by the addition of 100 μL substrate (cAMP or cGMP) and allowed to proceed for a
appropriate time period (i.e., reaction was linear for the time period used) at 30\(^{\circ}\). The reaction was stopped with 100 \(\mu\)L of 55% TCA. At this point, if a precipitate was observed, the sample was centrifuged and 500 \(\mu\)L of the supernatant was placed into a test tube. To the supernatant, 500 \(\mu\)L of ammonium molybdate and 50 \(\mu\)L of reducing agent was added. (If no precipitate was observed, the centrifugation step was omitted and 1 mL of ammonium molybdate and 100 \(\mu\)L of reducing agent was added to the sample). The reaction mixture was incubated at 30\(^{\circ}\) for 10 minutes after which the absorbance is read at 660 nm. The reducing agent was prepared by dissolving 500 mg of 1-amino-2-naphthol-4-sulphonic acid in 12% solution of sodium bisulphite containing 1.2% sodium sulphite. PDE activity is expressed as U/mg. A phosphate standard curve was used to calculate the amount of phosphate produced in the PDE assay (Appendix 2). A unit is defined as the amount of PDE catalyzing the hydrolysis of 1 \(\mu\)mol of cAMP or cGMP per minute. The amount of nucleotidase used in this assay was the same as dictated by the procedure of Sharma and Wang (1979). Although, it was also tested that the 5'-nucleotidase was present in sufficient amounts to catalyse the conversion of the 5'-AMP produced to phosphate and the corresponding nucleoside as increasing the amounts of the enzyme did not result in an increase in phosphate production.

2.1 SEPARATION OF GLUCOSYLATED PROTEIN FROM NON-GLUCOSYLATED PROTEIN VIA m-AMINOPHENYL BORONATE AFFINITY CHROMATOGRAPHY

A protein sample containing a mixture of glucosylated and non-glucosylated protein was applied over \(m\)-aminophenyl boronate gel (1 X 3 cm) which had previously been equilibrated with 250 mM ammonium acetate, pH 8.8 containing 50 mM MgCl\(_2\). After application of the sample the column was washed extensively with the equilibrating buffer to remove any non-specifically bound protein. The glucosylated protein was eluted with either 200 mM sorbitol or 10 mM acetic acid, pH 3.0
2.2 PROCEDURE FOR MBTH ASSAY

The procedure entails four major steps: (a) periodate oxidation of fructosamine releasing HCHO, (b) removal of protein and excess periodate by zinc precipitation, (c) conjugation of released HCHO with the dye MBTH, forming an azine, (d) production of the chromogen by reacting the azine with the FeCl₃ oxidized form of the dye.

Aliquots containing fructose standards or glucosylated protein were adjusted to pH 3.0 with 15 µL of 0.1 M HCl. The volume was adjusted to 1.005 mL with deionized distilled water. Oxidation was started by the addition of 150 µL of 10.0 mM NaIO₄ and allowed to proceed at room temperature for 5 min. The excess periodate was precipitated by addition of 300 µL of 50% (w/v) ice cold ZnSO₄ and 45 µL of 1.0 M NaOH. The sample was centrifuged at 15,000 X g for 2 min. To 400 µL of the supernatant, 100 µL of 0.1 M glycine-HCl, pH 2.6, was added followed by the addition of 100 µL of 10 mM MBTH. The sample was incubated for 20 minutes at room temperature after which 100 µL of 20 mM FeCl₃ was added. The color was allowed to develop for 5 minutes after which the reaction was quenched with 300 µL of acetone. The absorbance was read at 630 nm in a Shimadzu UV-160 spectrophotometer.

HCHO calibration curves were performed under similar conditions. HCHO was standardized by the method of Ma and Cheronis (1964).

2.3 PURIFICATION OF PLATELETS

Whole blood samples from ten diabetic patients were obtained from Salvation Army Grace Hospital. The patients were classified on the basis of percentage of glycated hemoglobin content in their blood (low <7%, High > 11%). The blood from five patients exhibiting low and high levels of glycated Hb was pooled separately and centrifuged at 150 X g for 20 minutes. The platelet rich plasma (PRP) was separated from the red blood cell layer by aspirating the top layer into a plastic tube and centrifuged again at 150 X g for 20 min to further remove any red blood
cell contamination. The platelet pellet was obtained by centrifuging the PRP at 1000 X g for 10 minutes. The pellet was washed with 0.85% saline followed by centrifugation at 1000 X g for 10 minutes. The wash step was repeated twice. The platelet pellet was then stored at -80° until further use.

2.4 PARTIAL PURIFICATION OF CaM ON ECONO-Q ANION EXCHANGE CARTRIDGE

A platelet pellet was obtained as described in the above procedure. 1 mL of deionized distilled water was added to the pellet and the cells were lysed by repeated freezing and thawing. The cells were frozen with liquid nitrogen and thawed at 37°; the procedure was repeated three times. The lysed platelet sample was then centrifuged at 15,000 X g for 10 min. The supernatant was applied to an Econo-Q anion exchange cartridge (1 X 4 cm) (BioRad) which had previously been equilibrated with 20 mM Hepes buffer, pH 7.0 containing 0.1 mM EGTA. The cartridge was washed with the equilibrating buffer. The protein was eluted from Econo-Q by application of a linear gradient of 0-0.7 M NaCl in 20 mM Hepes buffer, pH 7.0 (flow-rate 1mL/min). 1 mL fractions of the eluate were collected and checked for CaM activity.

2.5 PURIFICATION OF CALMODULIN USING AFFI-GEL HEPARIN

Platelets were purified by the procedure described above. The cells were lysed by freezing and thawing and the platelet extract (approximately 3 mL) was subjected to heparin-agarose affinity column (0.7 X 5 cm). The affi-gel heparin column had previously been equilibrated with 20 mM Hepes; pH 7.0. After application of the sample the gel was washed with about 7 mL of equilibrating buffer. The breakthrough and the wash from affi-gel heparin were collected and applied to phenyl-Sepharose (0.7 X 3 cm) which had previously been equilibrated with 20 mM Hepes, pH 7.0, containing 0.2 M NaCl and 0.1 mM CaCl₂. The column was washed
extensively with the same buffer and CaM was eluted with 20 mM Hepes (pH 7.0) containing 0.2 M NaCl and 1.0 mM EGTA.

2.6 DETERMINATION OF CaM ACTIVITY

The assay for measuring CaM activity was based on the ability of CaM to stimulate heart phosphodiesterase. 100 µL of each fraction of the Econo-Q eluate was subjected to 70° for 20 minutes, which destroys any PDE activity present in the sample. The tubes were then cooled and the PDE assay as described in section 2 of this chapter, was performed on the heat-treated Econo-Q eluate fractions. The only difference was that no exogenous CaM was added to the assay tubes. The reaction mixture contained constant amount of heart PDE.

2.7 PROTEIN DETERMINATIONS

Protein concentration was determined according to the method of Bradford (1976). A Bovine serum albumin standard curve was used for estimation of uterine PDE, bovine heart and brain PDEs and calcineurin concentrations. CaM concentrations were estimated by either Bradford assay using a CaM standard curve or by measuring its absorbance at 274 nm (molar extinction coefficient of 1% CaM solution is 2.1 at 274 nm).
CHAPTER IV-Results and discussion

1.0 KINETIC CHARACTERIZATION OF THE MONOCLONAL ANTIBODY PURIFIED
BOVINE BRAIN AND HEART PHOSPHODIESTERASES

This study was initiated as a follow up on the results obtained in our laboratory
(Karuppih, 1987) where the three Ca\(^{2+}\)/CaM-dependent PDEs, low (60 kDa) and high (63 kDa)
M\(_r\) isoforms from the brain and 59 kDa isoform from the heart, were purified with the aid of
conformation specific monoclonal antibodies. Kinetic studies with fluorescent analogues of cAMP
and cGMP (Karuppih, 1987) indicated that these isoforms were kinetically distinct from those
previously isolated by Sharma et al. (1988); however a direct comparison of the isoforms was not
possible as native substrates were not used in Karuppih's work.

In the present study, the three bovine PDEs have been purified by similar procedures as
previously used in our laboratory and kinetics were performed with native substrates. The two
Ca\(^{2+}\)/CaM-dependent PDEs from the bovine brain were separated by using ACC-1 and ACAP-1
monoclonal affinity chromatography. These antibodies specifically recognize the conformation of
the phosphodiesterase when it is bound to CaM (Hansen and Beavo, 1986). The antibodies
have little affinity for CaM-free PDE. ACC-1 binds both the low and high M\(_r\) PDEs whereas,
ACAP-1 binds the low M\(_r\) PDE and the heart 59 kDa PDE. Previous studies have demonstrated
that the low M\(_r\) brain and the heart PDE are immunologically related and are homologous in their
amino acid sequence over all the regions except the N-terminal (Beavo, 1988).

The SDS-PAGE of the bovine PDE isozymes (Figure 10) indicates that the PDEs eluted
in ACAP-1 chromatography have a slightly higher electrophoretic mobility in comparison to the
high M\(_r\) (63 kDa) isozyme. The latter does not bind to ACAP-1 column. The low M\(_r\) weight band
(about 17,000) observed in each lane is CaM which is copurified by the two monoclonal antibody
columns.
Figure 10: 10% SDS-PAGE of bovine PDE (20 μg) isoforms. Lane 1- Heart ACAP-1 eluate; lane 2- Brain ACAP-1 eluate (Low M<sub>r</sub> Brain PDE); Lane 3- ACAP-1 breakthrough of ACC-1 purified brain PDEs (high M<sub>r</sub> Brain); lane 4- Molecular weight markers, BSA (66 kDa), Ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa). The electrophoresis was performed according to the method of Laemmli (1970).
The activities of all three bovine PDEs were stimulated by approximately 10-fold in the presence of saturating amounts of Ca$^{2+}$ and CaM. The low $M_r$ brain and heart isoforms exhibited specific activities of about 30 U/mg whereas, the high $M_r$ isoform exhibited a specific activity of 15 U/mg (using 1mM cAMP and no imidazole). Imidazole has been shown to increase both $K_m$ and $V_{max}$ values of the Ca$^{2+}$/CaM-PDEs. The physiological significance of this activator is unknown, and therefore it has been omitted from the assays in the present study. The specific activities of the low $M_r$ Brain and heart PDE were about 13-fold lower than the values originally reported for these isoforms (400 U/mg) purified by using the same monoclonal antibodies (ACAP-1 and ACC-1) (Hansen and Beavo, 1986). The specific activity of the high $M_r$ was about 5-fold lower than the previously reported value for this PDE (70 U/mg).

The steady-state kinetics were performed in the presence of saturating amounts of Ca$^{2+}$ and CaM, this was necessitated by the fact that an accurate determination of the basal hydrolysis rates, particularly at low substrate concentrations, is not possible with the assay employed. The three bovine PDEs exhibited linear Lineweaver-Burk plots with the native substrates, cAMP and cGMP (Figures 11-13). The kinetic parameters were calculated from a simplex fit (Appendix 1) of the kinetic data to the Michaelis-Menten equation and are summarized in Table-I.

It was expected that the three isoforms purified in the present study would have similar kinetic properties to those purified by Sharma et al. (1988), using different monoclonal antibodies. Contrary to our expectation, the enzymes purified in this study were distinct. The three enzymes were indistinguishable with respect to their affinity for cAMP and cGMP and exhibited Michaelis constants of approximately 50 μM for cAMP and 20 μM for cGMP (Table-I). In comparison, the PDEs isolated by Sharma et al. (1988) exhibited about 10-fold greater affinity for cGMP than for cAMP; the $K_m$s for cGMP exhibited by heart, low $M_r$ and high $M_r$ brain PDEs were 5 μM, 2.7 μM and 1.2 μM and for cAMP, 35 μM, 32 μM and 11 μM, respectively.

The $V_{max}/K_m$ ratios of the heart and brain low $M_r$ PDE in the present study were about unity indicating that both the enzymes are equally specific for cAMP and cGMP (Table-I). Only
Figure 11: Kinetics of bovine heart CaM-dependent PDE with cAMP (●) and cGMP (▲), in the presence of saturating amounts of Ca\(^{2+}\) and CaM. Kinetics were performed at 30° for half hour incubations which were stopped by the addition of 5.5% TCA. The reaction mixture contained 1.0 mM Ca\(^{2+}\), 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase and 1.66E-8 M heart PDE. The solid line is the theoretical best-fit of the kinetic data via the simplex method (Appendix I) to the Michaelis-Menten equation. All points represent triplicate determinations. The error bars representing 2X the standard error of each mean are omitted when they fall within the marker symbols.
Figure 12: Kinetics of bovine brain low Mr PDE with cAMP (○) and cGMP (▲), in presence of saturating amounts of Ca²⁺ and CaM. Kinetics were performed at 30⁰ for half hour incubations which were stopped by the addition of 5.5% TCA. The reaction mixture contained 1.0 mM Ca²⁺, 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase and 7.5E-8 M low Mr PDE. The solid line is the theoretical best-fit of the kinetic data via the simplex method (Appendix I) to the Michaelis-Menten equation. All points represent triplicate determinations. The error bars representing 2X the standard error of each mean are omitted when they fall within the marker symbols.
Figure 13: Kinetics of bovine brain high M_r PDE with cAMP (●) and cGMP (▲), in presence of saturating amounts of Ca^{2+} and CaM. Kinetics were performed at 30° for half hour for incubations which were stopped by the addition of 5.5% TCA. The reaction mixture contained 1.0 mM Ca^{2+}, 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase and 8.3E-9 M high M_r PDE. The solid line is the theoretical best-fit of the kinetic data via the simplex method (Appendix 1) to the Michaelis-Menten equation. All points represent triplicate determinations. The error bars representing 2X the standard error of each mean are omitted when they fall within the marker symbols.
TABLE I: Kinetic parameters for CaM-dependent bovine heart and Brain PDE isozymes with cGMP and cAMP.

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<td>$V_{max}$ (U/mg)</td>
<td>34±4</td>
<td>22.6±4</td>
<td>16.3±3</td>
<td>113±5</td>
<td>32±1.5</td>
<td>26.5±3</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>0.75</td>
<td>0.97</td>
<td>0.31</td>
<td>4.7</td>
<td>0.62</td>
<td>1.3</td>
</tr>
</tbody>
</table>
the high Mₘ brain isoform exhibited specificity towards cGMP, the $V_{\text{max}}/K_{\text{m}}$ ratios of this enzyme for cAMP and cGMP being 0.31 and 4.7, respectively. In Sharma's study, the brain enzymes exhibited $V_{\text{max}}/K_{\text{m}}$ ratios of about 10-fold or greater for cGMP as compared to cAMP. Figure 14 represents a comparison of the kinetic properties for the three enzymes purified in the present study and by Sharma et al. (1988). Clearly, the enzymes purified in our laboratory are distinct from those purified by Sharma and coworkers. In view of the very large range of kinetic parameters reported for the CaM-dependent PDEs, the existence of multiple kinetic forms of this enzyme is quite possible. For example, literature $K_{\text{m}}$ values for cAMP estimated for the bovine brain CaM-dependent PDE range between 10 µM to 200 µM (Sharma et al., 1984; Shenolikar et al., 1985; Morrill et al., 1979; Tucker, et al., 1981). It is possible that the activities of these isoforms are dependent on the age of the tissue. This could potentially account for the variation in the activity of the three enzymes studied in comparison to those originally reported for ACC-1 and ACAP-1 monoclonal antibody purified PDEs.

Further distinction between the three PDEs could be made by taking into account the predicted structures of the pseudosubstrates and the kinetic parameters obtained with the same (study done by Karuppiah). The steady state kinetic parameters obtained with the pseudosubstrates are represented in bar graph format in Figure 15. The structures of these pseudosubstrates were predicted with the aid of energy minimization calculations (PC MODEL). The pseudosubstrates are 2'-O-anthranyl- (ANT) and 2'-O-(N-methylanthraniloyl)-(MANT) derivatives of cAMP and cGMP (Figure 16, A). Kinetics with the pseudosubstrates indicated that the major effect of the 2'-O-substitutions was on the $V_{\text{max}}$ the values for which were 1-11% of the native substrates. Thus, the 2'-O-substitutions lower the catalytic efficiency of the enzymes presumably by interfering in the interactions of active site residues with the substrates. The $K_{\text{m}}$ values obtained for the three PDEs with pseudosubstrates were similar indicating that the substitutions at the 2'-O-position have little affect on the binding affinity of the PDEs.
COMPARISON OF THE KINETIC PARAMETERS OF THE BOVINE PDEs OBTAINED IN THE PRESENT STUDY AND IN SHARMA'S STUDY.

![Graphs showing kinetic parameters](image)

**Figure 14:** Kinetic parameters for cAMP and cGMP exhibited by the bovine PDE isoforms, in the present study and in Sharma's study. Upper panel: present study; Lower panel: Sharma's study. cAMP: , cGMP: .
Figure 15: Kinetic parameters for the ANT- and MANT-derivatives of the cAMP and cGMP exhibited by the PDE isoforms expressed in bar graph format (experiments done by Karupplah, 1987).

A: exhibits the $K_m$ values, the dotted lines represent the $K_m$ values obtained with the natural substrates.

B: exhibits the $V_{max}$ values.
**Figure 16:** The structures for ANT- and MANT- derivatives of the cNMPs. (A) Minimum energy conformation predicted by the energy minimization programme PC MODEL for ANT-cAMP. (B); and MANT- cAMP. (C); (---) indicates an H-bond with a predicted distance of 2.2 Å.
The low Mₐ brain PDE exhibited higher Kₘṣ and Vₘₐxₜ for ANT-derivatives as compared with the MANT-derivatives whereas, the high Mₐ brain PDE exhibited such a trend only with the cGMP derivatives. The slight structural differences of 2'-O-substituents had no effect on the heart enzyme. The differential response of the three isoforms with the ANT- and MANT-derivatives, is probably due to the differences in the active-site environment of these enzymes. Further, the fact that the heart and low Mₐ brain enzyme associate with same monoclonal antibodies but, are affected to varying extents by the pseudosubstrates indicates that the two enzymes are immunologically related but are kinetically distinct. The above assumption is corroborated by previous studies where it was demonstrated that the two enzymes have similar immunological properties but differ with respect to their CaM affinities and kinetic properties (Hansen and Beavo, 1986). Partial amino acid sequencing of the heart and the low Mₐ brain PDE has been obtained and it was observed that the heart enzyme is identical to the low Mₐ brain enzyme in all the regions examined except for small region in the amino-terminus (Beavo, 1988).

The predicted structures of the ANT-derivatives show that an intramolecular H-bond can be formed between 3'-O of the cyclic phosphate and ANT- amino hydrogen (Figure 16, B). The H-bond distance is predicted to be 2.2 Å. If the O atoms of the cyclic phosphate take part in catalysis, formation of such a H-bond would interfere with the attack of the active site residues. Interactions of cyclic O atoms with active site residues have been demonstrated in cGMP-PDE from rat liver (Erneux et al., 1981). This PDE contains two sites, an activating site exhibiting specificity for cGMP and a catalytic site. Using analogues of cAMP and cGMP, a model of cGMP interactions at the activating site was made. According to this model, the exocyclic O atoms of the cyclic ribose phosphate participate in ionic interactions with the amino acid side chains of the activating site whereas, the cyclic O atoms were shown to be involved in H-bonding with the side chains of the active site residues of the PDE (Erneux et al., 1981). If similar interactions take place in the Ca²⁺/CaM-PDEs, the higher Kₘ observed with ANT derivatives
could be explained on the basis of the above discussion i.e. the formation of an H-bond sterically
hinders the interaction of O atoms of cyclic phosphate with the active site residues.

The H-bond formation could limit the motion of the ANT substituent by making the cyclic
phosphate more accessible to the active-site residues taking part in catalysis. This in turn would
account for the high \( V_{\text{max}} \) observed with these derivatives. In the energy minimized structures
of the MANT derivatives, the H-bond was absent which is probably due to the steric constraints
introduced by the methyl group (Figure 16, C). The insensitivity of the heart enzyme to the
derivatives also indicates that the active site is large enough that small structural alterations of
cAMP and cGMP do not affect the interactions of the active site with the substrates. On the
other hand, the altered kinetic properties of the brain enzymes indicate that their active sites are
smaller and structural changes in substrate structure affect substrate binding.

The high \( M_f \) brain isoform exhibits higher \( K_m \) and \( V_{\text{max}} \) values only for ANT-cGMP
derivatives, indicating that the interaction of the guanine (and not adenine) at the purine binding
domain can induce conformational changes at the cyclic phosphate binding region of the enzyme.
Previous studies have shown that cGMP-derivatives with substitutions at the C-8 of the guanine
portion are potent inhibitors of CaM-dependent brain PDE activity. The same substitution on
cAMP had little effect on the catalytic activity of the enzyme (Couchie et al., 1983). It was
postulated that C-8 hydrogen of guanine is involved in H-bonding with the active-site residues
and that such a H-bond is not formed in case of cAMP interaction at the active-site.

In summary, the three isoforms purified in this study are kinetically distinct from the
enzymes previously isolated by Sharma et al. (1988). Discussion of kinetics obtained with the
pseudosubstrates in conjunction with predicted structures of ANT and MANT- derivatives allowed
speculation on the heterogeneity in the active site environment of these enzymes. The existence
of multiple kinetic forms of the Ca\(^{2+}\)/CaM-dependent PDEs is not unexpected as kinetic
parameters reported for the brain PDE vary considerably. The literature values of \( K_m \) for cAMP
for this enzyme range between 10 \( \mu \text{M} \) to 200 \( \mu \text{M} \) (Sharma and Wang, 1986; Shenolikar et al.,
1985; Morrill et al., 1979; Klee et al., 1980).
The altered kinetic properties observed with these three isoforms could possibly be a result of posttranslational modification like, phosphorylation. However, Sharma and coworkers (1985) have reported that phosphorylation of the low M_r brain PDE decreases its affinity for CaM, without affecting the kinetic properties of the enzyme. Modification of these isoforms by proteolysis is also unlikely in view of the fact that all the three enzymes could be purified using ACC-1 and ACAP-1 monoclonal antibodies, which recognise the CaM-PDE complex. It has been demonstrated that proteolysis of PDE abolishes its ability to bind and be stimulated by CaM (Kincaid and Vaughan, 1986). The activity of all the three enzymes were stimulated by addition of saturating amounts of Ca^{2+} and CaM which further indicates that the three PDEs were not proteolysed. The possibility that CaM is associated with the PDE during the purification and protects the CaM-binding domain from being proteolysed is also highly unlikely as EGTA was present in all the purification steps with the exception of the immunoseparation of the enzymes.

On the other hand, if these PDEs are actually different gene products, what is their in vivo significance? Sharma et al., (1988) postulated a scheme for regulation of the high M_r brain isoform by Ca^{2+} and cyclic nucleotide levels. In this scheme, an initial increase in Ca^{2+} concentration results in the activation of the CaM-dependent protein kinase. This enzyme phosphorylates the high M_r isoform rendering it inactive (Figure-6, B). This leads to a sharp increase in cyclic nucleotide levels. At a later stage, the Ca^{2+} ion concentration increases and the phosphatase, calcineurin (CaN) is activated. CaN dephosphorylates the PDE which can now hydrolyse the cNMPs. For this hypothesis to work it is necessary that the protein kinase is activated at lower concentrations of Ca^{2+} as compared to CaN and PDE.

The various isoforms of the Ca^{2+}/CaM-PDEs differ with respect to their CaM affinities (Sharma and Wang, 1986; Hansen and Beavo, 1986; Rossi et al., 1988). Furthermore, it has been postulated that Ca^{2+}-free CaM interacts with PDE but does not stimulate the enzyme. Ca^{2+}-binding to the CaM-PDE complex renders it active. Depending on the CaM affinities of the PDEs and the Ca^{2+}-sensitivity of the CaM-PDE complex, the various isoforms would be activated at different stages of cell stimulation with some PDEs requiring lower concentrations of
Ca$^{2+}$ and others requiring much higher Ca$^{2+}$ concentrations. This situation would be similar to the different Ca$^{2+}$-sensitivities observed for protein kinase and PDE in the hypothetical scheme presented by Sharma et al. (1988). The concerted action of the various Ca$^{2+}$/CaM-PDEs would ensure a transient increase of the cyclic nucleotide levels.
2.0 UTERINE Ca\textsuperscript{2+} /CALMODULIN-DEPENDENT PHOSPHODIESTERASE

2.1 PURIFICATION OF UTERINE Ca\textsuperscript{2+}/CaM-DEPENDENT PDE

Regulation of phosphodiesterase activity by pharmacological agents, provides an important point for controlling of cyclic nucleotide concentrations (Nicoisoon et al., 1991). PDEs have been isolated from the vascular, uterine and bronchial smooth muscles. Inhibition of cGMPDE from rat aorta by certain drugs like, amrinone, milrinone etc. has been associated with muscle relaxation (Kauffman et al., 1987). Uterus is composed of three layers the perimetrium, myometrium and the endometrium (Nalbandov, 1975). The myometrium is comprised of a massive coat of smooth muscle. Many PDE isoforms have been identified in uterine tissue from rhesus monkeys, rat, rabbit, bovine uterus and in cultured myometrial cells. No CaM-dependent phosphodiesterase was demonstrated in the above tissues. The only report of CaM-dependent PDE is from the human myometrium (Leroy et al., 1987).

Uterine Ca\textsuperscript{2+}/CaM-dependent PDE (Ut-CaM-PDE) was isolated from calf uterus. The enzyme was purified by a three step procedure using DEAE-cellulose, blue-Sepharose and CaM-agarose affinity chromatography (Figure 17). The major contamination in the purification of the Ca\textsuperscript{2+}/CaM-PDEs is by the CaM-dependent phosphatase, calcineurin (CaN), which exhibits a higher affinity towards CaM and competes with PDE for binding to a CaM-agarose column (Hubbard and Klee, 1987). Blue-Sepharose chromatography was used to separate PDE from calcineurin. Enzymes having nucleotide binding sites, like PDE, are retained on the column whereas calcineurin, devoid of the nucleotide binding site, is not adsorbed onto blue-Sepharose.

The purification of bovine Ut-CaM-PDE is summarized in Table II. Based on specific activity the enzyme was purified about 100 fold by DEAE-cellulose, blue-Sepharose and CaM-agarose affinity chromatography. The last step gave the highest degree of purification (30-fold). Furthermore, the activation by CaM is demonstrated only after the CaM-agarose purification step. The activity of the uterine enzyme was stimulated about 2-3-fold by CaM. The overall purification
Figure 17: Purification scheme of CaM-dependent uterine PDE from bovine uterus
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<th>Specific Activity (umol/min/mg)</th>
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TABLE II: PURIFICATION TABLE OF CM-DEPENDENT PDE FROM BOVINE UTERUS.
of the uterine PDE from 1 kg of uterine tissue resulted in yields of 2 to 3 mg of the partially purified enzyme.

SDS-PAGE of the partially purified PDE exhibited a major subunit at about 67 kDa (Figure 18). The subunit Mₚ's of other Ca²⁺/CaM-dependent PDEs range from 58 kDa to 63 kDa (Sharma et al., 1988), the two exceptions being the 75 kDa isozyme of bovine brain (Shenolikar et al., 1985) and the 68 kDa isozyme of mouse testis (Rossi et al., 1988).

One of the steps in the purification of uterine PDE involves blue-Sepharose chromatography. Previous studies have shown that bovine serum albumin (BSA), binds to blue-Sepharose (Morill et al., 1979) and is eluted under the same conditions as used for PDE. Furthermore, it was observed that BSA is non-specifically adsorbed onto CaM-agarose (unpublished results). In view of the above observations, plus the small difference in the Mₚ of BSA (66 kDa) and uterine PDE, it is possible that the major band observed on the 10% SDS-PAGE corresponds to BSA. The likelihood of the uterine isoform being BSA was investigated by immunoadsorption studies using anti-BSA (antibody to BSA) and ACC-1 (antibody to CaM-PDE complex).

Anti-BSA was coupled to the solid support protein A-Sepharose as described in the 'methods' section. 10 μg of BSA and the Ut-CaM-PDE were incubated with excess of protein A-anti-BSA gel. The samples were then centrifuged and the supernatants subjected to SDS-PAGE. The bands were subsequently analysed on thin layer chromatoscanner (Shimadzu, CS-930). BSA was >99% immunoadsorbed on the solid phase whereas about 50% of the Ut-CaM-PDE remained in the supernatant (Figure 19, lane 4). The distances migrated by the two proteins on 10% SDS-PAGE were also different. The Rₚ values for BSA and Ut-CaM-PDE were 0.245 and 0.216, respectively.

Further evidence for the 67 kDa band being uterine PDE was obtained by immunoadsorption experiments using ACC-1-protein-A Sepharose, prepared by coupling ACC-1 to protein-A Sepharose (procedure outlined in the 'methods' chapter). ACC-1 has 100 times higher affinity for CaM bound to brain Ca²⁺/CaM-dependent PDE than for free CaM (Hansen...
Figure 18: 10% SDS-PAGE of bovine uterus CaM-dependent PDE. Lane 1 & 6, Molecular weight markers, BSA (66 kDa), Ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa). Lane 2, uterine PDE (10 μg) elute off CaM-sepharose; lane 3, sample (20 μg) off blue-Sepharose; lane 4, sample (20 μg) off DEAE; lane 5, homogenate (20 μg). The electrophoresis was performed according to the method of Laemmli (1970).
Figure 19: 10% SDS-PAGE of BSA and Uterine PDE following immuno adsorption on anti-BSA-protein A Sepharose. Lane 1, mixture of 10 μg of BSA and protein A suspension (150 μL); lane 2, mixture of 10 μg of BSA and anti-BSA-protein A suspension (150 μL); Lane 3, mixture of 10 μg of uterine and protein A suspension (150 μL); Lane 4, mixture of 10 μg of uterine PDE and anti-BSA-protein A suspension (150 μL). Total suspension volume for all the samples was 350 μL. The electrophoresis was performed according to the method of Laemmli (1970).
and Beavo, 1986). A mixture of Ut-CaM-PDE and CaM was incubated with ACC-1-protein-A Sepharose at 4°C for 1 hour after which, the sample was centrifuged and the supernatant was subjected to SDS-PAGE. The Ut-CaM-PDE was >90% immunoadsorbed on the solid phase (Figure 20, lane 1). The adsorption was not due to non-specific binding of Ut-CaM-PDE to protein-A Sepharose, as the control experiment (uterine isoform and CaM incubated with protein-A Sepharose alone) showed that the majority of Ut-CaM-PDE is retained in the supernatant (Figure-20, lane 2). ACC-1 monoclonal antibody can also react with CaM-dependent enzymes, phosphorylase kinase and brain multiprotein kinase (Hansen and Beavo, 1986). But the likelihood of the 67 kDa band being a subunit of either of the kinases is minimal since, first, ACC-1 has very low affinity for the CaM complexes of the kinases as compared to that of PDE and secondly, the two kinases do not have subunits with molecular weight the range of 66-68 kDa.

Attempts were also made to correlate the activity of Ut-CaM-PDE with the major subunit observed on SDS-PAGE. This was done by subjecting the Ut-CaM-PDE to G-200 gel filtration. The chromatography was not successful as the protein applied on the gel is diluted to an extent that the elution of the PDE could not be detected by either monitoring its activity or absorbance at 280 nm.

The bovine Ut-CaM-PDE activity was stimulated about 2-fold by CaM (in the presence of saturating Ca^{2+}) with an apparent $K_{\text{activity}}$ of 10 nM (Figure 21). The affinity of Ut-CaM-PDE for CaM is 5-fold lower than other CaM-stimulated PDE isoforms ($K_{\text{activity}} \leq 2$ nM) (Sharma et al., 1980; Rossi et al., 1988). The low affinity observed could be due to presence of other CaM-binding proteins copurified with Ut-CaM-PDE. These proteins would compete with Ut-CaM-PDE for CaM and would effectively lower the concentration of CaM available for Ut-CaM-PDE. It has been shown that Ca^{2+}/CaM PDEs exhibit differential calmodulin affinities for example, the heart PDE has 5-fold greater affinity for the regulator protein as compared to the brain PDEs. In view of the above observation, it is possible that the uterine PDE is characterized by low CaM affinity as compared to other Ca^{2+}/CaM-PDEs. The CaM-dependent PDE from human myometrium also exhibits low affinity for CaM (Leroy et al., 1987).
Figure 20: 10 % SDS-PAGE of uterine PDE following immunoabsorption on ACC-1-protein A Sepharose. Lane 1, mixture of ACC-1-protein A suspension (100 μL) plus CaM (2 μg, containing 1mM Ca^{2+}) plus 5 μg of myometrial PDE; Lane 2, mixture of protein A suspension (100 μL) plus CaM (2 μg, containing 1mM Ca^{2+}) plus 5 μg myometrial PDE. The electrophoresis was performed according to the method of Laemmli (1970).
Figure 21: Titration of bovine CaM-dependent uterine PDE with CaM. The PDE activity is displayed as a function of [CaM]. Titrations were performed at 30°C for 20 minute incubations and stopped by the addition of 5.5% TCA. The reaction mixture contained 1.0 mM Ca²⁺, 1.0 mM cAMP, 0.2 U/mL 5'-nucleotidase and 15.0 µg/mL uterine PDE. All data points represent triplicate determinations. The error bars are not observed as they are obscured by marker symbols.
2.2 KINETICS OF UTERINE CaM-DEPENDENT PHOSPHODIESTERASE

Ut-CaM-PDE was assayed in the presence of saturating amounts Ca\(^{2+}\) and CaM. Lineweaver-Burk plots of cAMP and cGMP hydrolysis by Ut-CaM-PDE were linear (Figure 22 and 23). The steady-state kinetic parameters were estimated from a nonlinear regression (Marquardt-Levenberg algorithm; SigmaPlot 4.1, Jandel Scientific) fit of the kinetic data to the Michaelis-Menten-Henri equation and are summarized in Table-III. The bovine uterine PDE exhibited equal affinity for cAMP and cGMP, with apparent \(K_m\)s for the two substrates being 31.2 µM and 26.0 µM, respectively. In comparison to the Ca\(^{2+}\)/CaM-dependent PDE from human myometrium (Leroy et al., 1987) the \(K_m\) values of Ut-CaM-PDE are 7 to 9-fold higher. For cAMP and cGMP, the human myometrial isoform exhibited \(K_m\)s of 3.4 µM and 4.1 µM, respectively. The human myometrial isoform was purified by one step procedure using DEAE-affinity chromatography.

The Michaelis-Menten behaviour and equal affinity for cAMP and cGMP observed with the uterine isoform is in contrast to the kinetic behaviour observed with some other Ca\(^{2+}\)/CaM-dependent PDEs isolated from vascular smooth muscle. For example, the guinea pig and the canine aortic enzymes display concave downward curvature, from which both high affinity (cGMP, 0.29 µM-0.49 µM; cAMP, 0.35 µM-0.58 µM) and low affinity (cGMP, 7.3 µM-47 µM; cAMP, 32 µM) \(K_m\)s have been estimated (Silver et al., 1988). The bovine aorta CaM-PDE exhibits linear kinetics for cGMP hydrolysis with \(K_m\) of 0.8 µM. cAMP hydrolysis with this enzyme exhibited non Michaelian behaviour with a low and high affinity \(K_m\)s of 1 µM and 1-100 µM, respectively (Ahn et al., 1990; Lugnier et al., 1986).

For cAMP and cGMP the bovine Ut-CaM-PDE exhibited \(V_{max}\)s of 4.3 U/mg and 3.32 U/mg, respectively. These values are about 3000-fold higher than those reported for the human Ca\(^{2+}\)/CaM-dependent myometrial enzyme where \(V_{max}\) values of 0.0014 U/mg and 0.0007 U/mg were obtained with cAMP and cGMP, respectively (Leroy et al., 1987). In comparison to the high and low \(M_r\) brain and 59 KDa heart CaM-PDEs purified by Sharma et al. (1988), the \(V_{max}\) of the
TABLE III: KINETIC PARAMETERS OF Ca²⁺/CaM-DEPENDENT UTERINE PDE WITH cAMP AND cGMP

<table>
<thead>
<tr>
<th></th>
<th>cAMP</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$K_m$ (µM)</strong></td>
<td>31.2±4.0</td>
<td>26.0±6.0</td>
</tr>
<tr>
<td><strong>$V_{max}$ (U/mg)</strong></td>
<td>4.3±0.24</td>
<td>3.3±0.34</td>
</tr>
<tr>
<td><strong>$V_{max}/K_m$</strong></td>
<td>0.14</td>
<td>0.13</td>
</tr>
</tbody>
</table>
BOVINE UTERINE PDE cAMP KINETICS

Figure 22: Kinetics of uterine PDE with cAMP in the presence of saturating amounts of Ca\textsuperscript{2+} and CaM. Kinetics were performed at 30\textdegree for 10 minute incubations which were stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca\textsuperscript{2+}, 1.0 \mu M CaM, 0.2 U/mL 5'-nucletidase and 9 \mu g of uterine PDE. All points represent triplicate determinations. The vertical bars representing 2X the standard error of each mean are omitted when they fall within the marker symbols.
Figure 23: Kinetics of uterine PDE with cGMP in the presence of saturating amounts of Ca\textsuperscript{2+} and CaM. Kinetics were performed at 30\textdegree C for 10 minute incubations which were stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca\textsuperscript{2+}, 1.0 \mu M CaM, 0.2 U/mL 5'-nucleotidase and 9 \mu g of uterine PDE. All points represent triplicate determinations. The vertical bars representing 2X the standard error of each mean are omitted when they fall within the marker symbols.
UT-CaM-PDE is 2.2 to 36 fold lower. Whether this low specific activity is a characteristic of the myometrial enzyme or is an artifact of the isolation procedures cannot be ascertained. Although, low specific activity has also been observed for the other types of PDEs (not CaM-dependent) isolated from uterine tissue (Beatty et al., 1979).

2.3 INHIBITION STUDIES OF UTERINE CALMODULIN-DEPENDENT PDE

Various drugs have been shown to act as selective inhibitors of the different PDEs (Beavo, 1988; Nicholson et al., 1991). PDEs are known to be inhibited by xanthines (Kramer et al., 1977; Yamamoto et al., 1984). 3-isobutyl-1-methylxanthine (IBMX) derivatives having substitutions at the 8-position are more effective inhibitors of PDEs as compared to IBMX derivatives with substitutions at the 7-position (Figure 24) (Yamamoto et al., 1984). The inhibition profile of the UT-CaM-PDE with the drugs, 8-methoxymethyl IBMX, 7-benzyl IBMX, 8-methyl IBMX, Papaverine and rolipram are shown in Figures 25 to 29. 8-methoxy methyl IBMX (8-MeOMe), a specific inhibitor of Ca\(^{2+}\)/CaM PDEs (Beavo, 1988), and 8-methyl IBMX were more potent inhibitors as compared to 7-benzyl IBMX, rolipram and papaverine. For the uterine isoform, IC\(_{50}\)s of 35 \(\mu\)M and 12.5 \(\mu\)M were calculated with 8-MeOMe and 8-methyl IBMX, respectively. For 8-methyl IBMX, the IC\(_{50}\)s calculated for cAMP hydrolysis with various other Ca\(^{2+}\)/CaM-PDEs range from 1 \(\mu\)M to 21 \(\mu\)M (Kramer et al., 1977). 7-Benzyl IBMX was less potent than the 8-substituted xanthines and had an IC\(_{50}\) of 125 \(\mu\)M. Papaverine, a non-specific inhibitor of PDEs, gave an IC\(_{50}\) of 400 \(\mu\)M. Rolipram, inhibitor of low K\(_m\) PDEs, was a poor inhibitor of UT-CaM-PDE with an IC\(_{50}\) of >1 mM. Similar IC\(_{50}\)s (\(\geq\)1 mM) have been obtained with other Ca\(^{2+}\)/CaM-PDEs and rolipram (Beavo, 1988).

The drug sensitivity of UT-CaM-PDE and aortic CaM-PDE with various inhibitors of cyclic nucleotide PDEs was compared; results summarized in Table-IV (the comparison of drug sensitivity of the two PDEs were done by Dr. H-S Ahn at Schering-Plough). The IC\(_{50}\)s obtained for vinpocetine and 8-MeOMe IBMX, specific inhibitors of Calmodulin-dependent PDEs...
Figure 24: Structure of IBMX (3-isobutyl-1-methylxanthine) and 7-, 8-substituted derivatives.
(adapted from Yamamoto et al. 1984).
Figure 25: Titration of bovine uterine myometrial PDE with 8-MeOMe IBMX. The phosphodiesterase activity is displayed as a function of [8-MeOMe IBMX]. Titrations were performed at 30°C for 20 minute incubations and stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca^{2+}, 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase, 1.0 mM cAMP and 12 μg of bovine uterine PDE. All points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
INHIBITION OF BOVINE UTERINE PDE WITH 8-Me IBMX

\[ \text{IC}_{50} = 12 \ \mu\text{M} \]

Figure 26: Titration of bovine uterine myometrial PDE with 8-Me IBMX. The phosphodiesterase activity is displayed as a function of [8-Me IBMX]. Titrations were performed at 30° for 20 minute incubations and stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca^{2+}, 5.0 \mu M CaN, 0.2 U/mL 5'-nucleotidase, 1.0 mM cAMP and 12 \mu g of bovine uterine PDE. All points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
Figure 27: Titration of bovine uterine myometrial PDE with 7-benzyl IBMX. The phosphodiesterase activity is displayed as a function of [7-benzyl IBMX]. Titrations were performed at 30° for 20 minute incubations and stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca^{2+}, 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase, 1.0 mM cAMP and 12 μg of bovine uterine PDE. All points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
INHIBITION OF BOVINE UTERINE PDE WITH ROLIPRAM

![Graph showing inhibition of bovine uterine PDE with Rolipram. The x-axis represents the log of Rolipram concentration in M, and the y-axis represents the percentage of PDE activity. The graph shows a decreasing trend in PDE activity as the concentration of Rolipram increases, with an IC₅₀ value of greater than 1 mM.](image)

Figure 28: Titration of bovine uterine myometrial PDE with Rolipram. The phosphodiesterase activity is displayed as a function of [Rolipram]. Titrations were performed at 30° for 20 minute incubations and stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca²⁺, 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase, 1.0 mM cAMP and 12 μg of bovine uterine PDE. All points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
INHIBITION OF BOVINE UTERINE PDE WITH PAPAVERINE

Figure 29: Titration of bovine uterine myometrial PDE with Papaverine. The phosphodiesterase activity is displayed as a function of [Papaverine]. Titrations were performed at 30° for 20 minute incubations and stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca$^{2+}$, 5.0 μM CaM, 0.2 U/mL 5'-nucleotidase, 1.0 mM cAMP and 12 μg of bovine uterine PDE. All points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
### TABLE IV: COMPARISON OF DRUG SENSITIVITY OF BOVINE UTERINE AND AORTIC CaM-PDEs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Uterine PDE</th>
<th>Aortic PDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinpocetine</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>M&amp;B 22948</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>8-MeOMe MIX</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Trequinsin</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>440</td>
<td>118</td>
</tr>
<tr>
<td>MIX</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Quercetin</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>Fisetin</td>
<td>39&lt;sup&gt;*&lt;/sup&gt;</td>
<td>44&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Milrinone</td>
<td>20&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rolipram</td>
<td>12&lt;sup&gt;*&lt;/sup&gt;</td>
<td>35&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SQ 65442</td>
<td>113</td>
<td>68</td>
</tr>
</tbody>
</table>

Assay Conditions: Drug was incubated with enzyme at 25°C for 25 min in assay buffer (Tris-HCl, 50 mM; MgCl₂, 5 mM; cGMP, CaM, 1 µM; CaCl₂, 0.1 mM; pH, 7.5). The IC₅₀ values were determined by linear regression from the linear portion (15%-85% saturation) of the activity vs log₁₀ [inhibitor] response curves. The specific activity of the aortic enzyme was 14 nmol cGMP hydrolyzed/min/mg (with 1 µM cGMP).
(Beavo, 1988), were nearly identical with the two enzymes (for vinpocetine -30 μM and for 8-
MeOMe -12 μM). M&B 22948, an inhibitor of both cGMP-specific and CaM-dependent PDEs
(Nicholson et al., 1990), was 8 fold more potent against the aortic enzyme (IC50= 4 μM) as
compared to the myometrial enzyme (IC50= 32 μM). This could indicate that M&B 22948 binding
is sensitive to the heterogeniety in the drug interacting domains of the two enzymes. Overall, the
aortic and myometrial PDEs were inhibited similarly by most of the drugs tested. This is in
agreement with previous studies where the Ca2+/CaM-dependent PDEs from brain, coronary
artery and aorta exhibited similar inhibition profiles when tested with various PDE inhibitors (Ahn
et al., 1990; Ahn et al., 1989). The different kinetic properties but similar drug sensitivities
observed for various Ca2+/CaM-dependent PDEs indicates that these enzymes probably contain
two distinct sites, a catalytic site where cNMPs are hydrolysed and a regulatory site where the
drugs interact.

In summary, a Ca2+/CaM-dependent PDE has been partially purified from bovine uterus.
No CaM-dependent PDE activity has been previously described from this tissue. The kinetic data
obtained with the myometrial CaM-dependent enzyme indicated that the enzyme had similar
affinity for cAMP and cGMP. The myometrial and aortic CaM-dependent isoforms have similar
properties in terms of drug sensitivity. The availability of the myometrial CaM-PDE in larger
quantities relative to the aortic enzymes plus the fact that the drug sensitivity of the isoform is
similar to aortic CaM-PDE allows the use of the myometrial enzyme for screening of novel drugs.
3.0 NON-ENZYMATIC GLUCOSYLATION OF CALMODULIN

3.1 SEPARATION OF GLUCOSYLATED FROM NON-GLUCOSYLATED CALMODULIN

CaM was purified by a modified procedure of Sharma and Wang (1979). *In vitro* glucosylation of CaM is described in the methods section. After 10 days of incubation with glucose, the sample was extensively dialyzed against water to remove the sugar. The sample was then subjected to *m*-aminophenyl boronate affinity chromatography. This matrix can selectively bind *cis*-diols and is used to separate proteins to which the sugar residues are attached from those with no sugar residues (Mallia *et al.*, 1981). Glucosylated CaM was eluted with 200 mM sorbitol or 10 mM acetic acid, pH 3.0. Acetic acid was used to elute glucosylated CaM as sorbitol interferes with the MBTH assay used for quantitating CaM glucosylation. The elution profile of glucosylated CaM from *m*-aminophenyl boronate affinity chromatography is shown in Figure 30.

3.2 DETERMINATION OF THE EXTENT OF GLUCOSYLATION OF CALMODULIN

*m*-Aminophenyl boronate affinity chromatography provides an estimate of % glucosylated protein rather than the actual number of sugar residues incorporated in the protein. To quantitate the moles of fructosamine (1-amino-2-deoxyfructosamine; Amadori product of sugar with a amino group of the protein) per mole of CaM, the fluorescent assay based on periodate oxidation of fructosamine was used. This assay involves periodate oxidation of fructosamine releasing one mole of formaldehyde (HCHO) per mole of fructosamine. The HCHO released is complexed with acetylacetone and ammonia to produce the fluorophore, diacetylthiodyrolutidine (DDL). Fructose is used as a standard as it is structurally analogous to the fructose on the protein (Armbruster, 1987). Using this assay, amounts as low as 10 nmoles of fructosamine could be detected. These results were similar to the results obtained in the original assay. However, the
Figure 30: Elution profile of glucosylated CaM from m-aminophenyl boronate agarose. CaM (50 mg) was incubated with glucose, dialysed against water and applied to m-aminophenyl boronate agarose. The gel was washed with 250 mM of ammonium acetate buffer, pH 8.8. The glucosylated CaM (3 mg) was eluted with 250 mM of sorbitol or 10 mM acetic acid, pH 3.0.
extent of glucosylation of CaM with the above method was not possible due to high blanks obtained when protein was present. This was also observed in the case of alcohol dehydrogenase (Walton, unpublished results).

An alternative approach was to quantitate the HCHO released by other means. The reagent, 3-methyl-2-benzothiazolinone hydrazone (MBTH), has previously been used to quantitate formaldehyde (Capaldi and Taylor, 1985; Massamiri et al., 1979; Paz et al., 1965). Massamiri et al. (1979) used this reagent to quantitate sialic acid residues of enzymatically glycosylated membrane proteins. An attempt was made to apply the procedure used for the determination of sialic acid residues for quantitating non-enzymatically glucosylated CaM. The reaction scheme is outlined in Figure 31. The procedure entails four major steps:

(A) Periodate oxidation of the fructosamine releasing formaldehyde (HCHO).
(B) Removal of protein and excess periodate by precipitation with zinc sulphate.
(C) Conjugation of released HCHO with the dye MBTH forming an azine.
(D) Reaction of the azine with the FeCl₃ oxidized form of MBTH to produce the chromogen, a tetraazapentamethine dye (TPM).

The conditions for the colorimetric assay were optimized and are summarized in Table-V. Optimization studies were carried out using fructose standard. In the sialic acid procedure (Massamiri et al., 1979), mild periodate oxidation conditions were used so as to selectively release HCHO by cleavage of C₈-C₉ bond. In the present study, the oxidation was performed with 1.0 mM NaIO₄ at pH 3.0 for 5 minutes since, it has been established that the acidic conditions (pH 3.0-4.0) are required for the oxidation of more stable carbohydrates. Using NaIO₄ concentrations greater than 1.0 mM resulted in the formation of a precipitate at stage 4 (addition of FeCl₃) of the assay procedure. Periodate oxidation was essentially complete after 5 minutes. 2 moles of HCHO can be produced by periodate oxidation of fructose or fructosamine. One derived from the C-1 and the other from C-6 of fructose (or fructosamine). Gallop and coworkers (Gallop et al., 1981) demonstrated that periodate oxidation of fructosamine results in the formation of a lactone which is not hydrolyzed and only one equivalent of HCHO is released
Figure 31: The MBTH reaction scheme.

The MBTH assay involves the reaction of MBTH with different reagents and compounds.

Reaction 1:
MBTH + HCHO → MBTH + HCOOH
(10 mM Glycine, pH 2.6)

Reaction 2:
FeCl₃ + MBTH → MBTH

Reaction 3:
NaIO₄, pH 3.0, 5 minutes
<table>
<thead>
<tr>
<th>CONDITIONS OPTIMIZED</th>
<th>FINAL ASSAY CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaIIO₄</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>pH for periodate oxidation</td>
<td>3.0</td>
</tr>
<tr>
<td>Time of periodate oxidation</td>
<td>5.0 minutes</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>10%</td>
</tr>
<tr>
<td>[MBTH]</td>
<td>1 mM</td>
</tr>
<tr>
<td>pH of HCHO-MBTH incubation</td>
<td>2.6</td>
</tr>
<tr>
<td>Time of HCHO-MBTH incubation</td>
<td>20 minutes</td>
</tr>
<tr>
<td>[FeCl₃]</td>
<td>2 mM</td>
</tr>
<tr>
<td>Acetone</td>
<td>30%</td>
</tr>
</tbody>
</table>
(derived from C-1 of the fructosamine, Figure 31).

Another modification as compared to the sialic-acid procedure required the use of a buffer during formaldehyde-MBTH coupling step. When no buffer is used, addition of FeCl₃ during the HCHO-MBTH coupling step resulted in the formation of a precipitate. The possibility that the precipitation was caused by incomplete deproteinization or lack of removal of excess periodate was ruled out as the addition of a large excess of ZnSO₄ (10-fold over that used by Massamiri et al. (1979) failed to eliminate the problem. Lowering the pH to 2.2 (with HCl) eliminated the precipitate however, at this pH, the apparent extinction of the chromogen, tetraazapentamethine was decreased by about 50% (Figure 32). Use of glycine buffer (10 mM), pH 2.6 eliminated the precipitation problem and also restored the colour intensity of the chromogen (Figure 32).

Under the conditions employed, periodate oxidation of fructose released one mole of HCHO per mole of the sugar, as quantitated by comparison to standard curves obtained with authentic, standardized samples of HCHO. The calibration curve for HCHO is shown in Figure 33. An extinction coefficient of 66,000 M⁻¹ cm⁻¹ is calculated which is in agreement with the reported extinction coefficients (55,000-75,000 M⁻¹ cm⁻¹; Sawicki et al., 1961; Paz et al., 1965). With fructose, a linear response in colour production is observed for concentrations ranging between 1 μM and 10 μM (Figure 34). An average extinction coefficient of 67,000 M⁻¹ cm⁻¹ was calculated.

The MBTH assay was attempted on the non-enzymatically glucosylated protein of interest, CaM (Figure 35) and on another non-enzymatically glucosylated protein, bovine serum albumin (BSA) (Figure 36). In order to test for interference by non-glucosylated protein, the assay was performed in its presence where the absorbance was measured as a function of % glucosylated protein, i.e., varying amounts of glucosylated protein were supplemented with nonglucosylated protein to give a constant amount of total protein (150 μg). The calibration curve for CaM and BSA are shown in Figure 35 and 36. For CaM and BSA, the slope in the presence
Figure 32: Absorption spectra of tetraazapentamethine in 30% acetone as a function of pH. Fructose (10 μM) was subjected to periodate oxidation as described in "methods". The formaldehyde released was coupled with MBTH (1 mM). The reaction mixture was adjusted to pH 2.6 with 10 mM glycine-HCl (●); and to pH 2.2 with HCl (○), prior to the addition of FeCl₃ (2 mM). The reaction was allowed to proceed for 5 min. The spectra were taken subsequent to the quenching of the reaction by 300 μL acetone.
Figure 33: Calibration curve of formaldehyde, performed under the conditions described in 'methods'. The solid line represents the line of best-fit. All data point represent triplicate experiments. The error bars are not observed as they are obscured by the marker symbols ($m=0.066124 \ \mu M^{-1}; b=-0.010977; r=0.99967$).
Figure 34: Calibration curve of fructose, performed under the conditions described in 'methods'.

The solid line represents the line of best-fit. All data point represent triplicate experiments. The vertical error bars representing 2X the standard deviation of each mean are omitted when they fall within the marker symbols. \( m=0.067370 \mu M^{-1}; b=0.01109; r=0.99925 \).
Figure 35: Calibration curve of glucosylated CaM. Colour production was monitored as a function of % glucosylated CaM; varying amounts of glucosylated CaM were supplemented with nonglucosylated protein to give a constant amount of total protein (150 µg). The slope in the presence and absence of non-glucosylated CaM was 0.00900 µg %⁻¹ and 0.00902 µg %⁻¹, respectively. All data point represent triplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
Figure 36: Calibration curve of glucosylated BSA. Colour production was monitored as a function of % glucosylated BSA; varying amounts of glucosylated BSA were supplemented with nonglucosylated protein to give a constant amount of total protein (150 μg). The slope in the presence and absence of non-glucosylated BSA was 0.00846 μg %⁻¹ and 0.00852 μg %⁻¹, respectively. All data point represent triplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
nonglcosylated protein were same, i.e., 0.009 and 0.008, respectively. The fact that this assay is insensitive to non-glcosylated proteins means that it can be used to detect the glucosylated proteins without separating the two fractions. By this assay, 10 μg of glucosylated CaM and 5.7 μg of glucosylated BSA could be accurately quantitated.

The reliability of the MBTH assay was assessed by determining the between-run and within-run coefficient of variations (CVs). The between-run CVs for fructose and BSA gave values of 2.24 and 0.084, respectively. For glucosylated CaM, a between run CV of 12% was obtained. Taking into account that μg quantities of the protein are being quantitated, the higher CV seen with CaM as compared to fructose and glucosylated BSA, is quite acceptable. The within run CVs of less than one percent (0.54, 0.85 and 0.51 for fructose, glucosylated CaM and BSA, respectively) were obtained with fructose, glucosylated CaM and BSA.

m-aminophenyl boronate affinity chromatography was used to separate the glucosylated fraction from nonglcosylated fractions. For the MBTH assay, the glucosylated fraction was eluted with 10 mM acetic acid (pH, 3.0) instead of using sorbitol. Sorbitol will interfere in the detection of the fructosamine as it releases HCHO when subjected to periodate oxidation. Similar low pH conditions have previously been used to elute glucosylated proteins.

Comparison of the detection limits for various assays used to quantitate non-enzymatically glucosylated proteins show that the MBTH assay as currently modified can detect as low as 1.5 nmols of fructosamine. The TBA assay (Murtiashaw et al., 1983) and the fluorometric method (Gallop et al., 1981) can detect about 7 nmols of fructosamine whereas the lowest detection limit for the NBT assay (Johnson et al., 1982) has been shown to be about 1 μmole. However, the MBTH assay does not have the sensitivity (pmol range) of the HPLC-based furosine assay (Schleicher et al., 1981). The furosine assay requires 18 hour hydrolysis of fructosamine.

The disadvantages of the the MBTH assay are interference by sugars and other glycols present in the protein samples. However, a simple step of dialysis or gel filtration chromatography can eliminate the above mentioned interferences. Another disadvantage of this
assay and most of the other assays discussed before is the interference from enzymatically glycosylated proteins capable of releasing formaldehyde on mild periodate treatment. In this respect, the nitroblue tetrazolium assay is best since the enzymatically glycosylated proteins do not interfere, although the sensitivity of the assay is very low (lower limit of detection is 1 mM).

The MBTH assay was used to quantitate the extent of in vitro non-enzymatic glucosylation of CaM. 2.2 moles of glucose per mole CaM are incorporated when the protein was glucosylated in the presence of Ca\(^{2+}\). When glucosylation of CaM was performed in the absence of Ca\(^{2+}\) (incubation with EGTA), 1.5 moles of glucose per mole of CaM are incorporated. Similar results were obtained by Kowiluru et al., (1989) using [6-\(^{3}H\)] glucose where, 2.5 and 1.5 moles of glucose were incorporated per mole CaM in presence of Ca\(^{2+}\) and EGTA, respectively. Chemical modification studies have shown that the reactivity of the lysine residues is increased by the addition of Ca\(^{2+}\) (Winkler et al., 1987). An explanation for the altered reactivity of the lysine residues is that binding of Ca\(^{2+}\) to CaM induces a conformational change in the protein which in turn could alter the local environment of the lysine amino group making it more reactive. For example, the presence of amino acid residues with acidic side chains (carboxylate anions) in close vicinity to the lysine amino group could effectively lower the pK\(_a\) of the amino group thereby increasing its nucleophilicity and making it more reactive.

3.3 EFFECT OF CALMODULIN GLUCOSYLATION ON ITS FUNCTION

The effect of in vitro glucosylation of CaM on its ability to stimulate target proteins was studied on heart Ca\(^{2+}\)/CaM-dependent phosphodiesterase, bovine brain CaM-dependent phosphatase, calcineurin and red blood cell membrane (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase. Glucosylated CaM prepared in the presence of Ca\(^{2+}\) was used for these studies. Bovine heart PDE was purified using ion-exchange, blue-Sepharose and CaM-agarose affinity chromatography. The activity was determined by the phosphate assay. Titration of heart PDE with glucosylated and non-glucosylated CaM is shown in Figure 37. Half maximal activation of heart PDE with modified
Figure 37: Titration of bovine heart PDE with glucosylated (O) and non-glucosylated CaM (●). Titrations were performed at 30° for 30 minute incubations and stopped by the addition of 5.5% TCA. The assay mixture contained 1.0 mM Ca\textsuperscript{2+}, 1.0 mM cAMP, 0.2 U/mL 5'-nucleotidase and 3.7E-8 M of bovine heart PDE in 36.0 mM tris-HCl, pH 7.3. All points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols.
and native CaM were obtained at CaM concentration of 10 nM and 2.5 nM, respectively.

The concentrations of glucosylated and non-glucosylated CaM required for maximal stimulation of heart PDE were same (about 100 nM). The reported CaM concentration for half-maximal activation for monoclonal antibody purified heart PDE is about 0.1 nM (Hansen and Beavo, 1986). The non-glucosylated CaM used for the heart PDE titration was subjected to similar incubation conditions as the glucosylated CaM, the only difference being that glucose was omitted from the incubation mixture. The decreased affinity of non-glucosylated CaM for heart PDE indicates that the incubation conditions used for glucosylation alter CaM ability to stimulate the heart enzyme. Furthermore, presence of other CaM-binding proteins which are copurified with the heart PDE sample could also bind glucosylated CaM and lower the amount of modified protein available for the heart PDE. Comparing the activities at half saturating concentrations of glucosylated and non-glucosylated CaM, a 20% inhibition is observed.

The effect of glucosylated and non-glucosylated CaM with calcineurin and (Ca\(^{2+}\)+Mg\(^{2+}\))-ATPase is shown in Figures 38 and 39, respectively. For calcineurin, the apparent K\(_{\text{activity}}\) of glucosylated and native CaM were 76 nM and 42 nM, respectively. At half-saturating concentration of glucosylated CaM as compared to unmodified protein, there was a 16% reduction in its ability to stimulate calcineurin. Maximal stimulation of calcineurin required higher concentrations of the glucosylated protein (251 nM) as compared to the unmodified CaM (148 nM). (Ca\(^{2+}\)+ Mg\(^{2+}\))-ATPase activity was not effected by the glucosylation of CaM; half maximal activity of the enzyme required 5 nM of the glucosylated and non-glucosylated protein. A small decrease (16%) was observed in the maximum activity of the ATPase with glucosylated CaM as compared to unmodified protein.

Kowturu and coworkers (1989) report the effect of in vitro glucosylation of CaM on three enzyme systems: brain phosphodiesterase, membrane bound adenylate cyclase and protein kinase. The extent of inhibition of the three enzymes in Kowturu’s study is overall much higher than that observed in the present study. For brain phosphodiesterase, 70% inhibition in enzyme activity is obtained at half saturating concentrations of the modified CaM as compared to native
Figure 38: Titration of the bovine brain calcineurin with glucosylated (O) and non-glucosylated CaM (●). The initial rates were determined at 37°C for 200 seconds. The assay mixture contained 0.1 mM Ca²⁺, 2.0 mM MgCl₂, 10 mM p-nitrophenyl phosphate (PNPP), 150 mM KCl and 4.87E-8 M calcineurin in 100.0 mM tris-HCl, pH 7.0. All points represent triplicate experiments. The vertical bars representing 2X the standard error of each mean are omitted when they fall within the marker symbols.
Figure 39: Titration of (Ca$^{2+}$ + Mg$^{2+}$)-ATPase with glucosylated (O) and non-glucosylated CaM (●). These experiments were performed by Dr. Roufagalis at the University of Sydney.
CaM. Similarly, the extent of inhibition of adenylate cyclase and protein kinase was \(-40\%\). Kowluru et al. (1989) used phosphate as the buffer system for CaM glucosylation. It has previously been reported that phosphate and other anionic buffers enhance the rate of glucosylation (Baynes et al., 1989). Such an effect has been demonstrated in the protein hemoglobin (Shapiro et al., 1980) and the enzyme RNase (Brownlee et al., 1984). In case of the two proteins, the phosphate ion binds to the cationic pockets formed by basic amino acid residues of the two proteins. The bound phosphate enhances the rate of glucosylation possibly by lowering the pK\(_a\) of the amino group of Lys thus increasing its nucleophilicity. Similar cationic pockets are also observed in CaM structure (Klee and Vanaman, 1982). It is possible that the high level of inhibition observed in Kowluru's study is a result of increased rate of glucosylation due to the local catalysis by the phosphate group bound to CaM. In the absence of phosphate, the essential Lys residue of Hb and RNase were not glucosylated and the CaM-target protein interactions were not disturbed to a great extent.

Kowluru and coworkers also observed a 50% inhibition in Ca\(^{2+}\)-binding when calmodulin was glucosylated (Kowluru et al., 1989). The overall conformation of the protein remained the same for glucosylated and native CaM. This indicates that the conformational change in CaM structure, induced by Ca\(^{2+}\)-binding does not require four Ca\(^{2+}\) ions bound to it and binding of two Ca\(^{2+}\) is sufficient to induce the required conformational change. The Ca\(^{2+}\)-binding sites I and III were thought to be affected by glucosylation (Kowluru et al., 1989). Site I contains three lysine residues and site III contains one lysine residue which is located centrally in the domain such that glucosylation of this residue would effectively inhibit Ca\(^{2+}\)-binding.

CaM has eight Lys residues, of these the lysine at position 115 is trimethylated and cannot undergo glucosylation. Furthermore, the N-terminus of CaM is blocked and is not modified by glucose. Kowluru et al. (1989) subjected the glucosylated CaM to CNBr fragmentation and separated the various fragments by reverse phase HPLC (Kowluru et al., 1989). CNBr cleavage resulted in eight fragments of which four contain lysine residues. The four
fragments containing the lysine residues had also incorporated the [6-3H]-glucose. 2.5 moles of glucose are incorporated per mole of CaM. These results indicate that all lysine residues in CaM are available for glucosylation but no more than two or three are modified on any single calmodulin molecule.

The Amadori product on the protein can undergo a series reactions to produce advanced glucosylation end-products (AGEs) (Brownlee et al., 1988). These molecules are highly reactive and can form covalent crosslinks to other proteins. Such covalent crosslinks have been demonstrated in collagen whereby the protein forms polymers and becomes less soluble and less susceptible to collagenase (Schnider and Kohn, 1981). Similar protein crosslinking was observed for the enzyme, lysozyme, when incubated with 3-deoxyglucosone (3-DG), an advanced glucosylation product (Kato et al., 1989). The aliquots of protein were removed at certain time intervals and subjected to SDS-PAGE. A high molecular weight band (polymer of lysozyme) was observed after only 3 days. The intensity of the band increased with longer periods of exposure of the lysozyme to 3-DG. The possibility of AGEs being formed and causing protein crosslinking in case of CaM in vitro glucosylation, was tested. The protein was incubated with 0.5 M glucose for a period of 15 days. At certain time intervals (3 days), aliquots were removed and frozen at -80°C. At the end of 15 days, a constant amount of CaM from each incubation sample was subjected to SDS-PAGE. A high molecular band was not observed with CaM even after 15 days of incubation (Figure 40) indicating that AGEs are not formed when CaM is incubated with glucose.

3.4 ATTEMPTS TO PURIFY IN VIVO NON-ENZYMATICALLY GLUCOSYLATED CALMODULIN

The CaM levels in diabetic tissues have been quantitated and it was found that these tissues had higher levels of the calcium binding protein as compared to normal tissues (Morley et al., 1982; Perez de Gracia et al., 1980; G. Paolisso, 1986). Platelet CaM levels of patients with
Figure 40: 10% SDS-PAGE of CaM incubation with glucose (0.5 M), in presence of 1.0 mM Ca$^{2+}$, for 3 (lane B), 6 (lane C), 9 (lane D), 12(lane E) and 15 (lane F) days at 37$^\circ$ in 50 mM Hepes buffer, pH 8.0. Lane G, control CaM incubated for 15 days under same conditions (no glucose); Lane H, control CaM, no incubation; Lanes A, I and J, molecular weight standards (BioRad); rabbit muscle phosphorylase (97.4 kDa); bovine serum albumin (66.2 kDa); hen egg white ovalbumin (45 kDa); bovine carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa). The electrophoresis was performed according to the method of Lammeli (1970).
insulin dependent diabetes mellitus, are about 2-fold higher than the platelet CaM levels of normal individuals. The physiological significance of these observations is unknown. A possible explanation is that CaM is modified (maybe by glucosylation) which effects its binding to target proteins. The consequence of the altered binding characteristics would be to lower the effective concentrations of the protein which could result in impaired biological process. The increased levels of CaM observed in diabetics could result from the overexpression of the protein in response to the signals from the altered biological process.

Attempts were made to isolate in vivo non-enzymatically glucosylated CaM from diabetic patients. Platelets were chosen because of the availability of the tissue and also that platelet intracellular glucose concentrations can reach levels as high as the plasma glucose concentrations (Yardimci, 1980). Furthermore, it has been shown that in vitro glucosylation of CaM is essentially complete after 6 days. The fact that the life time of platelets is 8-10 days (Weiss, 1982), it is quite possible that CaM in platelets gets glucosylated.

Platelets were purified according to the method of Caines et al. (1988). Diabetic and control patients were classified on the basis of the extent of glucosylated hemoglobin (G-Hb) which has been used as an measure of glycemic condition in diabetics. Whole blood samples of five diabetic exhibiting high levels (≥ 11%) of glucosylated Hb (G-Hb) and five control patients exhibiting low levels (< 7%) of glucosylated Hb were used. The platelet extract from the above samples was subjected to Econo-Q cartridge (ion-exchange) chromatography. The cartridge was washed with buffer containing low salt (0.05 M). A linear gradient of 0.05 M to 0.6 M was applied and the elution followed by determining CaM activity (explained in methods). The elution profile as a function of CaM activity is shown in Figure 41. The activity versus elution profile shows that the samples from control patients (exhibiting low levels G-Hb) showed higher CaM activity as compared to samples from diabetic patients (exhibiting high levels of G-Hb) (Figure 41). 100 μL of each fraction was also subjected to SDS-PAGE. The gels of the low and the high run is shown in Figures 42 and 43. For the both runs, the CaM band observed on SDS-PAGE corresponded with the CaM activity eluted offf Econo-Q column. The low activity observed with the diabetic
Figure 41: Elution of CaM off Econo-Q. Fractions eluted off Econo-Q were assayed for CaM activity. 100 µL of the fractions were heated at 70ºC, cooled and the PDE assay was performed. Each sample contained 0.2 U/mL of 5'-nucleotidase, 1.0 mM Ca²⁺, 1.0 mM cAMP and 1.7E-07 M heart PDE in 36.0 mM tris-HCl; pH 7.3. The reaction was allowed to proceed at 30ºC for 30 minutes after which it was stopped with 5.5% TCA. The phosphate released was coupled to ammonium molybdate and 1-amino-2-naphtol sulphonic acid. The mixture further incubated for 5 minutes after which the absorbance was read at 660 nm.
Figure 42: 10 % SDS-PAGE of the fractions (exhibiting CaM activity) eluted off Econo-Q column. The platelets used were obtained from five diabetic patients exhibiting high glucosylated hemoglobin levels. 100 µL of the sample was subjected to TCA precipitation and subjected to SDS-PAGE after adjusting the pH of the sample to about 7.0 with 1.0 M tris-HCl (pH 7.3). The fractions are indicated by numbers. The electrophoresis was performed according to the method of Lammeli (1970).
SDS-PAGE OF CALMODULIN FROM PLATELETS OF PATIENTS EXHIBITING LOW GLUCOSYLATED HEMOGLOBIN

C-CaM 9 18 22 24 26 28 34 40 PLATELET EXTRACT

Figure 43: 10 % SDS-PAGE of the fractions (exhibiting CaM activity) eluted off Econo-Q column. The platelets used were obtained from five diabetic patients exhibiting low glucosylated hemoglobin levels. 100 μL of the sample was subjected to TCA precipitation and subjected to SDS-PAGE after adjusting the pH of the sample to about 7.0 by 1.0 M tris-HCl (pH 7.3). The fractions are indicated by numbers. The electrophoresis was performed according to the method of Lammeli (1970).
platelets was surprising as the electrophoresis showed a relatively pure band of CaM as compared to the normal platelet samples which had a high molecular weight contamination.

The next step was to obtain a homogenous CaM preparation from platelets. Attempts were made to obtain pure calmodulin from platelets, pooled from 20 diabetic patients exhibiting high levels of glucosylated Hb. Using the conventional procedure, the CaM purified exhibited a high $M_r$ protein contamination (Figure 44, lane 2). Thus, the procedure had to be modified to obtain homogenous CaM. Towards this end, platelets were purified (as described in the methods section) and lysed by freezing and thawing. The platelet extract was subjected to Affi-gel heparin affinity chromatography followed by phenyl Sepharose affinity chromatography. CaM was eluted from phenyl Sepharose with EGTA which was electrophoretically pure. SDS-PAGE of human platelet CaM is shown in Figure 44 (Lane 1). Heparin Agarose has been used for the separation of growth factors, coagulation factors, DNA and RNA specific enzymes, lipase and lipoproteins. The character of the high $M_r$ protein contaminations is not known but the use of affi-gel heparin eliminated their copurification with CaM. If these proteins are also present in other blood cells (red and white blood cells) then use of Affi-gel heparin provides a new means of isolating CaM from blood cells. The sample was not analyzed for glucosylated protein as the protein concentration was too low (the total amount of CaM purified by the above method was about 100 $\mu$g).

In summary, the in vitro glucosylation of CaM in the presence of Ca$^{2+}$ results in the incorporation of 2.2 moles of glucose per mole CaM as determined by the MBTH assay. In the absence of Ca$^{2+}$, 1.5 moles of glucose are incorporated per mole of CaM. These results were similar to the results obtained by Kowuru et al. (1989) using radioactive glucose. Thus the MBTH assay provides the same sensitivity as the radiometric method. Furthermore, the assay is economic, rapid and more sensitive than majority of the procedures currently employed for the quantitating glucosylated proteins. The modification of the lysine residues had little effect on the protein's ability to stimulate the target enzymes; heart phosphodiesterase, brain calcineurin and red blood cell membrane ATPase were inhibited by glucosylated CaM by 20%, 19% and 0%.
Figure 44: 10% SDS-PAGE of human platelet CaM (~10 μg). lane 1, CaM purified using heparin Agarose and phenyl sepharose chromatography; lane 2, CaM purified by DEAE and Phenyl Sepharose chromatography; Lane 3, bovine brain CaM; lane 4, the high M₇ proteins which bind to heparin Agarose and are eluted with high salt. The electrophoresis was performed according to the method of Lammeli (1970).
respectively. SDS-PAGE of CaM incubation with glucose indicates that protein crosslinking due to formation of advanced glucosylation end-products does not occur with CaM.
CHAPTER V- Conclusions

The present study provides evidence for the existence of three distinct phosphodiesterase isoforms, two in the bovine brain (low and high $M_r$) and one in bovine heart. These enzymes are kinetically different from those isolated by Sharma et al., (1988) from the brain and heart. The PDEs, in the present study were purified using conformationally specific monoclonal antibodies which recognise the CaM-PDE complex. In Sharma's study, the PDEs were isolated using monoclonal antibodies (ACC-1 and ACAP-1) directed to the PDE polypeptide chain. The three isoforms isolated in the present study were CaM dependent since addition of CaM resulted in 10-fold stimulation of the activity. Furthermore, the fact that these enzymes could associate with the monoclonal antibodies indicates that the enzymes are capable of binding CaM and have not been modified by proteolysis. The kinetic studies with native substrates, cAMP and cGMP, indicated that the heart enzyme and the low $M_r$ brain enzyme are equally specific for the two substrates. The high $M_r$ enzyme was different from the other two in that it exhibited specificity towards cGMP. In comparison, all the three PDEs isolated by Sharma exhibited specificity towards cGMP as compared to cAMP. These observations lead to the conclusion that the isoforms isolated in the present study are kinetically distinct from ones isolated by Sharma et al., (1988).

The use of pseudosubstrates allowed further distinction between the three enzymes (the kinetic study done by Karuppiah, 1987). The main effect of these substitutions was on the $V_{max}$, i.e., the substituents lowered the catalytic efficiency of the enzymes and had little effect on the binding ability for the pseudosubstrates. It was also observed that the ANT-derivatives gave rise to higher kinetic parameters as compared to the MANT-derivatives. This effect is thought to be related to the H-bond formed in the ANT derivatives (Figure 16, B). The H-bond formation, possibly limits the motion of the ANT group in a way that the pseudosubstrate cyclic phosphate is placed in a catalytically favourable position. This effect results in the higher $V_{max}$ observed with the ANT derivatives. The heart enzyme was not affected by the 2'-O- substitutions,
indicating that the active site of this enzyme differs from the brain enzymes. The high Mr brain PDE exhibited higher $K_M$ and $V_{max}$ with only cGMP derivatives indicating that the active site of this enzyme is sensitive to guanine and not adenine binding. The derivatives primarily affected the kinetic parameters of the low Mr brain PDE; it is possible that the active site of this enzyme is small and cannot accommodate the structural alterations of the substrates. On the other hand, the kinetic properties of the heart enzyme were not altered by the 2'-O-substituents, indicating that the active site of this enzyme is large enough to accommodate structural alterations of the native substrates.

The second project on the CaM-dependent phosphodiesterase describes the isolation of a CaM-dependent PDE from the bovine uterus. This isoform exhibits equal affinity for cAMP and cGMP and has low affinity for CaM as compared to most of the other CaM-dependent PDEs. The enzyme was stimulated 2-fold by CaM. The properties of the bovine uterine PDE is similar to the CaM-dependent PDE isolated from human myometrium (Leroy et al., 1987). The human myometrial enzyme also exhibits low affinity towards CaM and is equally specific for cAMP and cGMP. SDS-PAGE experiments indicate that the uterine calmodulin-dependent PDE has a major subunit with $M_r$ of about 67 kDa. A comparison of the inhibition studies of the uterine PDE with the CaM-dependent aortic enzyme indicated that both the enzymes had similar drug sensitivities. These results are in agreement with previously reported observations that the various CaM-dependent PDEs differ with respect to their kinetic properties but have similar drug sensitivities (Beavo, 1988). In view of the fact that vascular tissues have low amounts of PDE and that there is a limited supply of the tissue, the use of bovine uterine CaM-dependent PDE provides an alternative means for screening new pharmacologically important agents.

Finally, it has been demonstrated that CaM undergoes in vitro non-enzymatic glucosylation. 2 mols of glucose are incorporated per mol of CaM when the latter is incubated with the sugar in the presence of Ca$^{2+}$. In the absence of Ca$^{2+}$, 1.5 mol of glucose residues are incorporated per mole CaM. The MBTH assay was developed to quantitate the extent of non-enzymatic glucosylation of CaM. The conditions of the assay were optimized and the method
tested on two proteins, CaM and BSA. The advantage of this assay was that the interference from non-glucosylated proteins is minimal. The latter is of importance since it eliminates the step of removal of glucosylated protein from non-glucosylated protein. Furthermore, this assay is more sensitive than most of the other assays currently employed for quantitating non-enzymatic glucosylation.

The incorporation of 2 mols of glucose per mol of CaM does not drastically alter the ability of CaM to stimulate its target enzymes, heart PDE, calcineurin and (Ca$^{2+} +$ Mg$^{2+}$)-ATPase. It has been previously demonstrated that the Amadori product on the protein can undergo a series of reactions to form advanced glucosylation endproducts which in turn cause protein crosslinking. Under the conditions used in the present study for CaM glucosylation, the advanced glucosylation end-products are not formed and intermolecular crosslinking of CaM does not occur.
APPENDIX-I

STEADY STATE KINETICS OF THE CALMODULIN-DEPENDENT PHOSPHODIESTERASES

The steady state kinetic parameters for the hydrolysis of the substrates, cAMP and cGMP, by the calmodulin-dependent phosphodiesterases were estimated from a simplex fit of the kinetic data to the Michaelis-Menten equation:

\[ v_0 = \frac{V_{\text{max}} \times [S_0]}{[S_0] + K_m} \]

The simplex fit is described (Noggle, 1985; reproduced with permission)
The Simplex Method


A simplex is a figure that has one more vertex than the dimension of the space in which it exists. For two dimensions, the space for two parameters \((a, b)\), the simplex is a triangle. If there are \(NP\) parameters, the number of vertices of the simplex \((NV)\) is \(NV = NP + 1\). The initial locations of the vertices are determined somewhat arbitrarily. It is helpful to have some idea of the value of the parameters before starting; this will also help to avoid false minima. Such estimates can sometimes be obtained by some other method; for example, for Eq. 8.3 the double reciprocal plot could be used for this purpose. Often a thoughtful inspection of the data will produce reasonable estimates. Then, for each vertex, the response is calculated and the best response (BR) and worst response (WR) are identified.

This is most easily pictured with two parameters; see Figure 8.2. For two parameters, there are 3 vertices that can be ranked as to response as best \((B)\), second best \((S)\), and worst \((W)\). The centroid \((C)\) of all points but the worst is calculated as the sum of coordinates \((a, b)\) of the points:

\[
C_a = \frac{1}{(NV - 1)} \sum_{w \neq w} a_w \quad C_b = \frac{1}{(NV - 1)} \sum_{w \neq w} b_w \quad \text{etc. (8.5a)}
\]

The worst point is then reflected through the centroid to produce the reflected point \((1)\); for each coordinate, we calculate the reflected value:

\[
\text{reflected coordinate} = C + F(C - W) \quad (8.5b)
\]

\((F = 1\) in Eq. 8.5b, at this stage). The response is then calculated at the reflected point. Calling this response \(R_1\), the procedure below is followed:

If the reflected point is better than the best \((R_1 < BR)\), then the simplex is expanded using Eq. 8.5b with \(F = 2\) (point 2 on Figure 8.2). The
Figure 8.2: The Simplex.
response at this point is measured (R2). If this is better than the reflected point, R2 < R1, the expanded point is accepted in place of the worst point, and the new simplex will be \((B, S, 2)\). If not, the reflected point is accepted in place of the worst and the new simplex is \((B, S, 1)\).

If the reflected point is not as good as the best \((R1 > BR)\), but is better than the worst \((R1 < WR)\), then the reflected point is accepted in place of the worst point and the new simplex is \((B, S, 1)\).

If the reflected response is worst of all, \(R1 > WR\), then a contracted point \((3, \text{ on Figure } 8.2)\) is calculated using Eq. 8.5b with \(F = -0.5\). If this response \((R3)\) is better than the worst \((R3 < WR)\), it is accepted in place of the worst and the new simplex is \((B, S, 3)\). If not, the original simplex is contracted toward the best point.

After the new simplex is defined (by one of the criteria discussed above), the procedure is repeated. The program that follows implements the simplex method outlined above:

PROGRAM SIMPLEX

1 ' PROGRAM SIMPLEX
10 DEFINT I-N
20 DIM S(6,7),T(6),TT(6),R(7),C(8),X(30),Y(30)
30 NP=3; NV=NP+1 ' SET NUMBER OF PARAMETERS = NP
40 DEF FNY(X)= T(1) + T(2)*EXP(T(3)*X)
50 DATA COOPER.8,1.2,3.30, 2.3.74, 3.5.01, 4.6.74, 5.9.06, 6.12.2
51 DATA 7.16.43, 8.22.14
59 DATA END,0
100 CLS' COMMAND CENTER
110 PRINT *;ITER=*:K1:*/:KX:*R=*:BR:*:/:EP:*PTS:*M,TT$
120 PRINT*;PARAMETERS:*;FOR I=1 TO NP; PRINT S(I JB):;NEXT
130 PRINT*
  <I> DATA INPUT
  <G> INPUT GUESSES
  <P> PRINT RESULTS

126
<C> CALCULATE
<F> FUNCTION CALC
<Q> QUIT

140 PRINT"**:GOSUB 150: J=INSTR(\"I1GPQF\",A$)
150 ON J GOTO 200,300,400,1000,900,800
160 GOTO 100
170 A$=INKEY$: IF A$="*"
   THEN GOTO 190
   ELSE PRINTA$;"**: RETURN
200 READ T$I,M$: IF T$I="*END"
   THEN GOTO 100
210 FOR I=1 TO M: READ X,Y
220 X(I)=X: Y(I)=Y
230 NEXT
240 GOTO 100
300 FOR I=1 TO NP:PRINT I;*: INPUT*VALUE, INC*Z,D
310 FOR J=1 TO NV
320 S(I,J)= Z - D*(I>=J)-D/2
330 NEXT
340 NEXT
344 GOSUB 350: GOTO 400
350 FOR I3=1 TO NV
360 FOR J3=1 TO NV: T(J3)=S(J3,I3): NEXT
370 GOSUB 500: R(I3)=R
380 NEXT: RETURN
400 PRINT*RESPONSE/PARAMETERS*
405 FOR I=1 TO NV:PRINT R(I);*: /
410 FOR J=1 TO NP
420 PRINT S(I,J);*: NEXT
430 PRINT: NEXT
480 PRINT"***:GOSUB 190
490 GOTO 100
500 R=0 'CALCULATE RESPONSE'
510 FOR IS=1 TO M
520 R=R+(Y(IS)-FNY(X(IS)))^2
530 NEXT
540 R=SQR(R/(M-1)): RETURN
600 ' FIND NEW VERTEX (T)
610 FOR J6=1 TO NP: SU=0
615 IF F<>.1
620 THEN GOTO 660
625 FOR K6=1 TO NV 'CALC CENTROID
630 IF K6<>.JW
635 THEN SU=SU+S(J6,K6)
639 NEXT
640 C(J6)=SU/NP
650 T(J6)= C(J6)+1+F*F*F(J6,JW)
660 NEXT
670 GOSUB 500: PRINT"/ F=",F;"R=",R;
690 RETURN
700 ' EXCHANGE T FOR WORST
710 FOR I7=1 TO NP
720 S(I7,JW)=T(I7)
730 NEXT
740 R(JW)=R
750 GOTO 1050
800 INPUT 'X=';X
810 PRINT FNY(X)
820 GOTO 140
900 END: GOTO 100
1000 KL=0 ' ITERATION
1010 IF M=0 OR R(1)=0
    THEN GOTO 100
1020 INPUT MAX # ITER*;KX
1030 EP=1E-5
1040 IF BR<>0 AND BR<EP
    THEN EF=EP/10: GOTO 1040
1050 WR=R(1);SR=WR; JW=1; JB=1
1060 FOR I=2 TO NV
1070 IF R(I)>WR
    THEN WR=R(I); JW=1
1080 IF R(I)<BR
    THEN BR=R(I); JB=1
1090 NEXT
1100 IF KL>=KX OR BR<EP
    THEN PRINT]+$END$;GOSUB 190: GOTO 100
1110 IF (WR-BR)/BR < EP
    THEN PRINT: GOTO 400
1170 IF INKEY$"Q"
    THEN GOTO 100
1200 PRINT: KL=KL+1; PRINT*"#:KL;
1210 IF BR<>PR
    THEN PR=BR;PRINT$"NEW BEST";BR;"WORST";WR
1220 F=1: GOSUB 600  'NEW VERTEX
1230 IF R<BR
    THEN GOTO 1400  'EXPAND VERTEX
1240 IF R<WR
    THEN GOTO 700: 'EXCH FOR WORST
1250 F=-0.5: GOSUB 600  'CONTRACT
1260 IF R<WR
THEN GOTO 700 'EXCHANGE FOR WORST
1270 PRINT 'CONTRACT': CONTRACT ALL VERTICES
1280 FOR I=1 TO NP
1290 FOR J=1 TO NV
1300 IF J<>JB
    THEN S(I,J)=(S(I,JB)+S(I,J))/2
1310 NEXT J
1320 GOTO 1050
1400 RT=R:FOR I=1 TO NP: TT(I)=T(I): NEXT 'SAVE VERTEX
1410 F=2: GOSUB 600 ' EXPANDED VERTEX
1420 IF R<-=RT
    THEN GOTO 700 'ACCEPT EXPANDED VERTEX
1430 R=RT:FOR I=1 TO NP: T(I)=TT(I): NEXT 'RESTORE
1440 GOTO 700 'EXCHANGE W/ WORST
Figure 45: Calibration curve of phosphate. The assay conditions were similar to those described for the phosphodiesterase assay in the 'methods' section. The solid line represents the line of best-fit. All data points represent duplicate experiments. The error bars are not observed as they are obscured by the marker symbols. (m=0.00423, b=0.00332 and r=0.999).
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