Sulfhydryl modification of the calmodulin-stimulated phosphatase, calcineurin.

Jerome David. Wagner

University of Windsor

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SULFHYDRL MODIFICATION
OF THE
CALMODULIN-STIMULATED PHOSPHATASE,
CALCINEURIN

by
Jerome David Wagner

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

Windsor, Ontario, Canada
1990
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ABSTRACT

SULFHYDRYL MODIFICATION

OF THE

CALMODULIN STIMULATED-PHOSPHATASE,

CALCINEURIN

by

Jerome David Wagner

Calcineurin (CaN), a calmodulin stimulated protein phosphatase is a heterodimer composed of a 61kD catalytic subunit and a 19kD regulatory subunit. The larger subunit has three binding sites for transition metal ions and also contains the intrinsic metal ions, Zn²⁺ and Fe²⁺. The smaller subunit contains four "EF-hand" Ca²⁺ binding sites. All ten of calcineurin's cysteine residues are found on the catalytic subunit. This study explores the metal ion dependence of CaN using Ca²⁺, Mg²⁺, Mn²⁺ and Ni²⁺ as well as its thiol chemistry using Ellman's reagent, iodoacetate, iodoacetamide, glutathione and a novel thiol reagent introduced by this study, 1-p-chlorophenyl-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide (CDDP).

CaN was purified from bovine brain using DEAE-cellulose and calmodulin-agarose chromatography. Enzyme kinetics were performed with CaN in the presence of EDTA, Ca²⁺, Ca²⁺ and Mg²⁺, EGTA and Mg²⁺, Ca²⁺ and Mn²⁺ and Ca²⁺ and Ni²⁺, ± calmodulin (CaM).
The highest activity was observed when CaN was assayed in the presence of Ca\(^{2+}\), Mg\(^{2+}\) and CaM.

Thiol titrations of CaN revealed the presence of 4 exposed sulfhydryl groups on the native protein, and 9 sulfhydryl groups when the protein was denatured with SDS. Modification of CaN by Ellman's reagent, iodoacetate and iodoacetamide was accompanied by a sharp increase in activity followed by a decrease in activity. This suggests that one or more sulfhydryl groups are essential for the full expression of CaN's activity.

Attempts were made to locate the 4 exposed sulfhydryl groups on CaN by titrating the various binding sites with Ellman's reagent in the presence and absence of a blocking group. Free sulfhydryl groups were absent from the CaM binding site as well as from the active site, but titrations of the Mn\(^{2+}\) binding site in the presence and absence of Mn\(^{2+}\) revealed the presence of an additional sulfhydryl which may be located in this site.

CDDP, a Mannich base derivative of an \(\alpha,\beta\)-unsaturated ketone was found to react selectively with sulfhydryl groups over other groups found in proteins. Further study showed it was unique in that it could react irreversibly with protein thiols but reversibly with low molecular weight thiols.
Dedicated to my wife,

Gwenyth
ACKNOWLEDGMENTS

I would like to thank Dr. Mutus, my supervisor, for the guidance he has given me over the course of this project. In addition, I would like to thank my committee members, Dr. R. Thibert, Dr. L. Lee, Dr. D. Thomas and Dr. T. Draisey, for their support, especially during the year Dr. Mutus was away on sabbatical. Appreciation also goes to my external examiner, Dr. R. Khandelwal, for participating in my defense. To my friends, who have seen me through this degree, Jaswinder Grewal, Betsy Palmer, Chris Talpas, Ed Saravolac and John Williams, I remain indebted. Finally, I would like to thank my family for their prayers and encouragement, with special thanks to my father who has inspired me to attain this goal.
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ABBREVIATIONS

AC_{1/2} concentration required to achieve half maximal activity
β-ME beta-mercaptoethanol
BSA bovine serum albumin
CaM calmodulin
cAMP cyclic adenosine monophosphate
CaN calcineurin
CDDP 1-p-chlorophenyl-4,4-dimethyl-3-diethyl-amino-1-penten-3-one hydrobromide
DEAE diethylaminoethyl
DTNB 5,5′-dithiobis(2-nitrobenzoate)
EDTA ethylenediamine tetraacetic acid
EGTA ethyleneglycolbis(β-aminoethyl ether)
N,N,N′,N′-tetraacetic acid
EPPS N-(2-hydroxyethyl)piperazine-
N′-(3-propane-sulfonic acid)
GSH reduced glutathione
GSSG oxidized glutathione
I-1 inhibitor-1
I-2 inhibitor-2
IAc iodoacetate
IAM iodoacetamide
K_d dissociation constant
kD kiloDalton

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<tr>
<td>( K_{app} )</td>
<td>apparent ( K_a )</td>
</tr>
<tr>
<td>( M_r )</td>
<td>relative molecular weight</td>
</tr>
<tr>
<td>NM</td>
<td>neuromodulin</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>pNP</td>
<td>para-nitrophenoxide</td>
</tr>
<tr>
<td>pNPP</td>
<td>para-nitrophenyl phosphate</td>
</tr>
<tr>
<td>( P_i )</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PP</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>( PP_i )</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>PP</td>
<td>protein phosphatase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>( N,N,N',N'-\text{tetramethylethylene diamine} )</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>( V_{max , app} )</td>
<td>apparent ( V_{max} )</td>
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CHAPTER 1
INTRODUCTION

1.1. Overview

Calcineurin (CaN) is a Ca\textsuperscript{2+}/Calmodulin (CaM) stimulated protein phosphatase found in high concentration in nervous tissue (Klee et al., 1979). It is a heterodimer of a 61kD catalytic subunit (CaN A) and a 19kD regulatory subunit (CaN B), a CaM-like Ca\textsuperscript{2+} binding protein (Wallace et al., 1979). In vitro, CaN's phosphatase activity can be modified by Ca\textsuperscript{2+}/CaM (Wallace et al., 1980), metal ions (Pallen and Wang, 1984), limited proteolysis (Manalan and Klee, 1983), phospholipids (Politino and King, 1987) and various inhibitors (King and Huang, 1984a; Klee et al., 1983). Bovine brain CaN contains 10 sulfhydryl groups located on its catalytic subunit (Klee et al., 1983), one of which may be essential for expression of its Ni\textsuperscript{2+} stimulated activity (King, 1986). This dissertation explores both the metal ion dependence and the thiol chemistry of CaN.

1.1.1. Historical perspectives

Calcineurin was originally discovered in 1976 by Wang and Desai as a brain protein which inhibited modulator-activated cyclic nucleotide phosphodiesterase (PDE) activity (Wang and Desai, 1976). In the past fourteen years it has been referred to as: the "inhibitor protein", based on its ability to compete with PDE for calmodulin (CaM) (Wang and Desai, 1976);
the "modulator-binding protein", at a time when CaM was known as the Ca²⁺-modulator protein (Wang and Desai, 1977); Calcineurin, on the basis of its Ca²⁺-binding properties and its high concentration in the nervous system (Klee et al., 1979); CaM-BP₉₀, in reference to the relative molecular weight of the binding protein (Wallace et al., 1980); CaM-dependent protein phosphatase (Stewart et al., 1982); protein phosphatase 2B (PP-2B) (Stewart et al., 1982); and finally, as the CaM-stimulated phosphatase (Klee et al., 1983). I have chosen to use the most commonly used name, calcineurin (CaN), in this dissertation.

1.1.2. Protein Phosphatases

CaN is but one enzyme in a family of protein phosphatases. The protein phosphatases (PP) involved in cellular regulation were originally classified by Ingebritsen and Cohen in 1983 (Ingebritsen and Cohen, 1983a). Two classes of PP were recognized: type-1 PP, which had the ability to dephosphorylate the β-subunit of phosphorylase kinase and were inhibited by two thermostable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2); and type-2 PP, which had the ability to dephosphorylate the α-subunit of phosphorylase and were insensitive to I-1 and I-2 inhibition.

Type-2 PP's were subdivided into three categories: PP-2A, phosphatases which were active in the absence of metal ions; PP-2B, phosphatases which had an absolute requirement
for Ca$$^{2+}$$; and PP-2C, phosphatases which had an absolute requirement for Mg$$^{2+}$$ (Cohen, 1989). CaN is a type-2 PP, dependent upon Ca$$^{2+}$$ for activity and stimulated by CaM.

1.1.3. Protein kinases

Reversible phosphorylation of proteins has been recognized as a mechanism for metabolic control in cells since the mid 1950's when the enzymes involved in glycogen metabolism were discovered to be regulated by phosphorylation (Cohen, 1983). Since then, many enzymes have been found to be regulated by reversible phosphorylation.

The degree of phosphorylation of any enzyme in a cell is dependent upon the relative activities of the protein kinases and phosphatases (Cohen and Cohen, 1989) making them equally important in metabolic regulation. At present, much more is known about the protein kinases (Blackshear et al., 1988) than about the protein phosphatases (Cohen, 1989), but interest in the latter is on the increase.

1.2. Molecular properties of calcineurin

The molecular properties of CaN can be discussed in terms of its primary, secondary, tertiary and quaternary structure.

1.2.1. Primary structure

The primary structure of brain CaN has been determined in whole or in part in a number of species including: human,
both the A (two isoenzymes) (Guerini and Klee, 1989a) and the B subunits (Guerini et al., 1989b); cattle, the B subunit (Aitken et al., 1984); rabbit, the A subunit (da Cruz e Silva and Cohen, 1989); rat, the A subunit (two isoenzymes) (Ito et al., 1989; Kuno et al., 1990); and mouse, an incomplete A subunit (Kincaid et al., 1988) and an incomplete B subunit (Guerini et al., 1989b). A comparison of the sequences shows greater homology in the B subunits than in the A subunits. This is in agreement with antibody cross reactivity studies where the B subunit was recognized by a wide variety of species and tissue types, and cross reactivity of the A subunit was poor (Krinks et al., 1985).

Rat brain CaN A is strikingly similar to rabbit PP-1 and rat PP-2A with 42% and 45% identity, respectively. When conservative substitutions are included, the homology increases to 62% and 58% respectively. CaN A has no sequence similarity to PP-2C, the acid and alkaline phosphatases (Ito et al., 1989). The 2 isoenzymes of human CaN A (M, 58kD and 59kD) also show high similarity to rabbit PP-1 and rabbit PP-2A. The 2 human isoenzymes are identical with the exception of an 18 amino acid insert in the central part of the molecule and different carboxy terminal sequences (Guerini and Klee, 1989a). These differences have been explained on the basis of alternative splicing events. A third CaN A isoenzyme, found in rabbit brain, denoted as PP-2B, differs from the human isoenzymes primarily in the catalytic domain. It is
thought that this isoenzyme may have a distinct substrate specificity (da Cruz e Silva and Cohen, 1989).

Sequence analysis of Bovine brain CaN B indicates it is a member of the Ca\(^{2+}\) binding protein superfamily (Aitken et al., 1984). Bovine brain CaN B has an overall sequence identity of 35% with bovine CaM and 29% with rabbit troponin-C. The highest degree of homology is in the region of the putative Ca\(^{2+}\) binding loops. In this region, a 54% identity with bovine CaM is observed (Klee and Cohen, 1988). The B subunit is slightly larger than CaM with 9 and 10 additional residues at the carboxy and amino terminals, respectively.

Bovine brain CaN contains ~722 amino acids, 554-555 in the A subunit (M, 61,000) according to amino acid analysis (Klee and Cohen, 1988) and 168 in the B subunit (M, 19,000) according to its protein sequence (Aitken et al., 1984). Amino acid analysis has revealed the presence of 10 cysteinylin residues on the catalytic subunit of CaN and none on its regulatory subunit (Klee et al., 1983) however titrations of the B subunit with DTNB and iodoacetate have detected the presence of a sulfhydryl group, demonstrating that a single cysteine residue may reside on the B subunit (Guerini et al., 1989b). This would not be surprising since human CaN B also contains cysteinylin residues (Guerini et al., 1989b). In the rat brain, CaN A contains 12 cysteinylin residues (Ito et al., 1989), two more than is present in bovine brain CaN A.
Bovine brain CaN A contains 7-8 tryptophanyl residues (Klee and Cohen, 1988) while the B subunit completely lacks this amino acid (Aitken et al., 1984). Rat CaN A is reported to contain only 4 tryptophanyl residues (Ito et al. 1989). Bovine brain CaN contains 21 tyrosyl residues located mostly on the A subunit, giving the enzyme an extinction coefficient (E°$\text{cm}^{-1}$ cm$^{-1}$) of 9.7 (Wallace et al. 1979). CaN is also rich in aspartic and glutamic acids, as reflected by its low isoelectric point, 4.5-6.0 (Klee et al., 1983; Wallace et al., 1979).

The terminal amino acids of both bovine brain CaN’s A and B subunits are blocked. The identity of the blocking group on the A subunit is unknown (Klee et al., 1983). The B subunit was found to be blocked by myristic acid (Aitken et al., 1982), a 14 carbon saturated fatty acid thought to be involved in subunit interactions (Aitken, 1984), or membrane association (Armstrong, 1989). The amino terminal sequences of CaN A from human and bovine brain have been found to contain 11 consecutive proline residues. This rare sequence has been proposed as a structural element involved in CaN’s interaction with CaM (Guerini and Klee, 1989a).

1.2.2. Secondary structure

Circular dichroism of the CaN holoenzyme indicates it has a low $\alpha$-helical content and an appreciable random coil structure. The binding of metal ions to these regions causes
partial unfolding of the helices (Wolff and Sved, 1985). According to the primary sequence of bovine brain CaM B (Aitken et al., 1984), 54% of this subunit is predicted to contain α-helical structure and 13% β-pleated sheet (Aitken et al., 1984). The Ca\textsuperscript{2+} binding loops are predicted to be globular with high probability of a β-turn at the amino terminal; a feature characteristic of "EF-hand" Ca\textsuperscript{2+} binding loops (Klee and Cohen, 1988). In addition, three of the four loops are flanked by short stretches of α-helix similar to the loops of CaM.

Much less information is available on the secondary structure of the A subunit. The CaM binding domain, located 110 residues from the carboxy terminus, has been identified by a consensus sequence of amphipathic amino acids. This 24 amino acid region is mostly α-helical, containing apposed cationic and hydrophobic environments, a feature seen in other CaM stimulated enzymes (Kincaid et al., 1988).

Recently, a polyproline sequence of 11 residues has been discovered close to the amino terminal of human and bovine CaM A. This unique feature is thought to exist in an extended type II helix with the α-carbons in trans configuration, as this structure would precisely span the length of the central helix of CaM (Guerini and Klee, 1989a).

1.2.3. Tertiary structure

CaM is a globular protein (M\textsubscript{r}, 80,000) with a Stokes
radius of 4.05 nm and a frictional coefficient of 1.4 (Sharma and Wang, 1979). It is a metalloenzyme containing both Zn" and Fe" (King et al., 1984b). It has four binding sites for Ca" on the B subunit (Aitken et al., 1984) and three binding sites for transition metal ions on the A subunit, one high affinity site specific for Ni", another distinct high affinity site specific for Mn", and a third low affinity site capable of binding either metal ion (Pallen and Wang, 1986). The four Ca" binding sites on CaN B are "EF-hand" binding domains as defined by Kretsinger (Kretsinger, 1980). CaN contains 10 sulfhydryl groups, none of which are found as disulfides (King, 1986).

CaN A has been mapped by limited proteolysis using the proteases, clostripain (Hubbard and Klee, 1989a), calpain 1 (Wang et al., 1989), trypsin (Manalan and Klee, 1983) and chymotrypsin (Kincaid et al., 1986a). Five functional domains have been identified through amino acid sequence analysis (Ito et al., 1989; Guerini and Klee, 1989a) and a structural domain, whose function has not yet been established, has been identified with monoclonal antibodies (Hubbard and Klee, 1989b).

Limited proteolysis by the arginine specific protease, clostripain, has shown that CaN A contains at least four functional domains linked together by proteolytically sensitive hinge regions. FIGURE 1 is a schematic representation of the domain structure of CaN A. The domains,
FIGURE 1

Domain Organization of CaN A

\[ \text{Catalytic domain} \quad \text{CaN B} \quad \text{CaM} \quad \text{Inhibitory domain} \]

\[ \text{NH}_2 \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad - \text{COOH} \]

\[ \text{---} \quad 14\text{kD} \quad \text{---} \]

\[ \text{---} \quad 42\text{kD} \quad \text{---} \]

\[ \text{---} \quad 55\text{kD} \quad \text{---} \]

\[ \text{---} \quad 57\text{kD} \quad \text{---} \]

\[ \text{---} \quad 61\text{kD} \quad \text{---} \]

FIGURE 1: A schematic representation of the functional domains of CaN A. Cleavage of CaN A by clostripain in the presence of CaM is sequential. Cleavage of the carboxy terminal domain (the inhibitory domain) leaves a 57kD peptide that can bind CaM but is fully active in the absence of Ca\(^{2+}\) and CaM. Cleavage of the catalytic domain leaves a 55kD peptide which is essentially inactive. Cleavage between the CaN B and CaM binding domains leads to the formation of two inactive peptides, MW 42kD and 14kD. Lines connecting the domains represent hinge regions susceptible to proteolytic attack. (Adapted from Hubbard and Klee, 1989a.)
starting at the amino terminus, include the catalytic domain (which likely contains the metal ion binding sites (Martin and Graves, 1987; Hubbard and Klee, 1989a)) the CaN B binding domain, the CaM binding domain and the inhibitory domain (Hubbard and Klee 1989a).

In the presence of CaM, clostripain nicks CaN A sequentially. The 61kD subunit loses the inhibitory domain, a 4kD peptide located at the carboxy terminus, yielding a 57kD degradation product. This fragment is able to bind CaM but is fully active in the absence of Ca" or CaM. Removal of the catalytic peptide (2kD), leaves a 55kD peptide with essentially no activity (20% residual). A final cleavage between the CaM binding domain and the CaN B binding domain leaves two fragments, 42kD and 14kD. The larger peptide, has essentially no activity (7% residual) and the smaller peptide is capable of binding CaM in the presence of Ca" with low affinity (Klee et al., 1987).

In the absence of CaM the two small terminal domains are cleaved, leaving a 43kD peptide which retains its catalytic activity. This degradation product is 10-fold more active than intact CaN A in the absence of Ca" and 40-fold more active in its presence. The 43kD peptide is reduced to a 40kD and then to a 38kD fragment upon further incubation, resulting in loss of stimulation by Ca" and eventually, total loss of catalytic activity (Klee et al., 1987).
CaN B appears to be resistant to proteolytic attack. Limited proteolysis by the other proteases mentioned above give similar degradation products and activity profiles. This is not surprising since the hinge regions are more susceptible to protease treatment, regardless of the agent used.

The peptide sequence of the autoinhibitory domain has recently been discovered. The sequence I-T-S-F-E-E-A-K-G-L-D-R-I-N-E-R-M-P-P-R-R-D-A-M-P, 50-60 residues carboxy terminal to the CaM binding domain, completely inhibited the activity of CaN (Hashimoto et al., 1990). The autoinhibitory sequence apparently interacts directly with the active site of the phosphatase. Addition of Ca\textsuperscript{2+}/CaM relieves the inhibitory effect. This appears to be analogous to the autoinhibitory domain of the Ca\textsuperscript{2+}/CaM-dependent protein kinase II (Soderling, 1990). In this case, the binding of Ca\textsuperscript{2+}/CaM to the CaM binding domain causes a conformational change in the enzyme such that the inhibitory domain is removed from the catalytic site.

1.2.4. Quaternary structure

CaN is composed of two subunits in an α,β, configuration (Wallace et al., 1979) associated through hydrophobic interactions, possibly involving the myristic acid blocking group on CaN B (Aitken, 1984). The subunits of CaN have been dissociated using 6 M urea (Sharma and Wang, 1979; Merat et al., 1985; Gupta et al., 1986), SDS (Gupta et al., 1985a) and
monoclonal antibodies (Winkler et al., 1984). When assayed in isolation, CaN A has an absolute requirement for Mn$^+$ (Gupta et al., 1986; Winkler et al., 1984). Both CaM and CaN B are capable of stimulating CaN A but they cannot substitute for each other and their action is synergistic. CaM stimulates CaN A 2-fold, CaN B stimulates CaN A 13-fold and together they stimulate CaN A 21-fold. Only CaM is capable of stimulating the holoenzyme (Merat et al., 1985). It has been suggested that Ca$^+/CaM$ may be the in vivo regulator of the holoenzyme relieving the inhibitory effect of CaN B on CaN A in the holoenzyme (Gupta et al., 1986).

CaN A binds CaM in a 1:1 stoichiometric ratio (King et al., 1984b). The interaction between these two proteins is also hydrophobic (Gopalakrishna and Anderson, 1982). The polyproline helix located at the amino terminus of the A subunit (see section 1.2.2.) is thought to be involved in CaM binding (Guerini and Klee, 1989a).

1.3. Mechanism of catalysis

The mechanism of CaN's catalysis has been studied extensively by Martin and Graves (Martin et al., 1985; Martin and Graves, 1986; Martin and Graves, 1987; Martin and Graves, 1988). Based upon the findings that CaN catalysis does not involve a phosphoryl-enzyme intermediate and that the products of pNPP hydrolysis are competitive inhibitors of CaN's phosphatase activity, they suggest catalysis follows a rapid
equilibrium uni-bi reaction mechanism (see FIGURE 2) (Martin and Graves, 1986). The simplest model for the reaction fitting the available data is shown in FIGURE 3. The reaction involves a nucleophilic attack by water on the phosphoryl end of the phosphate ester substrate in concert with a proton transfer from a group in the active site of CaN to the leaving group (alcohol). The phosphoryl group acceptor (water) is oriented by a metal ion (shown in the diagram as Mn") and the proton donor is postulated to be histidine.

1.4. Regulation of CaN activity

CaN's phosphatase activity can be altered in a variety of ways in vitro. A number of these mechanisms have been proposed as in vivo modes of regulation. This section surveys several of the possibilities: Ca"/CaM, metal ions, limited proteolysis, reversible phosphorylation, interaction with phospholipids and product inhibition.

1.4.1. Regulation of CaN activity by metal ions

CaN interacts with metal ions on four levels of complexity: CaN is itself a metalloenzyme containing both Zn" and Fe"; CaN can be stimulated by several divalent cations including Mg"", Mn" and Ni"; the CaN B subunit is a Ca" binding protein; and CaN is stimulated by Ca"/CaM (Pallen and Wang, 1985a). The question of which divalent metal ion is responsible for in vivo activation of CaN remains unanswered.
FIGURE 2
Kinetic Scheme for Phosphate Ester Hydrolysis

FIGURE 2: A sequential kinetic scheme for the hydrolysis of phosphate esters by CaN phosphatase (a rapid equilibrium random uni-bi kinetic mechanism). Abbreviations: E, enzyme (CaN); ROP, substrate (phosphate ester); ROH, alcohol product; P_i, inorganic phosphate. (Adapted from Martin and Graves, 1985)
FIGURE 3: A proposed mechanism for the hydrolysis of a phosphate ester by CaN phosphatase. Abbreviations: \( \text{RPO}_4^{2-} \), substrate; \( \text{AH} \), proton donor in the catalytic site; Mn, metal ion required to orient a water molecule; \( \text{H}_2\text{O} \), phosphoryl acceptor. Hydrolysis is a concerted reaction in which a water molecule attacks the phosphoryl moiety of the substrate at the same time a proton is transferred to the leaving group by the enzyme. (Adapted from Martin and Graves, 1987)
at this time.

Table 1 summarizes five categories of metal ions used to determine CaN's in vitro specificity. In each case, CaN was assayed at neutral pH with the addition of metal ion at a concentration equal to or less than 1 mM. Mg$^{2+}$ is placed in a category of its own since it interacts with CaN differently than the transition metal ions. In order for Mg$^{2+}$ to be an effective activator, the pH and metal ion concentration have to be optimized. 20 mM Mg$^{2+}$ at a pH of 8.6 is required to achieve full activation (Li, 1984).

1.4.1.1. Regulation of CaN by endogenous metal ions

CaN is a metalloenzyme containing near stoichiometric quantities of Zn$^{2+}$ and Fe$^{2+}$ and sub-stoichiometric quantities of Al$^{3+}$ (King et al., 1984b). The function of these endogenous metal ions is not known. Zn$^{2+}$ is found in over 200 enzymes, including phosphatases e.g., alkaline phosphatase (Ochiai, 1988). It functions as a Lewis acid in some enzymes polarizing specific bonds in order to enhance the rate of attack by a nucleophile. It is also used to align substrates thereby facilitating catalysis. In most of these enzymes, the structure of the active site around Zn$^{2+}$ is essentially tetrahedral. Significant distortion to this geometry is possible however since the electronic configuration of Zn$^{2+}$ can assume a tetrahedral or octahedral structure. This means that the requirements for substrates binding to Zn$^{2+}$ in the active
<table>
<thead>
<tr>
<th>Category</th>
<th>Metal ion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Activator</td>
<td>Ni$^{2+}$</td>
<td>Most potent activator in the absence of CaM</td>
</tr>
<tr>
<td></td>
<td>Mn$^{2+}$</td>
<td>Activation enhanced in the presence of CaM</td>
</tr>
<tr>
<td></td>
<td>Co$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>2 Activator</td>
<td>Mg$^{2+}$</td>
<td>A unique activator *</td>
</tr>
<tr>
<td>3 Activator</td>
<td>Ca$^{2+}$</td>
<td>Less effective activator in the presence of CaM</td>
</tr>
<tr>
<td></td>
<td>Ba$^{2+}$</td>
<td>Little or no activity in the absence of CaM</td>
</tr>
<tr>
<td></td>
<td>Sr$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>4 Neutral</td>
<td>Al$^{3+}$</td>
<td>Non-activator in the presence or absence of CaM</td>
</tr>
<tr>
<td></td>
<td>Be$^{2+}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cu$^{2+}$</td>
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</tr>
<tr>
<td></td>
<td>Fe$^{2+}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe$^{3+}$</td>
<td></td>
</tr>
<tr>
<td>5 Inhibitor</td>
<td>Zn$^{2+}$</td>
<td>Potent inhibitor in the presence or absence of CaM</td>
</tr>
<tr>
<td></td>
<td>Cd$^{2+}$</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1:** A summary of the metal ion specificity of CaM (Pallen et al., 1989). Metal ions were tested at neutral pH at concentrations up to 1 mM. (Pallen and Wang, 1984). *, Mg$^{2+}$ was found to be an effective activator at a concentration of 20 mM and a pH of 8.6, in the presence of Ca$^{2+}$ (Li, 1984).
site of an enzyme are less rigid than they would be for other cations. In some cases, Mn\(^{2+}\) and Co\(^{2+}\) can substitute for Zn\(^{2+}\) without loss of enzyme activity since they can assume both a tetrahedral or octahedral conformation (Ochiai, 1988).

Fe\(^{3+}\) is also a common metal ion found in proteins. Its best known function is as a transporter of oxygen in hemoglobin. It is also found in enzymes such as cytochrome oxidase and cytochrome P-450 hydroxylase. In biological systems, Fe\(^{3+}\) (as well as Fe\(^{2+}\)) has a coordination number of 6 and forms octahedral complexes. Ni\(^{2+}\) can possess outer orbital bonding in which the coordination number is 6 and can also form octahedral complexes (Nielsen, 1984). Alternately, Ni\(^{2+}\) favours square coplanar complexes in biological systems. For this reason Ni\(^{2+}\) has been found to competitively interact with both Cu\(^{2+}\) (as well as Cu\(^{+}\)) and Fe\(^{3+}\) (as well as Fe\(^{2+}\)) but not with Zn\(^{2+}\).

1.4.1.2. Regulation of CaN by transition metal ions

Mn\(^{3+}\), Ni\(^{2+}\) and Co\(^{2+}\) are three transition metal ions found to activate CaN in the absence of CaM. These metal ions will support CaM activity in the absence of Ca\(^{2+}\) acting as allosteric activators. In addition, they can serve as cofactors for catalysis (Li and Chan, 1984). CaN A contains three binding sites for the transition metal ions. One site has a high affinity for Mn\(^{3+}\), a second distinct site has a high affinity for Ni\(^{2+}\) and the third site is low affinity site
capable of binding either Mn$^{2+}$ or Ni$^{2+}$ (Pallen and Wang, 1986). The binding of Ni$^{2+}$ and Mn$^{2+}$ cause conformational changes in CaN as demonstrated by monoclonal antibodies which inhibit the Ni$^{2+}$ conformation but not the Mn$^{2+}$ conformation (Matsui et al., 1985).

Neither Ni$^{2+}$ nor Mn$^{2+}$ are associated with CaN in vivo (Rao and Wang, 1989) however both metal ions are known constituents of other metalloenzymes. Mn$^{2+}$ is associated with pyruvate carboxylase, superoxide dismutase, arginase (Schramm, 1982) and glutamine synthase (Wedler et al., 1982) and Ni$^{2+}$ metalloenzymes include jack bean urease (Dixon et al., 1975), carbon monoxide dehydrogenase in \textit{Acetobacterium woodii} (Ragsdale et al., 1983) and the hydrogenase found in \textit{Rhizobium japonicum} (Arp, 1984).

1.4.1.3. Regulation of CaN by Magnesium

Mg$^{2+}$ is unique in the way it activates CaN. It does not bind to the B subunit of CaN (Aitken et al., 1984) or support CaM stimulated activation of CaN (Klee and Cohen, 1988; Cox, 1988) and therefore acts solely as a cofactor for catalysis. The transition ion, in contrast, substituting for Ca$^{2+}$, can bind to the B subunit of CaN and to CaM (Li, 1984). Mg$^{2+}$ can be removed from CaN by exhaustive dilution (Li, 1984) indicating its association is not very tight. The transition metal ions can not be removed in this manner, indicating they bind to CaN with high avidity (Pallen and Wang, 1984).
Approximately 10-fold higher concentrations of Mg^{2+} are required to activate CaN. The K_0 values have been estimated at 5, 0.1 and 0.04 mM for Mg^{2+}, Ni^{2+} and Mn^{2+}, respectively, indicating that the transition ions have a higher affinity for CaN than Mg^{2+} (Li, 1984). Mg^{2+} shifts the pH optimum of CaN towards the alkaline end of the scale whereas the pH optimum for the transition metal ions is in the neutral region. (Li, 1984).

Evidence supporting the suggestion that Mg^{2+} might be the physiological activator (cofactor) of CaN has come from several groups. Li first suggested Mg^{2+} as the physiological activator after sorting out the assay optimization discrepancy (Li, 1984). Chernoff reported the synergistic effect of Ca^{2+}, Mg^{2+} and CaM on in vitro assays (Chernoff et al., 1984) and Rao established that CaN does not contain tightly associated Ni^{2+} or Mn^{2+} in vivo (Rao and Wang, 1989).

1.4.1.4. Regulation of CaN by Calcium

CaN B is a Ca^{2+} binding protein similar in many respects to CaM. Both proteins have four "EF-hand" binding domains capable of binding Ca^{2+} with high affinity, with a K_0 in the μM region (Kincaid and Vaughan, 1986b; Li, 1984; Klee et al., 1979) and both are of similar size, 148 and 168 residues in CaM and CaN respectively. Stimulation of CaN by Ca^{2+} is cooperative. The exact number of Ca^{2+} molecules required to achieve association of CaN and CaM or to achieve full
activation of the complex is unknown (Klee and Cohen, 1988), however it has been postulated that CaM requires two Ca\textsuperscript{2+} molecules in order to associate with CaN, and additional Ca\textsuperscript{2+} to activate the enzyme (Kincaid and Vaughan, 1986b).

Ca\textsuperscript{2+} is, by itself, a relatively poor activator of CaN but in conjunction with CaM, it is able to stimulate phosphatase activity 10-fold. It is thought that Ca\textsuperscript{2+} acts solely as an allosteric activator of CaN (Li, 1984), Mg\textsuperscript{2+} acts solely as a cofactor for catalysis, and the transition metal ions can act in either capacity (Li and Chan, 1984).

1.4.2. Regulation of CaN activity by other mechanisms

CaN is a CaM stimulated phosphatase thought to be regulated \textit{in vivo} by Ca\textsuperscript{2+}/CaM. Other modes by which CaN may be regulated include reversible phosphorylation, regulation by phospholipids, chronic activation by limited proteolysis and product inhibition.

1.4.2.1. Regulation of CaN by Calmodulin

CaM is capable of stimulating CaN through a Ca\textsuperscript{2+} dependent association with the A subunit. CaM binds to CaN in a 1:1 complex (King \textit{et al.}, 1984b) with high affinity, $K_d \leq 0.1$ nM (Hubbard and Klee, 1987). The only CaM dependent enzyme with higher affinity for CaM than CaN is phosphorylase kinase (Hubbard and Klee, 1987). The reason for this high affinity may be explained by the types of binding sites on
phosphorylase kinase. In addition to interactions with a basic amphiphilic α-helix, as CaM does in a wide variety of target proteins (Kincaid et al., 1988), CaM interacts with a second non-conformant binding sequence in phosphorylase kinase (O'Neil and DeGrando, 1990). CaM is considered an integral part of phosphorylase kinase, analogous to CaN A/CaN B interaction.

CaM binds with its target proteins in different ways as demonstrated by affinity chromatography with various CaM fragments (Ni and Klee, 1985) and with monoclonal antibodies against specific conformations of CaM (Hansen and Beavo, 1986). CaM fragments however are not capable of stimulating CaM’s target enzymes (Minowa et al., 1988). This supports the idea of Kincaid (Kincaid and Vaughan, 1986b) that binding and activation of CaN by CaM are two separate events dependent upon the number of Ca" binding sites that are filled.

CaM affects phosphatase activity by increasing the V_{max} of CaN but not altering the K_{a}. Activation of CaM is followed by a time dependent deactivation (King and Huang, 1984a). This deactivation can be reversed by the addition of Mn" or Ni" (Pallen and Wang, 1984) or Mg" (Li and Chan, 1984) to the assay mixture which suggests that the binding of CaM causes a displacement of the metal ion (Li and Chan, 1984; Pallen and Wang, 1984; Pallen and Wang, 1986). The deactivation of CaN by CaM is not considered to be physiologically significant since the association of the proteins in vivo is thought to
occur transiently (Fallen et al., 1988).

1.4.2.2. Regulation of CaN by phosphorylation

Reversible phosphorylation of CaN has been postulated as a mechanism of in vivo regulation. CaN has been phosphorylated by CaM-dependent protein kinase II (Hashimoto et al., 1988; Martensen et al., 1989), protein kinase C (Tung, 1986; Hashimoto and Soderling, 1989) and glycogen synthase kinase-1 (Singh and Wang, 1987) and can be dephosphorylated by PP-2A and to a lesser extent by itself i.e., autodephosphorylation (Hashimoto et al., 1988).

The effect of phosphorylation by the CaM-dependent kinase II appears to be minimal, a 2-fold increase in the K_\text{m} or a 0.5-fold decrease in V_\text{max} depending upon the substrate used. Phosphorylation by this enzyme is dependent upon the presence of Ca^{2+} but inhibited by Ca^{2+}/CaM. This is because the phosphorylation site lies within the CaM binding site, a serine residue ~110 residues from the carboxy terminus. (Martensen et al., 1989; Hashimoto and Soderling, 1989).

Tung has phosphorylated CaN with protein kinase C and found it did not affect CaN activity (Tung, 1986). Singh found glycogen synthase kinase-1 was capable of phosphorylating CaN at two sites, but again, CaN activity was not affected by the modification (Singh and Wang, 1987).
1.4.2.3. Regulation of CaN by limited proteolysis

Limited proteolysis is used to regulate a wide variety of processes in eukaryotic cells including digestion and blood coagulation. The best known example of limited proteolysis is probably the activation of pancreatic zymogens e.g. trypsinogen to trypsin during digestion. The process is specific and irreversible. Another example of regulation by limited proteolysis is the process of blood coagulation. A cascade of clotting factors become activated ultimately leading to the conversion of fibrinogen to fibrin (Cohen, 1983).

Thrombin stimulated platelets contain P28, a phosphoprotein preferentially dephosphorylated by CaN in vitro (Pezzi et al., 1989). Platelets also contain the Ca$^{2+}$ activated neutral proteases Calpain I and II (Tallant and Cheung, 1985). These proteases are capable of irreversibly activating CaN (Tallant et al., 1988). It is not known whether limited proteolysis of CaN and dephosphorylation of P28 in this manner is a physiological phenomenon.

1.4.2.4. Regulation of CaN by phospholipids

Phosphatidylserine and phosphatidylglycerol (but not phosphatidylcholine or phosphatidylethanolamine) have been found to stimulate CaN phosphatase activity for phosphoprotein substrates in vitro, by as much as 23-fold (Politino and King, 1987).
Phosphatidylinositol can also stimulate CaN’s phosphatase activity. In addition, it is capable of modulating the response of the enzyme to CaM. CaN apparently has two sites for phosphoinositol binding; a high affinity site and a low affinity site. When the high affinity site is filled, the affinity of the enzyme for CaM is increased. When the low affinity site is filled, phosphatase activity is stimulated in the absence of CaM, and inhibited in the presence of CaM (Huang et al., 1989). The physiological role of phospholipids in the regulation of CaN has yet to be established.

1.4.2.5. Regulation of CaN by inhibitors

CaN is inhibited by a variety of reagents in vitro including: metal ion chelators such as EDTA and EGTA (Gupta et al., 1984) which take advantage of CaN’s absolute requirement for Ca$^{2+}$ (Cohen, 1989); trifluoperazine, an antipsychotic drug which binds to CaM (Zimmer and Hofmann, 1987) as well as CaN (Stewart et al., 1983); F$^{-}$ and Zn$^{2+}$ (Klee et al., 1983) and vanadate (Chan et al., 1986), traditional inhibitors of phosphatases; and competitive inhibitors of CaN such as P$_i$, PP$_i$, and pNP (Martin and Graves, 1986). Inhibition by P$_i$ and PP$_i$ may be physiologically significant (Martin and Graves, 1986; King and Huang, 1984a).
1.5. CaN physiology

CaN is a relatively new enzyme to the field of biochemistry. Questions about its physiological function remain unanswered, however clues about its role come from knowledge of its distribution and enzyme specificity. Studies dealing with the co-localization of CaN and its preferred substrates may eventually lead to the elucidation of its physiological role.

1.5.1. Distribution of CaN

CaN has been detected in a wide variety of eukaryotic species including chicken, rat, mouse, pig (Tallant and Cheung, 1983), human (Guerini and Klee, 1989a), rabbit (Stewart et al., 1983) cattle (Klee et al., 1979) frog, fish (Tallant and Cheung, 1983) and protozoa (Klumpp et al., 1983). This limited data suggests CaN’s distribution is ubiquitous throughout the eukaryotic world. Further testing is required to substantiate this claim and to explore the possibility that CaN may be present in the prokaryotic world. One would expect the distribution of CaN to follow that of CaM. No CaM has been found in prokaryotic organisms as of yet, but only a limited number of organisms have been tested (Klee and Vanaman, 1982).

Although the highest concentrations of CaN are found in nervous tissue, 600-800 mg/kg of wet brain (Krinks et al., 1985), lower concentrations have been found in liver
(Ingebritsen et al., 1983c), kidney, lung, spleen, testis, thyroid (Wallace et al., 1980) thymus (Krinks et al., 1985), placenta (Pallen and Wang, 1985b), pancreas (Burnham, 1985), adrenal tissue (Papadopoulos et al., 1989), adipose tissue (Ingebritsen et al., 1983c), tongue (Wallace et al., 1980), skeletal muscle (Stewart et al., 1983) smooth muscle (Krinks et al., 1985) and cardiac muscle (Manalan and Werth, 1987). In addition, CaN has been found in certain brain tumours (Goto et al., 1986a; 1987b), erythrocytes (Brissette et al., 1983), platelets (Tallant and Cheung, 1985), lymphocytes (Chantler, 1985; Alexander et al., 1988) and sperm (Tash et al., 1988). This indicates that CaN is not restricted to nervous tissue as previously thought (Klee et al., 1979).

Goto has studied the distribution of CaN in the brain on a regional, a cellular and a subcellular level (Goto et al., 1986b; Goto et al., 1987a). The extrapyramidal region of the brain contains the highest concentrations of CaN, the caudate nucleus and putamen contain especially high levels. CaN immuno-reactivity was found in neurons only. Non-neuronal cells (glial, meningeal, ependymal and vascular endothelial cells) were not associated with CaN immunoreactivity (Goto et al., 1986b). In addition, the CaN-positive cells were medium-sized densely spiny neurons (Goto et al., 1987a). In contrast to Goto’s findings, Kincaid suggests CaN is found in virtually all classes of neurons (Kincaid et al., 1987).
In the brain, CaN is distributed between the particulate and cytosolic fractions in approximately equal quantities (Tallant and Cheung, 1983). Within the neuron, CaN is localized in the post-synaptic densities, dendritic microtubules (Wood et al., 1980) as well as the presynaptic densities (Cooper et al., 1985). Goto determined that CaN was present in dendrites (including the post synaptic densities), in the somata spines, in the axons, and the nerve terminals (synaptic vesicle) (Goto et al., 1986b). Goto has also indicated that CaN is found in the peripheral nervous system (Goto et al., 1987b). Possibly CaN found outside of the CNS is still of neural origin.

1.5.2. Substrate specificity of CaN

CaN is capable of dephosphorylating a wide variety of substrates in vitro including both protein (at phosphoseryl, phosphothreonyl and phosphotyrosyl residues) and non-protein phosphate esters (Martin et al., 1985). CaN was considered to have the narrowest substrate specificity relative to the other protein phosphatases (Ingebritsen and Cohen, 1983a), but with further testing was found to have a much wider substrate specificity (Pallen and Wang, 1985a).

CaN is capable of dephosphorylating a number of non-protein low molecular weight phosphoesters. In order of effectiveness they are β-naphthyl phosphate > p-nitrophenyl phosphate > α-naphthyl phosphate >> tyrosine phosphate (Li,
1984). CaN showed no significant activity towards phosphoserine, phosphothreonine, β-glycerol phosphate, AMP, ADP, or ATP. This indicates that the direct attachment of the phosphate group to an aromatic ring may be a structural requirement for low molecular weight phosphoesters to serve as effective substrates (Li, 1984). Some 20 naturally occurring phosphocompounds were assayed for CaN’s phosphatase activity (Pallen et al., 1985c) but only phosphoenol pyruvate was dephosphorylated at a significant rate. The $K_v$ for this compound is 1 mM, indicating that it is not likely to be a physiological substrate (Pallen et al., 1985c).

That CaN could use tyrosine phosphate as a pseudo-substrate raised the possibility that the enzyme may be a phosphotyrosyl protein phosphatase. Indeed, CaN is capable of dephosphorylating phosphotyrosyl proteins including: casein, histone (Chernoff et al., 1984), epidermal growth factor receptor (Pallen et al., 1985b), smooth muscle myosin light chain, angiotensin 1 and a synthetic peptide matching the sequence of the autophosphorylation site of $R_1$ and the regulatory subunit of cAMP-dependent protein kinase (Chan et al., 1986). The fact that CaN can dephosphorylate proteins at tyrosyl residues does not mean it catalyzes this reaction in vivo nor does it mean that these specific proteins are its in vivo substrate. In fact, these substrates are not necessarily phosphorylated at tyrosyl residues in vivo (Lau et al., 1989). They serve only as a convenient assay for
phosphotyrosyl protein phosphatases. The fact that the phosphoseryl and phosphotyrosyl activities of CaN are similar seems to indicate that it is not a specific phosphotyrosyl phosphatase (Lau et al., 1989; Jones et al., 1989).

CaN is capable of dephosphorylating proteins at both phosphoseryl (Chan et al., 1986; King et al., 1984b; Li and Chan, 1984; Chernoff et al., 1984; Stewart et al., 1982) and phosphothreonyl (King et al., 1984b; Li and Chan, 1984) moieties. This implies that CaN might be involved in metabolic regulation, as most regulatory protein phosphorylation occurs on seryl and threonyl residues. Phosphorylation of protein tyrosyl residues on the other hand, are thought to play an important role in the action of growth factors and regulation of cellular growth (Lau et al., 1989).

Although CaN's in vivo substrates are not known, several proteins act in vitro as good substrates. CaN is able to dephosphorylate the phosphothreonyl residues of I-1 and the α-subunit of phosphorylase kinase (Ingebritsen and Cohen, 1983a) as well as the phosphoseryl residues of the G subunit of PP-1 (Hubbard and Cohen 1989c) at a significant rate in vitro indicating it may be involved in glycogen metabolism in skeletal muscle. In addition to I-1, the dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (Hemmings et al., 1984) and G-substrate (Aswad and Greengard, 1981) are inhibitors of PP-1. All three are phosphorylated on threonyl residues and are potentially good in vivo substrates for CaN.
Both the 60kD and the 63kD isoenzymes of CaM dependent PDE are dephosphorylated by CaN in vitro (Sharma and Wang, 1986) which may indicate CaN has a role in the regulation of cAMP concentrations in the cell. Dephosphorylation of neuromodulin suggests CaN may regulate the concentration of free CaM within the cell (Liu and Storm, 1989). CaN may be involved in regulating intracellular Ca²⁺ concentrations by dephosphorylating the Ca²⁺ channel (or a closely related protein) (Armstrong, 1989). CaN dephosphorylates a 65kD phosphoprotein in Paramecium which suggests it may be involved in exocytotic membrane fusion (Momayez et al., 1987) and a 28kD phosphoprotein in human platelets, the significance of which is not known (Pezzi et al., 1989).

Other threonyl or seryl phosphoproteins dephosphorylated by CaN include, myelin basic protein (Gupta et al., 1985b), protein K-F, synapsin 1 (King et al., 1984b), myosin light chain, casein (Chan et al., 1986), microtubule-associated protein-2 (MAP-2), tau factor and tubulin (Goto et al., 1985).

1.5.3. Physiological role of CaN

The physiological role of CaN has yet to be discovered. Educated guesses based upon co-localization of substrate and enzyme, as well as kinetic parameters for these substrates have suggested a variety of possibilities.

Sharma and Wang have suggested that CaN may be involved in the regulation of cAMP concentrations in the cell by
modulating the activity of the CaM-dependent PDE isoenzymes (Sharma and Wang, 1986). Dephosphorylation of phospho-PDE by CaN increases the affinity of PDE for CaM. In the presence of saturating concentrations of CaM however, this modification increases the sensitivity of PDE-CaM complex for Ca\textsuperscript{2+}. The working hypothesis is based upon the assumptions that there are discrete, sequential, transient increases in the levels of both cAMP and Ca\textsuperscript{2+} and that the various CaM-dependent enzymes have different sensitivities towards Ca\textsuperscript{2+} in vivo.

FIGURE 4 outlines the proposed mechanism of cAMP modulation. An external stimulus causes the production of cAMP from ATP by adenylate cyclase. The initial rise in cAMP levels causes the phosphorylation of the 60kD PDE isozyme causing it to be less active (thus facilitating the production of more cAMP). An increase in the Ca\textsuperscript{2+} concentration during the second phase of cell activation causes the dephosphorylation of the phospho-PDE by the Ca\textsuperscript{2+}/CaM dependent phosphatase, CaN. Activation of PDE causes the degradation of cAMP, terminating the signalling process. The presence of Ca\textsuperscript{2+}/CaM also serves to block phosphorylation of PDE increasing the effectiveness of CaN.

The situation for the 63kD PDE isoenzyme is similar to that for the 60kD isoenzyme, except that it is inactivated via the actions of CaM-dependent protein kinase during the initial rise in Ca\textsuperscript{2+} concentration. At higher levels of Ca\textsuperscript{2+}, CaN becomes active, and again dephosphorylates PDE initiating the
FIGURE 4

Regulation of cAMP Levels

EARLY EVENTS

CaM

+ 60kD or 63kD PDE (more active)

CaM-PK

CaM/PK

60kD or 63kD PDE-P (less active)

Proteins

Proteins-P

PP-1

I-1-P

I-1

LATE EVENTS

Ca²⁺

+ 60kD or 63kD PDE

CaN

CaN/Ca²⁺

FIGURE 4: Hypothetical scheme for the regulation of cAMP levels as mediated by CaN. Dotted lines indicate positive or negative effects as shown. Abbreviations: cAMP, cyclic adenosine monophosphate; Ca²⁺, ionized calcium; CaM, calmodulin; CaN, calcineurin; PP-1, protein phosphatase-1; I-1, inhibitor-1; PDE, CaM-dependent phosphodiesterase; CaM-PK, CaM-dependent protein kinase; cAMP-PK, cAMP-dependent protein kinase (Adapted from Pallen et al., 1988).
termination of the signal. Although this hypothesis has not been demonstrated in vivo it does present a possible explanation for the apparent antagonism between the CaM-dependent kinase and phosphatase.

Another example of antagonism between the two second messenger systems (cAMP and Ca") is CaN's possible involvement in glycogen metabolism in skeletal muscle. FIGURE 5 is an overview of a possible role for CaN in glycogen metabolism. Under the influence of Ca"/CaM, CaN dephosphorylates the glycogen binding subunit of PP-1 and I-1. This promotes reassociation of the G, glycogen binding and C, catalytic subunits of PP-1. PP-1 then is able to dephosphorylate phosphorylase a and glycogen synthase b thereby promoting glycogen formation (Hubbard and Cohen, 1989c). If this occurs in vivo it is an example of a protein phosphatase cascade system. This is not likely to be the major function of brain CaN since glycogen metabolism is relatively unimportant in the brain (Stewart et al., 1983).

CaN may also play a role in regulating the concentration of CaM in neuronal tissues (Liu and Storm, 1989). FIGURE 6 is a hypothetical scheme depicting the regulation of CaM by neuromodulin. Neuromodulin (NM), a neural-specific CaM binding protein found in high concentration in the brain, is unique in that it binds CaM with higher affinity in the absence of Ca" than in its presence. Phosphorylation of neuromodulin by protein kinase C completely abolished its
FIGURE 5

Regulation of Glycogen Metabolism

\[
\begin{align*}
\text{cAMP} & \quad + & \text{PrK} \\
\text{PhKb} & \quad \uparrow & \text{PrK} \\
\quad \downarrow \quad \quad \quad \text{P-PhKb} \\
\text{glycogen} & \quad \uparrow & \text{GSa} \\
\quad \downarrow & \text{Phb} \quad \downarrow & \text{Gsa} \\
\quad \text{Pha-P} & \quad \downarrow & \text{GSb-P} \\
\quad \downarrow & \text{glucose} & \\
\text{PP-1} & \quad \downarrow & \text{C-I-1-P} \\
\quad \text{(active holoenzyme)} & \quad \downarrow & \\
\text{G-P} & \quad \downarrow & \text{G} \quad \downarrow & \text{C} \\
\quad \text{C} & + & \text{I-1} \quad \downarrow & \\
\quad \text{CaN} & \quad \uparrow & \text{Ca^{2+}/CaM} \\
\end{align*}
\]

FIGURE 5: Hypothetical scheme showing the involvement of the cAMP and Ca^{2+} second messenger systems in glycogen metabolism. The action of CaN on PP-1 represents a possible phosphatase cascade. Dotted lines indicate positive effects. Abbreviations: PrK, protein kinase; PhKb, phosphorylase kinase b; Ph, phosphorylase (a and b); GS, glycogen synthase (a and b); PP-1, protein phosphatase 1; G and C, the glycogen binding and catalytic subunits of PP-1; I-1, inhibitor 1; CaN, calcineurin; CaM, calmodulin. (Adapted from Hubbard and Cohen, 1989c)
FIGURE 6

Regulation of Free CaM Levels

PK-C phosphorylates neuromodulin in or adjacent to its CaM-binding site which probably accounts for the decrease in affinity of neuromodulin for CaM (Apel et al., 1990). Dotted lines indicate positive or negative effects as shown. Abbreviations: NM, neuromodulin; CaM, calmodulin; CaN, calcineurin; PK-C, protein kinase C; PL-C, phospholipase C; PIPK, phosphatidylinositol-4-phosphate kinase; PIP, phosphatidylinositol-4-phosphate; PIP₂, phosphatidylinositol-4,5-bisphosphate; IP₃, inositol triphosphate; DAG, diacylglycerol; ER, endoplasmic reticulum. (Adapted from Liu and Storn, 1989)
affinity for CaM (Alexander et al., 1987).

An extracellular signal stimulates phospholipase C to degrade phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol triphosphate. Diacylglycerol activates protein kinase C to phosphorylate neuromodulin causing it to release its sequestered CaM. This allows both CaM and phospho-NM to carry out their biological roles. Phospho-NM is thought to inhibit protein kinase C and phosphatidylinositol phosphate kinase. These two feedback loops would ultimately terminate signal transduction. CaM would be free to interact with its target proteins. Inositol triphosphate stimulates the release of Ca$^{2+}$ from the endoplasmic reticulum, supplying Ca$^{2+}$ to CaM. Ca$^{2+}$/CaM could then interact with CaN which would dephosphorylate phospho-NM, making its association with CaM possible. When the concentration of Ca$^{2+}$ drops CaM would again be sequestered by NM (Liu and Storm, 1989).

1.6. Clinical Significance of CaN

Any diseased state which involves CaM may potentially involve CaN. If the ability of CaM to interact with its target enzymes is impaired a pathological condition may result. CaM has been implicated in psoriasis (Tucker et al., 1986), cystic fibrosis (Rupp, 1986), Ni$^{2+}$ toxicity (Raos and Kasprzak, 1990), malignant transformation (Hait and DeRosa, 1988) and in the complications of diabetes (Kowluru et al., 1989).
CaN is found in high concentration in the extrapyramidal regions of the brain (Wallace et al., 1980; Goto et al., 1986b). For this reason it has been proposed as a marker of extrapyramidal diseases such as Wilson’s disease (Sourkes, 1981), Huntington’s chorea (Goto et al., 1987a) and Parkinson’s disease (Goto et al., 1989). In addition, it has been suggested that CaN be used as a marker for neuronal brain tumours (Goto et al., 1986a).

1.7. Chemical modification of CaN

Chemical modification is a useful tool for studying enzymes. It is capable of providing both qualitative and quantitative information about reactive side chains. Methods have been developed to detect a wide variety of protein constituents including sulfhydryl, disulfide, thioether, amino, guanidino, imidizole, indole, phenolic and carboxy groups (Means and Feeney, 1971). This dissertation explores the sulfhydryl chemistry of CaN.

Not all methods for sulfhydryl titration have equal specificity. Sulfhydryl groups can be titrated with a wide variety of reagents from those with absolute specificity, such as Ellman’s reagent (thiol-disulfide exchange), to those of relatively high specificity, such as iodoacetic acid and its amide (alkylation) (Torchinski, 1981). Variations also exist in the sensitivity of these reagents. Fluorescent probes such as monobromobimane exhibit high quantum yields upon reaction
with thiols (Kosower et al., 1979). Ellman's reagent has an extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm (Ellman, 1958) which makes it very useful for quantitative work whereas N-ethylmaleimide's extinction coefficient is 620 M⁻¹ cm⁻¹ at 305 nm (Torchinski, 1981). Radio-labelling of this and other sulphydryl reagents have led to improved sensitivity.

Important characteristics of modifying reagent which require consideration include its size, charge and polarity (Means and Feeney, 1971). N-(1-pyrene)-maleimide is an example of a large hydrophobic thiol reagent. It is limited in that it may be too large to fit into the active site of an enzyme or into a hydrophilic area. Ellman's reagent is a medium-sized, negatively charged thiol reagent. Iodoacetic acid, a small negatively charged thiol reagent and iodoacetamide, its neutral counter part, are ideal reagents for probing the active sites of enzymes with respect to their size, but may not be able to penetrate into hydrophobic regions due to their polarity. The choice of reagent is dependent upon the purpose of the modification. Different reagents are used for titrating the native enzyme, for modifying the active site, or for use as an environmental probe.

The conditions required for modification also vary with respect to the integrity of the protein. Fairly harsh conditions can be used for detecting and quantitating specific groups in a protein when the native conformation is not a
concern, whereas modification of reactive groups for the purpose of assessing their effect on enzymatic activity must be performed under mild conditions. By avoiding or minimizing undesirable structural change (enzyme denaturation) the effect of the modifying group can be assessed fairly. Reversible modifying reagents such as Ellman's are ideal for this task. The modifying group can be removed quickly and easily by the addition of a reducing agent such as β-ME which will restore the original activity of the enzyme demonstrating that the effect seen is due to the modification rather than enzyme denaturation.

Chemical modification is capable of supplying more than the basic qualitative and quantitative information. As alluded to above, under denaturing conditions the total number of sulfhydryl groups can be titrated. Under non-denaturing conditions, the number of exposed sulfhydryls can be elucidated. In addition, the number of sulfhydryls titratable by various reagents may differ according to the characteristics of the titrating reagent as discussed above.

The presence of sulfhydryls in various binding sites (the substrate binding site, metal ion binding sites and the CaM binding site in the case of CaN) can be determined by thiol titrations in the presence and absence of the bound substance. The function of sulfhydryl groups in these binding sites can also be explored. i.e. sulfhydryl found in a particular binding site might be involved in binding or orienting the bound
substance. Blockage of these sulfhydryls, which prevents
binding, may indicate an essential role for such a sulfhydryl.
Alternately, it may indicate a secondary effect or the
presence of a "false essential group" (Means and Feeney, 1971)
i.e. blockage of a particular sulfhydryl group causes steric
hindrance of another essential group.

Reduction and oxidation of sulfhydryls may indicate a
structural role of cysteiny1 residues in disulfide bridges.
CaN is reported to have ten sulfhydryls all in the reduced
form (King, 1986). This may indicate that CaN is a fairly
flexible enzyme i.e. not constrained by disulfide bridges.
That CaN’s phosphatase activity is dependent upon free
sulfhydryl groups (Tallant and Cheung, 1984; Gupta et al.,
1984; King, 1986; Martin and Graves, 1987) makes it an
attractive area for research.

1.7.2. CDDP as a sulfhydryl titrant

1-p-Chlorophenyl-4,4-dimethyl-5-diethylamino-1-
penten-3-one hydrobromide (CDDP) is one of a series of related
Mannich base derivatives of α,β-unsaturated styryl ketones
which were originally synthesized as antineoplastic agents
(Dimmock and Taylor, 1975; Dimmock et al., 1983; Dimmock et
al., 1989). Its bioactivity is thought to be related to its
selective reaction with thiols (Dimmock et al., 1983;
Prendergast et al., 1983). This, in conjunction with the
large change in absorbance observed during nucleophilic
addition suggested it had the potential to be used as a sulfhydryl titrant. This dissertation examines the sulfhydryl specificity of CDDP as well as its reaction with a variety of thiols.

1.8. Purpose of the present study

This study can be divided into three sections. The first section deals with the metal ion dependence of CaN, the second is a study of CaN's thiol chemistry and the third involves the development of a novel thiol reagent.

It has been suggested that CaN may be activated in vivo by Mg²⁺ (Li, 1984), Mn²⁺ (Wolff and Sved, 1985; Pallen and Wang, 1986) or Ni²⁺ (King and Huang, 1983; Pallen and Wang, 1984). In this study, a kinetic analysis of CaN's phosphatase activity in the presence of these metal ions has been undertaken in order to determine which of these three metal ions is the best in vitro activator of CaN, and which is the most likely candidate for in vivo activation.

CaN activity is also dependent upon free sulfhydryl groups (Tallant and Cheung, 1984; Gupta et al., 1984; King, 1986; Martin and Graves, 1987). This prompted a more thorough study of the CaN's thiol chemistry. Of interest was the number and location of sulfhydryls on the native enzyme, as well as the effect of modifying these groups with a variety of thiol reagents. It was also of interest to determine if CaN could be regulated in vivo by a mechanism of thiol-
disulfide exchange with oxidized glutathione.

CDDP is one of a series of related Mannich derivatives whose bioactivity is thought to be related to its selective reaction with thiols (Dimmock et al., 1983). This has prompted a study of the potential for this compound to be used as a thiol reagent.
<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
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<tr>
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<tr>
<td>Ammonium persulfate</td>
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<td>Sigma</td>
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<tr>
<td>L-Aspartic acid</td>
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<td>Sigma</td>
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<tr>
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<td>Bio Design Inc.</td>
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<tr>
<td>Bis-acrylamide</td>
<td>BDH</td>
</tr>
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<td>CDR Meat Packers</td>
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<td>Sigma</td>
</tr>
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<td>Sigma</td>
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<td>Sigma</td>
</tr>
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</tr>
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<tr>
<td>Ethylenediaminetetraacetic acid</td>
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3-propane sulfonic acid Sigma
Ethylene glycol bis(β-aminoethyl ether)
N,N,N',N'-tetraacetic acid Sigma
Electrophoresis molecular weight markers Sigma
L-Glutamic acid Sigma
Glutathione (reduced) Sigma
Glutathione (oxidized) Sigma
Glutathione reductase Sigma
Glyceraldehyde-3-phosphate dehydrogenase Sigma
Glycerol BDH
Hydrochloric acid BDH
Iodoacetic acid Sigma
Iodoacetamide Sigma
Lauryl sulfate Sigma
L-Lysine GBI
Magnesium acetate Sigma
Manganese sulfate Sigma
Methanol BDH
L-Methionine Sigma
Nickel sulfate Sigma
p-Nitrophenyl phosphate BDH
Oncomodulin Gift from
Dr. MacManus
Phenyl-Sepharose Sigma
Phenylmethylsulfonyl fluoride Sigma
Potassium chloride
Protein-dye reagent
Sephadex G-25
L-serine
Sodium hydroxide
Sodium phosphate
N,N,N',N'-Tetramethylethylenediamine
Thioredoxin
Trizma base
DL-Tyrosine

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CHAPTE 3

APPARATUS

Balances
Mettler balance PC 4400
Mettler balance H16

Centrifuges
Beckman Model J-6B
Eppendorf Model 5414

Electrophoresis
Bethesda Research Laboratories
Vertical Electrophoresis System

Fraction collector
Pharmacia Fraction Collector Frac-100

Graphics program
Sigma plot Version 3.1.

Integrator
Shimadzu C-R3A Chromatopac Integrator

Lyophilizer
Labconco Model 75035

pH meter
Fisher Accumet Model 800

Protein monitor
Bio-rad UV-Monitor Model 1306

Spectrophotometer
Shimadzu UV-160 Spectrophotometer
(with temperature block)

Water purifier
Zenopure MEGA-90
CHAPTER 4

METHODS

4.1. Protein purification

4.1.1. Calcineurin

Calcineurin was purified by a modified procedure of Klee (Klee et al., 1983). All steps were carried out at 4°C. One kg of bovine brain was homogenized in 4 L of 20 mM tris-HCl (pH=7.3), 1.0 mM Mg(CH₂COO)₂, 10 mM β-ME, 40 µg/mL PMSF, (buffer A), and 1.0 mM EGTA in a Waring blender. The homogenate was centrifuged for 1 hr at 3,000 g in a Beckman model J-6B centrifuge. The supernatant was then passed through glass wool and applied over DEAE-cellulose (9.5 x 10 cm) and washed with 0.05 M KCl and 0.1 mM EGTA in buffer A. CaN was eluted with 0.13 M KCl and 0.1 mM EGTA in buffer A. CaCl₂ was added to the eluate to attain a final concentration of 1.0 mM. The solution containing CaN was then applied to CaM-agarose (2.3 x 3.5 cm) and washed with 0.13 M KCl and 0.1 mM CaCl₂ in buffer A. CaN was eluted with 0.1 mM EGTA and 0.13 M KCl in buffer A. The purified CaN was then concentrated by dialysis against 40% glycerol in buffer A and stored at -20°C.

4.1.2. Calmodulin

Calmodulin was purified by a modified procedure of Sharma
(Sharma and Wang, 1979). This procedure is identical to the one described above up to and including the elution of CaN from DEAE-cellulose. At this point, CaM was eluted with 0.50 M KCl and 0.1 mM EGTA in buffer A. This eluate was then dialyzed against 16 L of buffer A. CaCl₂ was added to the solution to attain a final concentration of 1.0 mM. It was then applied to phenyl-Sepharose (5 x 10 cm) and washed with 0.1 mM CaCl₂ and 0.20 M KCl in buffer A. Purified CaM was eluted with 0.5 mM EGTA and 0.20 M KCl in buffer A. It was then dialyzed against 16 L of deionized water, lyophilized and stored at -20°C.

4.2. Electrophoresis

The purity of CaN and CaM was assessed by 10% SDS-PAGE using the method of Laemmli (Laemmli, 1970). The gels were dried at room temperature, sandwiched between two layers of Biogelwrap.

4.3. Protein determination

Protein determinations were performed according to the method of Bradford (Bradford, 1976) using a BSA standard curve. A CaN standard curve was used for the determination of CaN concentrations for all the thiol chemistry work. An extinction coefficient of 9.7 (E₅₀₀nm) was used to determine the concentration of CaN in large volumes (Wallace et al., 1979).
4.4. Protein monitoring

Elution of protein from Sephadex G-25 gel filtration columns (1 cm x 25 cm) was monitored at 280 nm, using a Bio-rad UV-Monitor. The eluting buffer contained 150 mM KCl, the appropriate metal ions at optimal concentrations and 20 mM tris at the optimal pH for the metal ions being used. Protein peaks were collected using a Pharmacia fraction collector and recorded on a Shimadzu integrator.

4.5. Phosphatase activity assay

CaN's phosphatase activity (initial rate) was determined according to the method of Pallen (Pallen and Wang, 1984). The assay cocktail contained 150 mM KCl, 0.5-40.0 mM pNPP, 20 mM tris (unless otherwise specified) at the pH optimal for the metal ion combination being used, metal ions (Mg\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\) or Ca\(^{2+}\)) at their optimal concentrations or metal ion chelators (EDTA or EGTA) at 1 mM and 0.17 \(\mu\)M CaN (preincubated in the appropriate metal ions) ± excess CaM. CaN was preincubated in the appropriate metal ions or metal ion chelators for 24 hr prior to use. CaN and CaM were stored on ice and warmed to 30°C two minutes prior to use (unless otherwise specified). The hydrolysis of pNPP at 30°C was followed on a Shimadzu UV-160 spectrophotometer at 405 nm. The reaction was initiated by the addition of the enzyme. The rate of pNP production was determined using a molar-extinction coefficient of 17,500 M\(^{-1}\)cm\(^{-1}\) (Rao and Wang, 1989).
4.6. Assay optimization

4.6.1. Optimization of the calmodulin concentration

CaN was titrated with CaM in the presence of 1 mM Ca\(^{2+}\), 150 mM KCl, 10 mM pNPP and 20 mM tris (pH=7.9). Both the CaM and the CaN concentrations were determined using the method of Bradford (Bradford, 1976) on a BSA standard curve.

4.6.2. Optimization of pH

The optimal pH for CaN phosphatase assays were determined in the presence of: 1 mM EDTA; 1 mM Ca\(^{2+}\); 1 mM Ca\(^{2+}\) and 30 mM Mg\(^{2+}\); 1 mM EGTA and 30 mM Mg\(^{2+}\); 1 mM Ca\(^{2+}\) and 3 mM Ni\(^{2+}\); and 1 mM Ca\(^{2+}\) and 1 mM Mn\(^{2+}\). The assay cocktail contained 10 mM pNPP, 150 mM KCl, the metal ion combinations listed above and 20 mM tris. The pH of the assay cocktail (at 30°C) was altered by the addition of 0.1 M HCl or 0.1 M NaOH. The pH was monitored using a pH meter adjusted to read at 30°C.

4.6.3. Optimization of metal ion concentrations

CaN was titrated with Ni\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\) in order to determine the optimal concentration to be used in kinetic assays. The assay cocktail used for the Mg\(^{2+}\) titration contained 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=8.3), 1 mM EGTA and Mg\(^{2+}\) at the concentrations indicated by the graph. CaN was preincubated in Mg\(^{2+}\) (at the concentrations indicated by the graph) and 10 mM EGTA for 2 hr prior to the titration.
The assay cocktail used for the Ni\textsuperscript{2+} titration contained 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.9) and Ni\textsuperscript{2+} at the concentrations indicated by the graph. CaN was preincubated in Ni\textsuperscript{2+} (at the concentrations indicated by the graph) for 2 hr prior to the titration.

The assay cocktail used for the Mn\textsuperscript{2+} titration contained 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.3) and Mn\textsuperscript{2+} at the concentrations indicated by the graph. No preincubation of CaN with Mn\textsuperscript{2+} was necessary since linear rates were achieved with or without preincubation.

4.7. CaN kinetics

CaN kinetics were performed in the presence and absence of CaM. The pH and metal ion concentrations were optimized for each condition used. Assays were performed as stated above. The plots displayed in the results section were obtained from the average of three consecutive determinations. Error bars indicate standard deviations for the three determinations.

4.8. Sulfhydryl titrations

4.8.1. Sulfhydryl titration with DTNB

The free sulfhydryl content of CaN was determined by the method of Ellman (Ellman, 1958). CaN was reduced with β-ME and separated from the reducing agent by gel filtration.
chromatography. The reaction was initiated by the addition of 20 μL of DTNB (final concentration 1 mM) to 980 μL of the protein solution (or buffer, in the case of the blank). The reaction was monitored at 412 nm and allowed to proceed to completion. The sulfhydryl content was calculated using an extinction coefficient of 13,600 M⁻¹ cm⁻¹ (Ellman, 1958). DTNB titrations were performed at room temperature. The assay cocktail contained 20 mM pNPP, 150 mM KCl, metal ions or metal ion chelators at their optimal concentrations and 20 mM tris at the optimal pH for the metal ions being used. Titrations of other thiols took place in 20 mM sodium phosphate buffer (pH=7.4), with 1 mM EDTA and 0.1% SDS as required. These titrations were initiated by the addition of the thiol rather than by the addition of DTNB.

4.8.2. Selective modification by DTNB

Selective thiol modification with Ellman’s reagent were carried out by two methods. In the first, modification took place as above, except after 1 minute of incubation the DTNB was separated from the CaN by gel filtration chromatography. In the second, modification took place as above except that the final DTNB concentration in the cuvette was adjusted to be equimolar to the protein concentration such that only the activating sulfhydryl was labelled.
4.8.3. Sulfhydryl titration with CDDP

The free sulfhydryl content of CaN was determined with CDDP using the method of Mutus (Mutus et al., 1989). CaN was reduced with β-ME and excess reducing agent was removed by gel filtration chromatography. Zero-time absorbance values were estimated from the product of the absorbance of CDDP prior to the addition of thiol and the dilution factor. An identical amount of protein was added to the sample and reference cuvette to eliminate any absorbance due to protein. The reaction was initiated by the addition of 200 μL of the protein (final concentration 4 x 10^{-6} M) to 1000 μL of buffered thiol reagent (4 x 10^{-3} M) containing 0.1% SDS for the denaturing trials. The reaction took place at 25°C, was monitored at 308 nm and allowed to proceed to completion. The extinction coefficient for CDDP is ~25,000 M^{-1} cm^{-1} and the extinction coefficient for CDDP-SR is ~4,000 M^{-1} cm^{-1}. The concentration of free thiol can be determined from the change in extinction coefficient (21,000 M^{-1} cm^{-1}). CDDP was prepared fresh daily by dissolving one crystal of CDDP in 10 μL of methanol and diluting to 1.0 mL with deionized water. The concentration of the CDDP solution was determined spectrophotometrically using the extinction coefficient for the reagent, 25,000 M^{-1} cm^{-1}. Titrations of other thiols took place in 20 mM sodium phosphate buffer (pH=7.4), with 1 mM EDTA and 0.1% SDS as required.
4.9. Sulfhydryl modifications and time course studies

4.9.1. Sulfhydryl modification of CaN with DTNB

CaN was reduced with β-ME and separated from the excess reducing agent by gel filtration chromatography. The reduced CaN was divided into two aliquots, a control and a sample to be modified. Modification took place as stated in section 4.8.1.

4.9.2. Sulfhydryl modification with IAc and IAm

CaN was reduced with β-ME (final concentration 10 mM) and then modified with excess Iodoacetate or Iodoacetamide (16.6 mM final concentration) for the time periods indicated on the graphs. Unmodified CaN was used as the control. The assay cocktail contained 20 mM pNPP, 20 mM EPPS buffer (pH=8.7), 1 mM Ca²⁺, 30 mM Mg²⁺, 150 mM KCl and 10 mM β-ME.

4.9.3. Sulfhydryl modification with GSSG

CaN was reduced with β-ME and then separated from excess reducing agent by gel filtration chromatography. The reduced CaN was divided into two aliquots, a control sample and a sample to be modified. GSSG (2 mM final concentration) was added to one of the CaN aliquots. The assay cocktail contained 20 mM pNPP, 20 mM tris (pH=8.7), 1 mM Ca²⁺, 30 mM Mg²⁺ and 150 mM KCl.
4.10. Location of sulfhydryl groups

4.10.1. Assay in the CaM binding site

Sulfhydryl titrations of CaN were performed in the presence and absence of CaM as described above (section 4.8.1.). In addition, EGTA (4.76 mM final concentration) was added to the CaN/CaM cuvette at the end of the titration to determine if removal of CaM from the CaM binding site exposed any additional sulfhydryl groups.

4.10.2. Assay in the active site

Sulfhydryl titrations using DTNB were performed on CaN in the presence and absence of 13% K$_2$HPO$_4$ (used to stop phosphatase activity by competing for the active site of CaN (Pallen and Wang, 1984)). In addition, CaN modified in the presence of 13% K$_2$HPO$_4$ was subjected to gel filtration chromatography in order to remove phosphate from the active site and re-titrated with DTNB in order to determine whether an additional sulfhydryl group would be exposed.

4.10.3. Assay in the metal ion binding site

Sulfhydryl titrations using DTNB were performed on CaN in the presence and absence of Mn$^{2+}$. CaN was preincubated in 1 mM Mn$^{2+}$ and reduced with B-ME. Excess reducing agent and Mn$^{2+}$ were removed by gel filtration chromatography. The protein was then titrated with DTNB. At the end of the
titration, EDTA (1 mM final concentration) was added to the cuvette to determine if an additional sulfhydryl was exposed upon removal of Mn$^{2+}$ from the metal ion binding site. Mn$^{2+}$ is not removed upon exhaustive dialysis, but can be removed by EDTA (Pallen and Wang, 1984). As a control, CaN that had not been exposed to Mn$^{2+}$ was also titrated in the presence of 1 mM EDTA.

4.11 Purification of water

Type 1 water was obtained from a Zeropure water purifier. Distilled water was passed through two deionizing cartridges, a 2 μm filter and an activated charcoal filter. The purified water (resistivity ≥ 15 MΩ) was stored in 20 L plastic containers.
5.1. Purification of CaN

CaN was purified by a two step procedure using DEAE-cellulose followed by CaM-agarose chromatography (see FIGURE 7). Elution of CaN from DEAE-cellulose with 0.13 M KCl separated CaN from PDE, bypassing the need for Cibacron blue affinity chromatography (Wallace et al., 1979). The purification table (TABLE 2) shows the specific activity for all phosphatases present in bovine brain in the presence and absence of CaM. The activation ratio derived from this data, is an index of CaN activity. Note that in each step of the purification, the activation ratio increases such that the purified enzyme shows a 9-fold increase in activity in the presence of CaM. Based upon the specific phosphatase activity in the presence of CaM, CaN has been purified 21-fold. This underestimates the purification since the phosphatase assay is non-specific. Based upon the total protein, CaN has been purified 410-fold (0.244% yield). The end result of the purification is 10 to 20 mg of relatively pure enzyme (see FIGURE 8). Attempts were made to remove the contaminating bands with gel filtration chromatography (G-200, S-200 and P-150) but resolution of the holoenzyme from the low molecular weight bands was not realized.
FIGURE 7

Purification of CaN from Bovine Brain

1Kg TISSUE

HOMOGENIZATION

CENTRIFUGATION

PELLET (discard)

SUPERNATANT

DEAE-CELLULOSE CHROMATOGRAPHY

0.13 M KCl FRACTION

0.05 M KCl FRACTION (discard)

CALMODULIN AGAROSE CHROMATOGRAPHY

0.13 M KCl 0.1 M Ca\textsuperscript{2+} FRACTION (discard)

0.13 M KCl 0.1 M EGTA FRACTION

CONCENTRATE 40% GLYCEROL

15-20mg CALCINEURIN

FIGURE 7: Schematic diagram showing the steps involved in the purification of CaN from bovine brain.
### TABLE 2

**Purification of CaN from Bovine Brain**

<table>
<thead>
<tr>
<th>Step and Condition</th>
<th>Volume (mL)</th>
<th>Protein Concentration (mg/mL)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (nmol/min/mg)</th>
<th>Activation Ratio (+CaN/-CaN)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>2000</td>
<td>3.20</td>
<td>6400</td>
<td>48.8</td>
<td>1.06</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>-CaN</td>
<td></td>
<td></td>
<td>46.1</td>
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</tr>
<tr>
<td>DEAE-cellulose</td>
<td>650</td>
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<td>54.7</td>
<td>1.27</td>
<td>1.12</td>
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<td></td>
<td>43.0</td>
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<td></td>
</tr>
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<td>24.0</td>
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<tr>
<td></td>
<td>-CaN</td>
<td></td>
<td></td>
<td>157</td>
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<td></td>
</tr>
<tr>
<td>Dialysis</td>
<td>18.0</td>
<td>0.865</td>
<td>15.6</td>
<td>1040</td>
<td>9.45</td>
<td>21.3</td>
</tr>
</tbody>
</table>

**TABLE 2:** Purification table for CaN. CaN activity was assayed at each step of the purification. The assay cocktail contained 10 mM pNPP, 20 mM Mg²⁺, 0.1 mM Ca²⁺, 150 mM KCl, 10 mM B-HE and 20 mM tris (pH=8.5). Assays were performed at 30°C. Protein concentration was determined by the method of Bradford (Bradford, 1976) using a BSA standard curve.
FIGURE 8: 10% SDS-PAGE of CaN and CaM. Lane 1 and 4: Molecular weight markers; BSA (66kD), ovalbumin (45kD), glyceraldehyde-3-phosphate dehydrogenase (36kD), carbonic anhydrase (29kD), trypsinogen (24kD), trypsin inhibitor (20.1kD) and α-lactalbumin (14.2kD). Lane 2: 14μg CaM (19.1kD). Lane 3: 16.5 μg CaN; CaN A (52.9kD) and CaN B (16.9kD). Both proteins were electrophoresed in the presence of 0.1 mM EGTA.
5.2. Optimization of phosphatase activity

CaN's phosphatase activity was optimized for each condition used prior to kinetic analysis. The CaM concentration required to achieve maximal activity was equimolar with the CaN concentration (see FIGURE 9). The $K_d$ for the interaction was calculated to be 0.29 nM (see FIGURE 10) by fitting the data from the CaM titration (by the simplex method (Noggle, 1985)) to the Richards-Vithayathil equation (Richards and Vithayathil, 1959). The estimated value agrees well with that estimated by Hubbard, $K_d \leq 0.1$ nM (Hubbard and Klee, 1987).

The pH optimum for phosphatase activity for each metal ion condition was also determined. These conditions included the absence of metal ions, the presence of Ca$^{2+}$ alone, the presence of Mg$^{2+}$ alone (i.e., in the presence of EGTA) and the combination of Ca$^{2+}$ with Mg$^{2+}$, Mn$^{2+}$ or Ni$^{2+}$. The resulting titration curves are shown in FIGURES 11 to 16. A summary of the pH optima is shown in TABLE 3. The presence of Mg$^{2+}$ in the assay cocktail tends to shift the pH optimum towards the alkaline end of the scale. The highest optimum was 8.7, assayed in the presence of Ca$^{2+}$ and Mg$^{2+}$. The lowest optimum was 7.4, assayed in the presence of Ca$^{2+}$ and Mn$^{2+}$.

The optimal concentration for the metal ions, determined by titration, are shown in FIGURES 17 to 19. A summary of the results are shown in TABLE 4. The optima were arbitrarily chosen from the graphs at the point of apparent enzyme
FIGURE 9
CaM Titration of CaN

FIGURE 9: CaM titration of CaN. 0.175 μM CaN was titrated with increasing CaM concentrations. Optimal activity was achieved with equimolar CaM as indicated by the arrow. The assay cocktail contained 10 mM pNPP, 150 mM KCl, 1 mM Ca²⁺ and 20 mM tris (pH=7.9).
FIGURE 10
CaN/CaM $K_d$ Determination

FIGURE 10: The $K_d$ for the interaction between CaN and CaM was determined from the CaM titration in FIGURE 9. A $K_d$ of $2.9 \times 10^{-10}$ was determined by fitting the data from the CaM titration to the Richards-Vithayathil equation (Richards and Vithayathil, 1959), $a=q(r+1-K-[(r+1+k)^2-4r]^{1/2})$ using the simplex curve fitting (Noggle, 1985), where $a$=the activity as a fraction of the highest activity obtained, $r=[CaM]/[CaN]$ and $K=1/K_d$. 
FIGURE 11

pH Titration of CaN in the Presence of EDTA

FIGURE 11: The optimal pH for CaN's phosphatase activity in the presence of 1 mM EDTA was determined to be 7.5. The assay cocktail contained 0.17 μM CaN, 20 mM pNPP, 150 mM KCl, 1 mM EDTA and 20 mM tris at the pH's indicated on the graph. CaN was preincubated in 3 mM EDTA for 24 hr prior to the titration.
FIGURE 12: The optimal pH for CaN's phosphatase activity in the presence of 1 mM Ca\(^{2+}\) was determined to be 7.9. The assay cocktail contained 0.17 μM CaN, 20 mM pNPP, 150 mM KCl, 1 mM Ca\(^{2+}\) and 20 mM tris at the pH's indicated on the graph. CaN was preincubated in 1 mM Ca\(^{2+}\) for 24 hr prior to the titration.
FIGURE 13

pH Titration of CaN in the Presence of EGTA Mg²⁺

FIGURE 13: The optimal pH for CaN's phosphatase activity in the presence of 1 mM EGTA and 30 mM Mg²⁺ was determined to be 8.3. The assay cocktail contained 0.17 mM CaN, 20 mM pNPP, 150 mM KCl, 1 mM EGTA, 30 mM Mg²⁺ and 20 mM tris at the pH's indicated on the graph. CaN was preincubated in 1 mM EGTA and 30 mM Mg²⁺ for 24 hr prior to the titration.
FIGURE 14

pH Titration of CaN in the Presence of Ca" Mg"

FIGURE 14: The optimal pH for CaN's phosphatase activity in the presence of 1 mM Ca" and 30 mM Mg" was determined to be 8.7. The assay cocktail contained 0.17 mM CaN, 20 mM pNPP, 150 mM KCl, 1 mM Ca", 30 mM Mg" and 20 mM tris at the pH's indicated on the graph. CaN was preincubated in 1 mM Ca" and 30 mM Mg" for 24 hr prior to the titration.
FIGURE 15: The optimal pH for CaN's phosphatase activity in the presence of 1 mM Ca$^{2+}$ and 1 mM Mn$^{2+}$ was determined to be 8.7. The assay cocktail contained 0.17 μM CaN, 10 mM pNPP, 150 mM KCl, 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$ and 20 mM tris at the pH's indicated on the graph. CaN was preincubated in 1 mM Ca$^{2+}$ and 1 mM Mn$^{2+}$ for 24 hr prior to the titration.
FIGURE 16

pH Titration of CaN in the Presence of Ca$^{2+}$ Ni$^{2+}$

FIGURE 16: The optimal pH for CaN's phosphatase activity in the presence of 1 mM Ca$^{2+}$ and 3 mM Ni$^{2+}$ was determined to be 8.0. The assay cocktail contained 0.17 mM CaN, 20 mM pNPP, 150 mM KCl, 1 mM Ca$^{2+}$, 3 mM Ni$^{2+}$ and 20 mM tris at the pH's indicated on the graph. CaN was preincubated in 1 mM Ca$^{2+}$ and 3 mM Ni$^{2+}$ for 24 hr prior to the titration.
TABLE 3

Summary of the pH optima

<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>pH OPTIMUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>7.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>7.9</td>
</tr>
<tr>
<td>EGTA Mg²⁺</td>
<td>8.3</td>
</tr>
<tr>
<td>Ca²⁺ Mg²⁺</td>
<td>8.7</td>
</tr>
<tr>
<td>Ca²⁺ Mn²⁺</td>
<td>7.4</td>
</tr>
<tr>
<td>Ca²⁺ Ni²⁺</td>
<td>8.0</td>
</tr>
</tbody>
</table>

TABLE 3: Summary of the pH optima for CaN activity as determined by the titration curves in FIGURES 11 to 16.
FIGURE 17

Titration of CaN with Mg²⁺

FIGURE 17: CaN was titrated with Mg²⁺ in order to determine the optimal concentration to use in the kinetic assays. The assay cocktail contained 0.17 μM CaN, 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=8.3), 1 mM EGTA and Mg²⁺ at the concentrations indicated by the graph. The AC₁/₂ for the titration was 12.8 mM and optimal Mg²⁺ concentration was determined to be 30.0 mM. CaN was preincubated in Mg²⁺ (at the concentrations indicated by the graph) and 10 mM EGTA for 2 hr prior to the titration.
FIGURE 18: CaM was titrated with Ni$^{2+}$ in order to determine the optimal concentration to use in the kinetic assays. The assay cocktail contained 0.17 μM CaM, 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.9) and Ni$^{2+}$ at the concentrations indicated by the graph. The AC_{1/2} for the titration was 0.2 mM and optimal Ni$^{2+}$ concentration was determined to be 3.0 mM. CaM was preincubated in Ni$^{2+}$ (at the concentrations indicated by the graph) for 2 hr prior to the titration.
FIGURE 19: CaN was titrated with Mn$^{2+}$ in order to determine the optimal concentration to use in the kinetic assays. The assay cocktail contained 0.17 µM CaN, 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.3) and Mn$^{2+}$ at the concentrations indicated by the graph. The AC$_{1/2}$ for the titration was 0.1 mM and optimal Mn$^{2+}$ concentration was determined to be 1.0 mM.
<table>
<thead>
<tr>
<th>METAL ION</th>
<th>$AC_{1/2}$ (mM)</th>
<th>OPTIMUM (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>12.8</td>
<td>30.0</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>0.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

TABLE 4: Summary of the metal ion optima and $AC_{1/2}$ as determined by the titration curves in FIGURES 17 to 19.
saturation. The $AC_{1/2}$ were determined from the graphs as the point half way between the concentration of metal ion required for maximal and minimal activation. As is apparent from the concentration of metal ion required to achieve half maximal activation, the affinity of CaN for the transition metal ions is higher than the affinity of the enzyme for Mg$^{2+}$. Due to Ca$^{2+}$ contamination in the assay cocktail, Ca$^{2+}$ titrations (not shown) performed in the absence of EGTA-Ca$^{2+}$ buffering provided limited information. They showed that there was enough Ca$^{2+}$ in the assay cocktail to fully activate CaN and that the addition of 0.1 mM EGTA to the assay cocktail caused a 2-fold decrease in phosphatase activity. Since the objective of this titration was to optimize the phosphatase assay and not to determine the $K_s$ for the interaction of Ca$^{2+}$ and CaN, 1 mM Ca$^{2+}$ was arbitrarily chosen as the optimum (Pallen and Wang, 1984; Gupta et al., 1984; Pezzi et al., 1989). This concentration was known to exceed the requirements for Ca$^{2+}$ activation (the Ca$^{2+}$ concentration required for half maximal activity is 1 $\mu$M (Wolff and Sved, 1985)) and therefore increased the probability that Ca$^{2+}$, rather than a transition metal ion, would occupy the Ca$^{2+}$ binding sites.

5.3. CaN Kinetics

CaN kinetics were performed under the optimized conditions. FIGURES 20 to 25 show the reverse reciprocal plots for CaN kinetic assays under 6 sets of conditions.
FIGURE 20

CaN Kinetics in the presence of EDTA

FIGURE 20: Reverse reciprocal plot of CaN kinetics in the presence of EDTA. The reaction was started by the addition of 50 μL of CaN (0.17 μM final concentration) to 950 μL of assay cocktail (10 to 40 mM pNPP, 150 mM KCl, 27 mM Tris (pH=7.5) and 1 mM EDTA) at 30°C. CaN was preincubated in 3 mM EDTA 24 hr prior to the assay. This data represents the average of 3 determinations. Error bars indicate the standard deviation for the determinations and the solid line indicates the line of best fit to the data.
CaN Kinetics in the presence of Ca$^{2+}$

FIGURE 21: Reverse reciprocal plots of CaN kinetics in the presence of Ca$^{2+}$, + CaM (closed circles) and - CaM (open circles). The reaction was started by the addition of 50 µL of CaM to 950 µL of assay cocktail (10 to 40 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.9) and 1 mM Ca$^{2+}$ ± 0.21 µM CaM) at 30°C. Half as much CaM was used in the presence of CaM as in its absence (0.08 µM and 0.17 µM final concentrations respectively). CaN was preincubated in 1 mM Ca$^{2+}$ for 24 hr prior to the assay. This data represents the average of 3 determinations. Error bars indicate standard deviation for the determinations and the solid lines indicate the lines of best fit to the data. Error bars not observed are obscured by the data points.
FIGURE 22

CaN Kinetics in the presence of EGTA Mg²⁺

FIGURE 22: Reverse reciprocal plots of CaN kinetics in the presence of EGTA Mg²⁺, + CaM (closed circles) and - CaM (open circles). The reaction was started by the addition of 50 μL of CaN to 950 μL of assay cocktail (10 to 40 mM pNPP, 150 mM KCl, 20 mM tris (pH=8.3), 1 mM EGTA and 30 mM Mg²⁺ ± 0.21 μM CaM) at 30°C. Half as much CaN was used in the presence of CaM as in its absence (0.08 μM and 0.17 μM final concentrations respectively). CaN was preincubated in 1 mM EGTA and 30 mM Mg²⁺ for 24 hr prior to the assay. This data represents the average of 3 determinations. Error bars indicate standard deviation for the determinations and the solid lines indicate the lines of best fit to the data. Error bars not observed are obscured by the data points.
FIGURE 23: Reverse reciprocal plots of CaN kinetics in the presence of Ca$^{2+}$ Mg$^{2+}$, + CaM (closed circles) and - CaM (open circles). The reaction was started by the addition of 50 μL of CaN to 950 μL of assay cocktail (10 to 40 mM pNPP, 150 mM KCl, 20 mM tris (pH=8.7), 1 mM Ca$^{2+}$ and 30 mM Mg$^{2+}$ ± 0.21 μM CaM) at 30°C. Half as much CaN was used in the presence of CaM as in its absence (0.08 μM and 0.17 μM final concentrations respectively). CaN was preincubated in 1 mM Ca$^{2+}$ and 30 mM Mg$^{2+}$ for 24 hr prior to the assay. This data represents the average of 3 determinations. Error bars indicate standard deviation for the determinations and the solid lines indicate the lines of best fit to the data. Error bars not observed are obscured by the data points.
FIGURE 24

CaN Kinetics in the presence of Ca\(^{2+}\) Mn\(^{2+}\)

FIGURE 24: Reverse reciprocal plots of CaN kinetics in the presence of Ca\(^{2+}\) Mn\(^{2+}\), + CaM (closed circles) and - CaM (open circles). The reaction was started by the addition of 50 µL of CaN to 950 µL of assay cocktail (10 to 40 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.4), 1 mM Ca\(^{2+}\) and 1 mM Mn\(^{2+}\) ± 0.21 µM CaM) at 30°C. Half as much CaN was used in the presence of CaM as in its absence (0.08 µM and 0.17 µM final concentrations respectively). CaN was preincubated in 1 mM Ca\(^{2+}\) and 1 mM Mn\(^{2+}\) for 24 hr prior to the assay. This data represents the average of 3 determinations. Error bars indicating the standard deviation for the determinations are obscured by the data points. The solid lines indicate the lines of best fit to the data.
FIGURE 25

CaN Kinetics in the presence of Ca²⁺ Ni²⁺

FIGURE 25: Reverse reciprocal plots of CaN kinetics in the presence of Ca²⁺ Ni²⁺, + CaM (closed circles) and - CaM (open circles). The reaction was started by the addition of 50 μL of CaN to 950 μL of assay cocktail (0.5 to 10 mM pNPP, 150 mM KCl, 20 mM tris (pH=7.4), 1 mM Ca²⁺ and 1 mM Ni²⁺ ± 0.21 μM CaM) at 30°C. Half as much CaN was used in the presence of CaM as in its absence (0.08 μM and 0.17 μM final concentrations respectively). CaN was preincubated in 1 mM Ca²⁺ and 1 mM Ni²⁺ for 24 hr prior to the assay. This data represents the average of 3 determinations. Error bars indicating the standard deviation for the determinations are obscured by the data points. The solid lines indicate the lines of best fit to the data.
A summary of the kinetic parameters is shown in TABLE 5. Note that the addition of CaM to the reaction mixture increases the \( V_{\text{app}} \) in each case while having little effect on the \( K_{\text{app}} \). The \( V_{\text{app}} \) to \( K_{\text{app}} \) ratio is highest in the presence of \( \text{Ca}^{2+} \), \( \text{Ni}^{2+} \) and CaM (584 x \( 10^{-6} \) L min\(^{-1}\) mg\(^{-1}\)) which suggests this combination is a good in vitro activator of CaN (see TABLE 6). Maximal activation is achieved in the presence of \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \) and CaM. Here a 67-fold increase over the basal (EDTA) rate is observed. The greatest percentage increase in activity is seen upon addition of CaM to CaN in the presence of \( \text{Ca}^{2+} \) alone (676% increase) but the magnitude of change (the numerical difference between \( V_{\text{app}} \) values) is largest upon addition of CaM to CaN in the presence of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) (a difference of 8094 nmol min\(^{-1}\) mg\(^{-1}\)). These results suggest that \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \) and CaM is the best combination of effectors for the in vitro activation of CaN.

5.4. Thiol chemistry

5.4.1. Thiol titrations

Thiol titrations of CaN with DTNB and CDDP in the presence of 0.1% SDS revealed the presence of 9.5 and 8.9 sulfhydryl groups respectively (TABLE 7). This closely approximates the number of cysteine residues determined by performic oxidation, 10 (Klee et al., 1983) and that determined by reductive carboxymethylation in guanidine hydrochloride, 9 (King, 1986). CDDP was useful for titrating
<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>$K_a$, app (mM)</th>
<th>$V_{max}$, app (nmol min$^{-1}$ mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>12.5</td>
<td>170</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>38.7</td>
<td>356</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2405</td>
</tr>
<tr>
<td>EGTA Mg$^{2+}$</td>
<td>70.5</td>
<td>1955</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2221</td>
</tr>
<tr>
<td>Ca$^{2+}$ Mg$^{2+}$</td>
<td>94.6</td>
<td>3345</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11439</td>
</tr>
<tr>
<td>Ca$^{2+}$ Mn$^{2+}$</td>
<td>25.5</td>
<td>1004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4203</td>
</tr>
<tr>
<td>Ca$^{2+}$ Ni$^{2+}$</td>
<td>2.0</td>
<td>1955</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3095</td>
</tr>
</tbody>
</table>

**TABLE 5:** Summary of the apparent kinetic parameters for the hydrolysis of pNPP by CaN under optimized conditions as determined by the reverse reciprocal plots in FIGURES 20 to 25.
### TABLE 6

**Analysis of Kinetic Parameters**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Kinetic Parameter Ratio</th>
<th>Fold Activation over Basal Activity</th>
<th>Fold Activation by CaH</th>
<th>Absolute Change in Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max, app}}/K_{\text{m, app}}$</td>
<td>(%)</td>
<td>(%)</td>
<td>(nmol min$^{-1}$ mg$^{-1}$)</td>
</tr>
<tr>
<td>+Ca$^2+$</td>
<td>+Ca$^2+$</td>
<td>+Ca$^2+$</td>
<td>EDTA</td>
<td>+Ca$^2+$</td>
</tr>
<tr>
<td></td>
<td>(L min$^{-1}$ mg$^{-1}$) $\times 10^6$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>63</td>
<td>14.1</td>
<td>6.76</td>
<td>2049</td>
</tr>
<tr>
<td>EGTA Mg$^{2+}$</td>
<td>32</td>
<td>13.1</td>
<td>1.14</td>
<td>266</td>
</tr>
<tr>
<td>Ca$^{2+}$ Mg$^{2+}$</td>
<td>133</td>
<td>67.3</td>
<td>3.42</td>
<td>8094</td>
</tr>
<tr>
<td>Ca$^{2+}$ Mn$^{2+}$</td>
<td>178</td>
<td>24.7</td>
<td>4.19</td>
<td>3199</td>
</tr>
<tr>
<td>Ca$^{2+}$ Ni$^{2+}$</td>
<td>584</td>
<td>18.2</td>
<td>1.58</td>
<td>1140</td>
</tr>
</tbody>
</table>

**TABLE 6:** An analysis of the kinetic parameters summarized in TABLE 5.
TABLE 7

Titration of Free Sulfhydryls on CaN

<table>
<thead>
<tr>
<th>Thiol reagent</th>
<th>Condition</th>
<th>Trials</th>
<th>Sulfhydryls ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Titration of denatured CaN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDDP</td>
<td>EDTA/SDS</td>
<td>n=5</td>
<td>8.9 ± 0.35</td>
</tr>
<tr>
<td>DTNB</td>
<td>EDTA/SDS</td>
<td>n=5</td>
<td>9.5 ± 0.18</td>
</tr>
<tr>
<td></td>
<td><strong>Titration of native CaN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDDP</td>
<td>EDTA</td>
<td>n=3</td>
<td>0.2 ± 0.18</td>
</tr>
<tr>
<td>DTNB</td>
<td>EDTA</td>
<td>n=4</td>
<td>5.0 ± 0.20</td>
</tr>
<tr>
<td>DTNB</td>
<td>Ca²⁺</td>
<td>n=4</td>
<td>4.1 ± 0.55</td>
</tr>
<tr>
<td>DTNB</td>
<td>Ca²⁺ Mg²⁺</td>
<td>n=4</td>
<td>4.1 ± 1.08</td>
</tr>
<tr>
<td></td>
<td><strong>Titration of binding sites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTNB</td>
<td>Ca²⁺/CaM</td>
<td>n=4</td>
<td>5.3 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>+EGTA</td>
<td>n=4</td>
<td>5.3 ± 0.87</td>
</tr>
<tr>
<td>DTNB</td>
<td>Ca²⁺/PO₄³⁻</td>
<td>n=3</td>
<td>4.8 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>-PO₄³⁻</td>
<td>n=3</td>
<td>4.8 ± 0.35</td>
</tr>
<tr>
<td>DTNB</td>
<td>Ca²⁺ Mn²⁺</td>
<td>n=4</td>
<td>2.3 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>+EDTA</td>
<td>n=4</td>
<td>3.4 ± 0.30</td>
</tr>
</tbody>
</table>

**TABLE 7:** CaN sulfhydryl titrations were performed with 1 mM DTNB or 40 µM CDDP under the conditions listed. The assay cocktail contained 4 µM CaN, 20 mM tris (at the optimal pH according to the metal ions present), 150 mM KCl (or 150 mM NaCl when SDS was used), where indicated, 0.1% (v/v) SDS, 1 mM EDTA, 1 mM Ca²⁺ (except in the presence of phosphate, when 0.05 mM Ca²⁺ was used), 30 mM Mg²⁺ and stoichiometric Mn²⁺. In order to determine if sulfhydryls existed in the binding sites two titrations were performed, one with the binding site blocked, the other when the blocking group was removed. The numerical difference between these titrations was considered the number of sulfhydryls present.
CaN in the presence of a denaturing reagent but not in its absence.

The presence of certain metal ions (Hg^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Hg^+, Ag^+, Cu^+ and Au^+) in the assay cocktail may bind sulphydryl groups interfering with the titration or cause auto-oxidation of these groups (Torchinski, 1981; Friedman, 1973). The effects of Ca^{2+} and Mg^{2+} are minimal since they bind sulphydryls very weakly (Huxtable, 1986). Titrations of native CaN in the presence of EDTA, Ca^{2+} and Ca^{2+} Mg^{2+} revealed the presence of 5.0, 4.1 and 4.1 exposed sulphydryl groups respectively. Similar results have been published for the titration of CaN in the presence of Ni^{2+} with p-hydroxymercuribenzoic acid; 4 to 5 sulphydryl groups were titrated (King, 1986).

The binding sites of CaN were titrated with DTNB in a two step process. The titration took place in the presence of a blocking group and continued once the blocking group was removed. The CaM site was blocked with CaM in the presence of Ca^{2+} and was removed with excess EGTA. No increase in absorbance was observed upon removal of the CaM therefore the conclusion drawn was that there were no sulphydryls in the CaM binding site. This conclusion is based upon the assumption that the proteins dissociate upon addition of the Ca^{2+} chelator. Evidence that the association of CaM and CaN is reversible and Ca^{2+} dependent comes from studies in which CaN is immobilized on nitrocellulose (Hubbard and Klee, 1987) and
CaM is immobilized on Sepharose 4B (Sharma et al., 1976).

The catalytic site was also assayed for the presence of sulfhydryl groups. The substrate binding site was blocked with 13% (v/v) NaH₂PO₄ (13% PO₄⁻³ is used to terminate CaN catalysis (Pallen and Wang, 1984) by competing with the substrate for the active site (Martin and Graves, 1986)) and removed by gel filtration chromatography. No increase in absorbance was observed upon re-titration of the enzyme. This appears to indicate that no sulfhydryls are present in the active site of the enzyme. This conclusion must be considered in light of the following assumptions: phosphate, a relatively small blocking group, completely blocks the active site; the phosphate was removed from the active site during the chromatography step, and the alleged sulfhydryl did not oxidize before it could be titrated the second time.

In order to determine if there was a sulfhydryl group in a transition ion binding site, sulfhydryl titrations were performed in the presence and absence of Mn²⁺. Mn²⁺ can be removed from its binding site with EDTA but is not removed by extensive dialysis (Pallen and Wang, 1984). In order to do this without the risk of metal ion induced oxidation of the free sulfhydryl groups, CaN was incubated in the presence of Mn²⁺ and then passed over a gel filtration column to remove excess, unbound Mn²⁺. This enzyme was titrated with DTNB and found to contain 2.3 sulfhydryl groups. Addition of EDTA to the reaction mixture caused an increase in absorbance
equivalent to the exposure of an additional sulfhydryl (3.4 sulfhydryl groups final). This sulfhydryl group may be involved in binding the transition metal ion or may be exposed as a result of a conformational change upon loss of the metal ion. Neither possibility can be ruled out at this time. However, it is apparent from a comparison of the titrations in the presence of EDTA, Ca" (Ca" Mg") and Ca" Mn" that different conformations of the enzyme exist in these states since 5.0, 4.1 and 2.3 sulfhydryl groups are exposed respectively.

5.4.2. Functional consequences of thiol modification

Modification of CaN with DTNB in the presence of Ca" reveals the presence of 4 sulfhydryl groups on the native protein. A time course of the activity of the enzyme during the modification (FIGURE 26) shows an initial 10-fold increase in activity, followed by a gradual decrease in activity. Even after 60 minutes of incubation with DTNB, the phosphatase activity of the modified enzyme remains higher than the control value. Similar results are observed when CaN is modified in the presence of Ca" and Mg" (FIGURE 27). This time, however, modification causes nearly total inhibition of the enzyme.

CaN is modified by DTNB more quickly in the presence of Ca" alone than in the presence of both Ca" and Mg". Modification in the presence of Ca" appears to be biphasic.
FIGURE 26

Time Dependent Modification of CaN with DTNB
in the Presence of Ca²⁺

FIGURE 26: A time course for modification of CaN by DTNB in the presence of Ca²⁺. Three graphs have been plotted in this figure: the thiol titration (closed squares), the activity of the enzyme as it is being modified by the thiol reagent (open circles) and the activity of the control (unmodified CaN) (closed circles). The assay cocktail contained 4 μM CaN, 20 mM tris (pH=7.9), 150 mM KCl, 1 mM Ca³⁺, and 20 mM pNPP (for the activity assays) and 1 mM DTNB (for the thiol titration and the modified CaN activity assay). CaN (preincubated in 1 mM Ca³⁺) was reduced with B-ME prior to the modification and separated from the reducing agent by gel filtration chromatography. The assays took place at room temperature.
FIGURE 27

Time Dependent Modification of CaN with DTNB in the Presence of Ca²⁺ Mg²⁺

FIGURE 27: A time course for modification of CaN by DTNB in the presence of Ca²⁺ Mg²⁺. Three graphs have been plotted in this figure: the thiol titration (closed squares), the activity of the enzyme as it is being modified by the thiol reagent (open circles) and the activity of the control (unmodified CaN) (closed circles). The assay cocktail contained 4 μM CaN, 20 mM Tris (pH=7.9), 150 mM KCl, 1 mM Ca²⁺, 30 mM Mg²⁺ and 20 mM pNPP (for the activity assays) and 1 mM DTNB (for the thiol titration and the modified CaN activity assay). CaN (preincubated in 1 mM Ca²⁺ and 30 mM Mg²⁺) was reduced with β-ME prior to the modification and separated from the reducing agent by gel filtration chromatography. The assays took place at room temperature.
The second order rate constants for this modification, estimated from the pseudo first order plot of the time course, are 1007 and 72 L mol\(^{-1}\) min\(^{-1}\) (see FIGURE 28). In the presence of Ca\(^{2+}\) Mg\(^{2+}\), all 4 sulphydryls appear to be equally accessible to the thiol reagent. The second order rate constant is estimated to be 59 L mol\(^{-1}\) min\(^{-1}\) (see FIGURE 29). This may indicate that the enzyme's Ca\(^{2+}\) conformation is different than its Ca\(^{2+}\) Mg\(^{2+}\) conformation.

Activation of CaN is not restricted to DTNB alone. Other thiol reagents including iodoacetate, iodoacetamide and GSSG activate the enzyme. Iodoacetate activates and inactivates CaN in a time dependent manner similar to that seen with DTNB, but on a smaller scale (FIGURE 30). The extent of the activation is less than 1.5-fold. Iodoacetamide appears to activate CaN initially and then inhibits the enzyme (FIGURE 31). Iodoacetamide may alkylate the same cysteine residue that DTNB exchanges with (in the presence of Ca\(^{2+}\) Mg\(^{2+}\)) since in both cases an inhibition below the control value is observed. GSSG both activates and stabilizes CaN activity (FIGURE 32).
Figure 28: The DTNB titration data from Figure 26 was plotted according to the pseudo first order rate equation \( \ln (A_{\infty} - A) = -kt \). Modification of CaN in the presence of Ca\(^{2+}\) appears to be biphasic. The second order rate constants for the modification were estimated to be 1007 and 71.8 L mol\(^{-1}\) min\(^{-1}\).
FIGURE 29

Pseudo First Order Rate Plot for the Titration
of CaN with DTNB in the Presence of Ca²⁺ Mg²⁺

FIGURE 29: The DTNB titration data from FIGURE 27 was plotted according to the pseudo first order rate equation (ln (A₀-A)=-kt). Modification of CaN in the presence of Ca²⁺ Mg²⁺ appears to be linear. The second order rate constant for the modification was estimated to be 59 L mol⁻¹ min⁻¹.
FIGURE 30
Time Dependent Modification of CaN with IAc in the Presence of Ca$^{2+}$ Mg$^{2+}$

FIGURE 30: A time course for the modification of CaN by iodoacetate in the presence of Ca$^{2+}$ Mg$^{2+}$. Two graphs have been plotted in this figure: the activity of the modified enzyme (closed circles) and the activity of the control (unmodified enzyme) (open circles). The assay cocktail contained 20 mM EPPS (pH=8.7), 150 mM KCl, 1 mM Ca$^{2+}$, 30 mM Mg$^{2+}$, 10 mM B-HE and 20 mM pNPP. 4 μM CaN preincubated in Ca$^{2+}$ and Mg$^{2+}$ was reduced with B-HE (10 mM final concentration) prior to the assays. Iodoacetate (16.6 mM final concentration) was added to an aliquot of CaN and allowed to incubate for the times indicated in the graph. The activity assay was started by adding the modified CaN to the assay cocktail. The assays took place at 30°C.
FIGURE 31

Time Dependent Modification of CaN with IAM
in the Presence of Ca$^{2+}$ Mg$^{2+}$

FIGURE 31: A time course for the modification of CaN by iodoacetamide in the presence of Ca$^{2+}$ Mg$^{2+}$. Two graphs have been plotted in this figure: the activity of the modified enzyme (closed circles) and the activity of the control (unmodified enzyme) (open circles). The assay cocktail contained 20 mM EPPS (pH=8.7), 150 mM KCl, 1 mM Ca$^{2+}$, 30 mM Mg$^{2+}$, 10 mM β-ME and 20 mM pNPP. 4 μM CaN preincubated in Ca$^{2+}$ and Mg$^{2+}$ was reduced with β-ME (10 mM final concentration) prior to the assays. Iodoacetamide (16.6 mM final concentration) was added to an aliquot of CaN and allowed to incubate for the times indicated in the graph. The activity assay was started by adding the modified CaN to the assay cocktail. The assays took place at 30°C.
FIGURE 32
Time Dependent Modification of CaN with GSSG
in the Presence of Ca\(^{2+}\) Mg\(^{2+}\)

FIGURE 32: A time course for the modification of CaN by oxidized glutathione in the presence of Ca\(^{2+}\) Mg\(^{2+}\). Two graphs have been plotted in this figure: the activity of the modified enzyme (closed circles) and the activity of the control (unmodified enzyme) (open circles). The assay cocktail contained 20 mM tris (pH=8.7) 150 mM KCl, 1 mM Ca\(^{2+}\), 30 mM Mg\(^{2+}\), 20 mM pNPP and 2 mM GSSG. CaN (preincubated in the presence of Ca\(^{2+}\) and Mg\(^{2+}\)) was reduced with B-ME prior to the modification and excess reducing reagent was removed by gel filtration chromatography. The assays took place at room temperature.
5.5. CDDP

CDDP is a Mannich base derivative of an α,β-unsaturated styryl ketone (FIGURE 33) which absorbs at 308 nm with an extinction coefficient of 25,000 M⁻¹ cm⁻¹ (FIGURE 34). The reactivity of CDDP towards a variety of functional groups was tested by incubating it with a 10-fold molar excess of arginine, lysine, aspartic acid, glutamic acid, methionine, serine, tyrosine and cysteine. Of the 8 amino acids tested, only cysteine was found to alter CDDP's absorption spectrum. This indicates that sulfhydryls are the only protein functional groups that react with CDDP at physiological pH. Increasing the cysteine concentration to a 40-fold excess decreased the absorbance of CDDP by 84% but additional cysteine, up to 100-fold excess, did not reduce the absorbance any further. This indicates that the CDDP-cys adduct also absorbs at 308 nm with an extinction coefficient of 4,000 M⁻¹ cm⁻¹.

CDDP was tested for its usefulness as a sulfhydryl titrant on both protein and non-protein thiols (TABLE 8). Although CDDP was as precise as the reference method (DTNB) when measuring protein thiols, it grossly underestimated the sulfhydryl concentration of the low molecular weight non-protein thiols (FIGURE 35). This is thought to be the result of a base catalysed reversal of the thiolation reaction. When EDTA was omitted from the assay cocktail the time course for the reaction of CDDP with low molecular weight thiols showed
FIGURE 33

Structure of CDDP

\[
\begin{align*}
\text{Cl} & \quad \text{C} = \text{C} - \text{C} - \text{C} - \text{CH}_2\text{N(C}_2\text{H}_5)_2\text{.HBr} \\
\text{H} & \quad \text{H} \quad \text{CH}_3
\end{align*}
\]

FIGURE 33: Structure of CDDP (1-p-Chlorophenyl-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide).
FIGURE 34

UV Absorption Spectrum of CDDP and CDDP-CYS Adduct

FIGURE 34: UV Absorption Spectrum of CDDP and CDDP-CYS Adduct. Two graphs have been plotted in this figure: the ultraviolet absorption spectrum of $2.2 \times 10^{-5}$ M CDDP in 100 mM sodium phosphate buffer (pH=7.4) (open circles) and the ultraviolet absorption spectrum of $2.2 \times 10^{-5}$ M CDDP and a 40-fold excess of cysteine in the same buffer (closed circles). CDDP has an extinction coefficient of $-25,000 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 308 nm and the CDDP-CYS adduct has an extinction coefficient of $-4,000 \text{ L mol}^{-1} \text{ cm}^{-1}$ at the same wavelength.
TABLE 8

Titration of Free Sulfhydryls on Various Thiols

<table>
<thead>
<tr>
<th>Thiol</th>
<th>mol Sulfhydryls / mol Thiol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
</tr>
<tr>
<td>8-Mercaptoethanol</td>
<td>1</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1</td>
</tr>
<tr>
<td>Ovomodulin</td>
<td>1</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>4</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>2</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>2</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>1</td>
</tr>
</tbody>
</table>

TABLE 8: Titrations of free sulfhydryls on protein and non-protein thiols by DTNB and CDDP. The number of accessible sulfhydryls per protein are given. Ovomodulin (MacManus and Whitfield, 1983), thioredoxin (Porque et al., 1970) and bovine serum albumin (Wilson et al., 1979) are monomers. Glyceraldehyde-3-phosphate dehydrogenase (Friedman, 1973) is a tetramer with 1 accessible sulfhydryl per subunit. Glutathione reductase (Torchinski, 1981) is a dimer with 1 accessible sulfhydryl per subunit. Thioredoxin and bovine serum albumin had to be denatured with 0.1% SDS before they would react with CDDP. Standard deviation for quadruplicate determinations are indicated. Assay cocktail contained 1 \times 10^{-3} M DTNB and 1 \times 10^{-5} M thiol or 4 \times 10^{-3} M CDDP and 2 \times 10^{-4} M thiol, 100 mM sodium phosphate buffer (pH=7.4), 1 mM EDTA and 0.1% SDS (for the titrations of BSA and thioredoxin).
FIGURE 35: Time course for the reaction of CDDP with low molecular weight thiols. Three graphs have been plotted in this figure: the reactions of CDDP with β-mercaptoethanol (open circles), with cysteine (open diamonds) and with glutathione (open squares). The assay cocktail contained $4 \times 10^{-6}$ M CDDP, $4 \times 10^{-6}$ M free sulfhydryl (determined by DTNB titration), 100 mM sodium phosphate buffer (pH=7.4) and 1 mM EDTA.
an initial decrease in absorbance followed by a slower increase in absorbance (see FIGURES 36 and 37). This is believed to be due to metal ion catalyzed oxidation of the thiol which causes a shift in the equilibrium from the thiol adduct to free thiol and CDDP. The reaction scheme for the thiolation and dethiolation is shown in FIGURE 38 (Mutus et al., 1989).

In contrast to the reaction of CDDP with low molecular weight non-protein thiols, the reaction of CDDP with protein thiols appears to be irreversible. Unfortunately, CDDP does not react with all protein thiols in their native state. Both BSA and thioredoxin had to be denatured before they would react with the reagent. The reaction of BSA in its native and denatured form with CDDP is shown in FIGURE 39.

The data accumulated thus far suggests that CDDP reacts reversibly with low molecular weight non-protein thiols and irreversibly with protein thiols. To further test this hypothesis, iodoacetamide, an irreversible thiol reagent, was added to the reaction of CDDP with a low molecular weight thiol and to the reaction of CDDP with a protein thiol at the apparent end point of the reactions. If the reaction of the thiol with CDDP is reversible, the predicted outcome is a shift in the equilibrium from the CDDP-thiol adduct to CDDP, which is accompanied by an increase in absorbance at 308 nm. Conversely, if the reaction is irreversible, no increase in absorbance is expected. The time course for the reaction of
FIGURE 36

Time Course for the Reaction of CDDP with Cysteine in the Absence of EDTA

FIGURE 36: Time course for the reaction of CDDP with cysteine in the absence of a metal ion chelator. The assay cocktail contained $4 \times 10^{-5}$ M CDDP, $4 \times 10^{-5}$ M cysteine (determined by DTNB titration) and 100 mM sodium phosphate buffer (pH=7.4).
FIGURE 37

Time Course for the Reaction of CDDP with Glutathione in the Absence of EDTA

FIGURE 37: Time course for the reaction of CDDP with glutathione in the absence of a metal ion chelator. The assay cocktail contained $4 \times 10^{-5}$ M CDDP, $4 \times 10^{-6}$ M glutathione (determined by DTNB titration) and 100 mM sodium phosphate buffer (pH=7.4).
FIGURE 38

Reaction Scheme for Thiolation and Dethiolation of CDDP

FIGURE 38: Reaction scheme for the thiolation (A) and dethiolation (B) of CDDP (Mutus et al., 1989).
FIGURE 39

Time Course for the Reaction of CDDP with BSA

FIGURE 39: Time course for the reaction of CDDP with BSA in the absence and presence of 0.1% SDS. Two graphs have been plotted in this figure: the time course for the CDDP titration of native BSA (closed circles) and the time course for the CDDP titration of denatured BSA (open circles). The assay cocktail contained $4 \times 10^{-9}$ M CDDP, $4 \times 10^{-6}$ M free sulfhydryl (determined by DTNB titration of BSA in the presence of 0.1% SDS), 100 mM sodium phosphate buffer and 1 mM EDTA in the presence or absence 0.1% SDS.
CDDP with BSA and glutathione are shown in FIGURES 40 and 41. As predicted, CDDP reacted irreversibly with BSA and reversibly with glutathione.
FIGURE 40
Effect of the Addition of IAm to the Reaction of CDDP with BSA

FIGURE 40: Effect of the addition of IAm to the reaction of CDDP with bovine serum albumin. At the time indicated by the arrow, 10 µL of IAm (100 µM final concentration) was added to the reference and sample cuvettes. The reference cuvette contained $3.61 \times 10^{-5} \text{ M CDDP, } 6 \times 10^{-5} \text{ M BSA (determined by DMEB titration), 100 mM Tris (pH=8.8), 0.1 mM EDTA and 0.1% SDS.}$ The reference cuvette contained $6 \times 10^{-6} \text{ M BSA, 100 mM Tris (pH=8.8), 0.1 mM EDTA and 0.1% SDS.}$
FIGURE 41

Effect of the Addition of IAm to the Reaction of CDDP with GSH

FIGURE 41: Effect of the addition of IAm to the reaction of CDDP with glutathione. At the time indicated by the arrow, 10 μL of IAm (100 μM final concentration) was added to the reference and sample cuvettes. The reference cuvette contained 3.66 x 10^{-5} M CDDP, 3.5 x 10^{-6} M glutathione (determined by DTNB titration), 100 mM tris (pH=8.8) and 0.1 mM EDTA. The reference cuvette contained 100 mM tris (pH=8.8) and 0.1 mM EDTA only.
CHAPTER 6
DISCUSSION

6.1. Purification

Bovine brain was used as the starting material for the purification of CaN since it contains the highest concentrations of the phosphatase, 600-800 mg/kg of wet brain (Krinks et al., 1985). Purification was based upon a two step procedure involving anion exchange chromatography and CaM-affinity chromatography. KCl rather than NaCl was used throughout the purification and subsequent assay procedures since it is the physiologically relevant salt. The CaN eluted from CaM-agarose was dialyzed against 40% glycerol buffer in order to concentrate the enzyme and to stabilize it during storage (Gupta et al., 1985). Purifications were performed under refrigeration (4°C) in the presence of the protease inhibitor, PMSF, in order to decrease proteolytic digestion of the enzyme.

The average yield of CaN from 1 kg of bovine brain was 15 mg, considerably less than that detected immunologically. This may be due in part to the subcellular distribution of CaN (Tallant and Cheung, 1983). Only the cytosolic fraction of CaN was purified by the method used in our laboratory (see Figure 7). Two additional procedures were attempted to purify further the protein. An attempt was made to purify monoclonal antibodies raised against CaN, but this project was terminated.
due to extenuating circumstances. Sepacryl S-200 and Sephadex G-200 were also tried in an attempt to resolve CaN from contaminating low molecular weight bands but both gels were ineffective. It is possible that these lower bands were degradation products of CaN (Manalan and Klee, 1983; Kincaid et al., 1986a; Tallant et al., 1988; Hubbard et al., 1989a).

6.2. Optimization of phosphatase activity

Assay optimization is routinely carried out as a preliminary step in the in vitro study of enzymes. The purpose of optimization is to maximize the activity in order to increase the sensitivity of the assay. Unfortunately, physiological conditions are not always used in the optimized assay. Many of these conditions however are essential to keep the enzyme active. The use of non-physiological substrates (pNPP), temperatures (30°C), buffers (tris-HCl), reducing agents (β-ME) and so forth, all define the system as artificial. In this context, the use of non-physiological pH and metal ion concentrations seems reasonable. In vitro assays are limited in that they only reflect what is actually happening within a cell.

In this study, the pH as well as the concentration of CaM and the metal ions were optimized. Optimal activity was achieved when CaM was equal to or greater than the CaN concentration (King et al., 1984b), therefore a slight excess of CaM was regularly used. The K_i for the interaction between
CaN and CaM was determined to be 0.289 nM by fitting the data from the CaM titration of CaN to the Richards-Vithayathil equation (Richards and Vithayathil, 1959). This value is in close agreement with the literature value determined by equilibrium competition experiments, \( K_d \leq 0.1 \) nM (Hubbard and Klee, 1987).

The concentration of the metal ions which gave maximal activity was also determined by titrations. A literature value for the Ca\(^{2+}\) concentration was used since titrations with Ca\(^{2+}\) did not show typical sigmoidal curvature. The lack of response of CaN to the Ca\(^{2+}\) titration and the fact that addition of EGTA to the assay mixture significantly reduced the activity of the enzyme, is indicative of Ca\(^{2+}\) contamination. Even very low concentrations of Ca\(^{2+}\) can activate CaN since the concentration of Ca\(^{2+}\) required to achieve half maximal activity is 1 \( \mu \)M (Wolff and Sved, 1985). The concentrations of Mg\(^{2+}\), Ni\(^{2+}\) and Mn\(^{2+}\) required for maximal phosphatase activity were determined to be 30 mM, 3 mM and 1 mM respectively.

The pH optimum for CaN varies depending upon the metal ion associated with the enzyme (Li and Chan, 1984). This is not unreasonable considering various metal ions are thought to cause different conformations of the enzyme (Matsui et al., 1985; Li, 1984). These conformations may require alterations in hydrogen bonding in order to accommodate the various metal ions or may expose different ionizable groups in the active
site of the enzyme. The pH optima for CaN ranges from 7.4 to 8.7 (see TABLE 3).

6.3. CaN kinetics

CaN kinetics were performed in the presence of five metal ion combinations and in the absence of metal ions. The results (TABLES 5 and 6) indicate that one or more metal ions are required for maximal activity. The highest activity is achieved in the presence of Ca\(^{2+}\), Mg\(^{2+}\) and CaM: the activity of this combination is much greater than the activity of the enzyme in the presence of Mg\(^{2+}\) alone (i.e., in the presence of EGTA and Mg\(^{2+}\)). This data supports the suggestion made by Li that CaN may be regulated in vivo by Ca\(^{2+}\)/CaM and that Mg\(^{2+}\), which does not bind to the B subunit of CaN or support CaM stimulated activation of CaN (Li and Chan, 1984; Aitken et al., 1984), is a cofactor for the reaction (Li and Chan, 1984).

The kinetic parameters from this study are comparable with those from other sources. Under optimized conditions, 50 mM tris (pH=8.7), 20 mM Mg\(^{2+}\) and 0.1 mM Ca\(^{2+}\), Li determined the \(K_s\) for CaN's hydrolysis of pNPP to be 55 mM in both the absence and presence of CaM and the \(V_{max}\) to be 3,333 and 25,000 nmol/min/mg in the absence and presence of CaM, respectively (Li, 1984). TABLE 5 lists the \(K_{app}\) from this study to be 70 mM in the absence and presence of CaM, and the \(V_{max, app}\) to be 3,345 and 11,439 nmol/min/mg in the absence and presence of
CaM, respectively. The lower $K_a$ value obtained by Li may be due to the use of a lower substrate concentration range, 2 to 20 mM pNPP as opposed to 10 to 40 mM used in this study. This substrate concentration range is closer to the $K_{a_{app}}$. Bracketing the $K_{a_{app}}$ was not possible due to the high absorbance of concentrated pNPP solutions.

In the presence of 50 mM tris (pH=7.4), 0.1 mM Ca$^{2+}$, 0.5 mM Mn$^{2+}$ and CaM, a $K_a$ of 13 mM and $V_{max}$ of 3,030 nmol/min/mg were determined (Li, 1984). These values compare favourably with a $K_{a_{app}}$ of 24 mM and a $V_{max_{app}}$ of 4,203 nmol/min/mg obtained in this study. Again, the higher $K_{a_{app}}$ in this study is probably a reflection of the higher substrate concentration used. In the absence of CaM and Ca$^{2+}$, and in the presence of 20 μM EGTA, a $K_a$ of 12.5 mM and a $V_{max}$ of 1,087 nmol/min/mg were obtained by Li. These values correspond to the $K_{a_{app}}$ of 25.5 mM and $V_{max_{app}}$ of 1004 nmol/min/mg obtained in this study in the absence of CaM and presence of 1.0 mM Ca$^{2+}$. The use of EGTA in Li's paper is questionable considering EGTA binds Mn$^{2+}$ 10-fold more strongly than it binds Ca$^{2+}$ (Dawson *et al.*, 1986).

A comparison of the Ca$^{2+}$ Ni$^{2+}$ kinetics are also available if the effect of EGTA added by Li is considered to be negligible. In the presence of 50 mM tris (pH=7.4), 0.1 mM Ca$^{2+}$ (or 20 μM EGTA) and 0.5 mM Ni$^{2+}$, a $K_a$ of 3.5 mM and 3.1 mM was determined in the absence and presence of CaM, respectively. The corresponding $V_{max}$ values were 2,057 and 7,690 nmol/min/mg in the absence and presence of CaM.
respectively (Li, 1984). The $K_{app}$ and $V_{max, app}$ values determined in this study were 2.0 mM and 5.3 mM in the absence and presence of CaM, respectively and 1955 nmol/min/mg and 3095 nmol/min/mg in the absence and presence of CaM, respectively. The substrate concentration range used was 0.5 to 10 mM pNPP.

The affinity of CaN for phosphoprotein substrates is much greater than its affinity for pNPP. The $K_a$'s for inhibitor-1 (Stewart et al., 1983), G-substrate, DARPP-32 (King et al., 1984b), casein (Chernoff et al., 1984), histone (King and Huang, 1983), myelin basic protein (Wolff and Sved, 1985) and myosin light chain (Klee et al., 1983) have all been reported to be in the µM range. This suggests that CaN’s in vivo substrate is a phosphoprotein. Whether Ca", Mg", Mn" or Ni" activates CaN in vivo is not known.

The classical model of the Ca" second messenger system is well known. The concentration of intracellular Ca" increases transiently from <10^-7 M to >10^-6 M in response to a variety of "first messengers" including noradrenaline, acetylcholine, serotonin, histamine, somatostatin, and thrombin (Tanaka, 1988). The increased Ca" concentration is responsible for the activation of CaM, a specific Ca" binding protein. Activated CaM is then capable of interacting with its target enzymes and the physiological effects take place. When the Ca" concentration in the cytosol falls, Ca" is thought to dissociate from CaM which in turn causes CaM to dissociate from its target enzymes. In this way Ca" is able
to turns on and turn off a series of metabolic processes (Rasmussen, 1989).

The intracellular concentration of Mg\(^{2+}\), in contrast to that of Ca\(^{2+}\), is relatively constant. It varies within a very narrow dynamic range, around the 1 mM level (Alvarez-Leefmans et al., 1987). Mg\(^{2+}\) is considered a static regulator of cellular function rather than a dynamic regulator, exerting fine control in metabolic activity by acting as a set point for hundreds of metabolic reactions (Alvarez-Leefmans et al., 1987). The potential role of Mg\(^{2+}\) as an intracellular regulator of metabolic activity is unclear due to lack of information regarding the levels of free Mg\(^{2+}\) in cells and whether these levels change in response to physiological conditions. There is however, a body of evidence that suggests Mg\(^{2+}\) may be involved specifically in the regulation of the Na\(^+-K^+\) pump, Ca\(^{2+}\) ATPase, adenylate cyclase (Alvarez-Leefmans et al., 1987) and the putative Na\(^+\) uniporter in mitochondria (Bernardi et al., 1990). Whether Mg\(^{2+}\) acts as a specific activator of CaN or acts in a more general capacity, fixing the set point for the enzyme has yet to be established.

The intracellular concentration of Mn\(^{2+}\) is ~10,000-fold lower than the intracellular concentration of Mg\(^{2+}\). The highest concentrations are found in hepatocytes, 0.2 to 1 µM (Schramm, 1982), while other tissues are reported to have less than 0.06 µM Mn\(^{2+}\) (Keen et al., 1984). Mn\(^{2+}\) serves as a cofactor for a number of enzymes including pyruvate
carboxylase, superoxide dismutase and arginase (Schramm, 1982). The role of Mn\textsuperscript{2+} in enzyme regulation has not yet been fully elucidated. In order for Mn\textsuperscript{2+} to regulate an enzyme, it must first bind to the enzyme and second, cause a metabolically significant response in its catalytic activity. Schramm has determined four conditions which must be met to permit interaction between Mn\textsuperscript{2+} and a target enzyme in vivo:

1) the kinetic constants for the interaction of Mn\textsuperscript{2+} must approximate the intracellular free Mn\textsuperscript{2+} concentrations at physiological substrate concentrations, 2) the binding sites must discriminate strongly against Mg\textsuperscript{2+}, 3) the amount of intracellular exchangeable Mn\textsuperscript{2+} must approximate or exceed the molarity of target enzymes required for observed metabolic flux and 4) the intracellular free Mn\textsuperscript{2+} must change in response to altered physiological or hormonal states (Schramm, 1982).

CaN has been reported to bind Mn\textsuperscript{2+} with high affinity but the dissociation constant for this interaction has not been calculated since the affinity of CaN for Mn\textsuperscript{2+} changes with time (Pallen and Wang, 1986). Therefore, it is not known whether the first criterion for an in vivo Mn\textsuperscript{2+}/enzyme interaction is met. It is also not known whether the "Mn\textsuperscript{2+} site" binds Mn\textsuperscript{2+} in preference to Mg\textsuperscript{2+} in vivo. Evidence exists which suggests the opposite. Neither Mn\textsuperscript{2+} nor Ni\textsuperscript{2+} were detected on immunoprecipitated CaN from bovine brain extracts (Rao and
Wang, 1989). In considering the third criteria, it is
doubtful whether the concentration of exchangeable Mn$^{2+}$ in the
cell exceeds the concentration of CaN in nervous tissue since
the total Mn$^{2+}$ concentration is $<0.06 \mu M$ (Freiden, 1984) and
the CaN concentration is $\sim 1 \mu M$ (Liu and Storm, 1989).
Finally, it is not known whether there is a change in the free
Mn$^{2+}$ concentration in the brain in response to altered
physiological or hormonal states. It is known however that
Mn$^{2+}$ can accumulate in the brain (via inhalation) since brain
mitochondria possess a mechanism for Mn$^{2+}$ influx but no
mechanism for clearance other than a slow Na$^+$-independent
mechanism (Gavin et al., 1990). The end result of continued
exposure to Mn$^{2+}$ is Mn$^{2+}$ toxicity or manganism therefore, it is
unlikely that the Mn$^{2+}$ concentration increases significantly
above normal.

It appears as though the requirements for the regulation
of CaN in the brain by Mn$^{2+}$ may not be met. These requirements
may however be met in the liver where the concentration of CaN
is much lower (Ingebritsen et al., 1983c) and the Mn$^{2+}$
concentration is known to decrease 3-fold in response to
fasting (Ash and Schramm, 1982).

Ni$^{2+}$ is an essential trace element found in concentrations
slightly higher than those of Mn$^{2+}$. Its concentration ranges
from 0.2 to 15.1 $\mu M$ in human fetal tissues e.g., heart, liver,
lung, bone, brain and skeletal muscle (Fallen and Wang, 1984;
Casey and Robinson, 1978). Ni$^{2+}$ is associated with a number
of enzymes including jack bean urease (Dixon et al., 1975), carbon monoxide dehydrogenase in *Acetobacterium woodii* (Ragsdale et al., 1983) and the hydrogenase found in *Rhizobium japonicum* (Arp, 1984). In addition, Ni\(^{2+}\) can activate many enzymes *in vitro*.

Ni\(^{2+}\) is not believed to serve as an *in vivo* regulator for CaN due to the high concentrations required to cause activation (Pallen and Wang, 1986) and because it is not found to be associated with CaN *in vivo* (Rao and Wang, 1989). The fact that Ni\(^{2+}\) binds to CaN with high affinity and activates CaN to a high degree suggests that a physiological role may yet be discovered.

Zn\(^{2+}\) and Fe\(^{2+}\) are endogenous metal ions associated with CaN. Their role in the enzyme has not yet been defined. They may participate in the active site of the enzyme or may serve in a structural capacity. Due to the low activity of CaN in the absence of exogenous metal ions it seems unlikely that Zn\(^{2+}\) or Fe\(^{2+}\) would function alone in phosphoester hydrolysis.

6.4. Thiol chemistry

CaN was titrated with both DTNB and CDPD (TABLE 7) under denaturing conditions and was found to contain 9.5 and 8.9 sulfhydryl groups respectively. This is in close agreement with the 10 cysteine residues determined by performic oxidation (Klee et al., 1983) and the 9 cysteine residues determined by reductive carboxymethylation under denaturing
conditions (King, 1986). DTNB titrations of the native enzyme in the absence of metal ions revealed the presence of 5 free sulfhydryl groups while titrations in the presence of Ca\textsuperscript{2+} or Ca\textsuperscript{2+} Mg\textsuperscript{2+} revealed the presence of 4 free sulfhydryl groups. The difference may be due to one of the metal ions forming a mercaptide with a free sulfhydryl, due to exposure of a sulfhydryl as a result of a conformational change in the enzyme, or it may be due to the error associated with the measurement. The conclusion drawn from these titrations is that the native enzyme does not expose all 10 of its free sulfhydryls in the native form.

The results of the titrations aimed at exploring the binding sites of CaN indicate that there are no free sulfhydryl groups in the CaM binding site. This is reasonable considering that CaM does not contain cysteine and hence does not form disulfide bonds when interacting with CaN. In contrast, the Mn\textsuperscript{2+} binding site may have a free sulfhydryl group. Titrations of CaN with Mn\textsuperscript{2+} in the active site indicate the presence of 2.3 sulfhydryl groups. Titrations of this CaN after the addition of 1 mM EDTA, which presumably removes the Mn\textsuperscript{2+} from the binding site, revealed the presence of 3.4 sulfhydryl groups. This additional sulfhydryl may be in the Mn\textsuperscript{2+} site, accounting for the high avidity of the metal ion for the site (Pallen and Wang, 1986), or it may be exposed elsewhere on the protein as a result of a conformational change in the enzyme. The active site was also titrated in
the presence and absence of a blocking group. The results indicate that there are no sulfhydryl groups in the active site, however, this must be considered in light of the problems associated with the experimental design. It is not known whether phosphate, the blocking group, was able to completely block the enzyme’s active site nor is it known whether the phosphate was removed from the active site by the gel filtration chromatography step. In addition it is doubtful that the enzyme remained in its reduced form by the time the second titration took place.

The time courses for the modification of CaN with DTNB in the presence of Ca\(^{2+}\) alone or in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) (FIGURES 26 and 28) indicated an initial increase in phosphatase activity followed by a decrease in activity. The increase in activity is thought to be the result of a conformational change which favours increased catalytic activity. It is possible that the activation is a result of the formation of a mixed disulfide in one of the transition ion binding sites mimicking mercaptide formation. The decrease in activity is thought to result from the formation of mixed disulfides which interfere with catalytic activity, perhaps in the active site but equally possible elsewhere on the protein. The rate of modification of the enzyme in the presence of Ca\(^{2+}\) alone indicates that one or more sulfhydryls are more accessible to the thiol reagent under these conditions and hence, the enzyme must exist in different
conformations under these two conditions. Observations that the deactivation of CaN is faster and more extensive in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) support this conclusion.

In addition to DTNB, both iodoacetate and iodoacetamide activate CaN (FIGURES 30 and 31). The extent of the activation is not as great as that by DTNB, nevertheless, the same pattern of activation and inhibition is observed. It is of interest to know that CaN is not the only enzyme to be activated by a thiol reagent. Glutamine synthetase has also been activated in this manner with iodoacetamide (Deuel, 1971; Nakano and Kimura, 1987).

CaN was also modified with oxidized glutathione in order to explore the possibility that CaN might be regulated in vivo by this low molecular weight non-protein thiol (Ziegler, 1985). Our results indicate that GSSG does modify the enzyme as indicated by the stabilized activity in the time course study (FIGURE 32). That GSSG can stabilize the phosphatase activity suggests CaN may exist as a mixed disulfide in vivo. The number of sulfhydryls modified by GSSG could not be determined directly, therefore attempts were made to synthesize the mixed disulfide GSSNB by the reaction of GSH and DTNB (Wong et al., 1988). This compound could have modified CaN with glutathione at the same time the release of thio-nitrobenzoate was being monitored. Unfortunately, a pure preparation of the mixed disulfide could not be attained. The physiological significance of the modification of CaN in the
brain by GSSG is questionable at best since glutathione is absent from neuronal stroma (Coles and Ketterer, 1990).

Several other investigators have modified CaN's sulfhydryl groups and observed an inhibition of enzyme activity. Phosphatase activity was inhibited with both p-hydroxymercuribenzoate (Gupta et al., 1984; Tallant and Cheung, 1984; King, 1986) and N-ethylmaleimide (Tallant and Cheung, 1984; King, 1986). King modified CaN with a number of other thiol reagents including, iodoacetate, iodoacetamide, iodosobenzoate, 5,5'-dithiobis-(2-nitrobenzoic acid) and 4,4'-dithiodipyridine, which were reported to have very little effect on the phosphatase activity. These results are based upon phosphatase assays conducted after either 15 or 30 minutes of incubation with the thiol reagent. Slight inhibition was observed with iodoacetate, iodoacetamide and DTNB while slight activation is recorded for the reaction with 4,4'-dithiodipyridine. The activation observed in our lab was seen within the first 10 minutes. The reason King did not see an activation is probably due to the timing of the assays.

King reported that the modification of CaN with N-ethylmaleimide was biphasic; an initial fast reaction followed by a second slower reaction. This second slower modification was correlated with inhibition, but the initial modification was not found to be associated with an activation. The alkylation of additional sulfhydryls by N-ethylmaleimide in the initial phase of modification, masking the activation, may
be the reason behind this. In our lab, DTNB was found to modify 1 to 3 sulfhydryls in the initial fast reaction, whereas 5 to 6 sulfhydryls were modified in King's experiment. The overall results indicate that one or more sulfhydryl groups are essential for the catalytic activity of CaN.

6.5. CDDP

CDDP, like other α,β-unsaturated ketones, reacts selectively with sulfhydryl groups. That nucleophilic addition of CDDP to a thiol is accompanied by a large decrease in absorbance at 308 nm, suggests that CDDP has the potential to be used as a sulfhydryl titrant. In order to further explore this possibility, CDDP was used to titrate a variety of protein and non-protein thiols.

The reaction of CDDP with protein thiols, oncomodulin (M, 11.7kD) (MacManus and Whitfield, 1983), glyceraldehyde-3-phosphate dehydrogenase (M, 140kD), glutathione reductase (M, 100kD), thioredoxin (M, 12.6kD) and bovine serum albumin (M, 66kD) was stoichiometric (TABLE 8), see equation 1.

\[
RSH + CDDP \rightarrow RS-CDDP
\]  (1)

Both the precision and the accuracy of the titration was similar to that of the reference method (DTNB). In contrast, the reaction of CDDP with low molecular weight thiols, cysteine, glutathione, and β-mercaptoethanol was less than
stoichiometric (TABLE 8), see equation 2.

\[ \text{RSH} + \text{CDDP} \leftrightarrow \text{RS-CDDP} \]  \hspace{1cm} (2)

This is thought to be due to the establishment of an equilibrium between free and bound thiol. CDDP reacts with each low molecular thiol to a different extent (FIGURE 35) reflecting the fraction of the deprotonated thiol in each case (Mutus et al., 1989).

In the absence of metal ion chelators (FIGURES 36 AND 37), not only is the reaction sub-stoichiometric, the equilibrium for the reaction is pulled to the left by the auto-oxidation of free thiol, see equation 3.

\[ \text{RSH} + \text{CDDP} \leftrightarrow \text{RS-CDDP} \]  \hspace{1cm} (3)

\[ \downarrow \]
\[ \text{RSSR} \]

CDDP can be used in this way to continuously monitor the oxidation of low molecular weight thiols by UV spectroscopy. In principle, this reaction can be applied to enzyme systems such as the ribonucleotide reductase assay where GSH is used as a cofactor (Holmgren, 1979).

In order to test the hypothesis that CDDP reacts irreversibly with protein thiols and reversibly with non-
protein thiols, iodoacetamide, an irreversible thiol reagent, was added to the apparent end point of the reaction of CDDP with a protein thiol and a non-protein thiol. The predicted results were a shift in the equilibrium to the left if the reaction was reversible, see equation 4,

\[
RSH + \text{CDDP} \rightleftharpoons RS\text{-CDDP} + \text{IAM}
\]

and no shift (no increase in absorbance) if the reaction was irreversible. The results of this experiment (FIGURES 40 and 41) show unequivocally that the reaction of CDDP with BSA is irreversible and that with GSH is reversible, thus substantiating the hypothesis.

There are two possible explanations for the characteristic reaction of CDDP with protein and non-protein thiols. Firstly, the base catalyzed dethiolation reaction may be more difficult for protein thiols than for low molecular weight thiols if the C₁ proton is shielded from the hydroxyl ion by the protein (FIGURE 38). This explanation is less likely when the reaction takes place with denatured protein since all side chains are expected to show normal chemistry beyond the β-carbon. The second explanation concerns the
"movement" of the double bond from the C₂₋,C₂ position to the C₃₋,C₃ position during the last step of dethiolation. In order for this to occur, C₁, C₂, and C₃ must assume an anti-periplanar conformation. This is easily achieved by low molecular weight thiols adducts, but not by protein thiol adducts, due to steric hinderance.

The net result of the CDDP work is the introduction of a novel thiol reagent which is easily prepared, water soluble, and thiol specific. This reagent can be used to label cysteine residues in proteins, quantitate the number of sulfhydryls in protein thiols and be used to continuously monitor the oxidation of low molecular weight thiols in solution.
CHAPTER 7

CONCLUSION

The results from this study suggest that CaN may be regulated \textit{in vivo} by Ca$^{2+}$ and its mediator in signal transduction, CaM. Evidence for the regulation of CaN by divalent metal ions appears to be weak. The role of Mg$^{2+}$ as a cofactor for the reaction is favoured over other divalent metal ions, including Mn$^{2+}$ and Ni$^{2+}$ for physiological reasons. Mg$^{2+}$ is more available than these ions intracellularly, and evidence exists which indicates neither of the other metal ions are bound to CaN \textit{in vivo} (Rao and Wang, 1989).

Thiol titrations of CaN with DTNB and CDPD reveal the presence of 9 or 10 free sulfhydryls in the denatured enzyme. Native CaN in contrast, exposes only 4 of these groups. Modification of CaN in the presence of Ca$^{2+}$ by DTNB (FIGURE 26) is accompanied by a sharp increase in enzymatic activity as one or more sulfhydryls are blocked, and a slow decrease in activity as the remaining sulfhydryls become modified. Similar results are observed when modification takes place in Ca$^{2+}$ and Mg$^{2+}$ (FIGURE 28) except that the decrease in activity occurs more quickly, and the modified enzyme’s activity falls below the control value. Modification of CaN with iodoacetate and iodoacetamide also cause a time dependent activation and inhibition of enzyme activity. This suggests that one or more sulfhydryl groups are essential for full expression of CaN’s
phosphatase activity. Modification of CaN with GSSG was found to stabilize enzyme activity which suggests the enzyme may exist as a mixed disulfide in vivo.

The location of the 4 sulfhydryl groups was also addressed in this study. Free sulfhydryl groups were absent from both the CaM binding site and the active site, but titrations of the Mn" site indicated the presence of a sulfhydryl group in this site or an exposure of a sulfhydryl elsewhere on the protein as a result of a conformational change resulting from the removal of the ion from its binding site.

Finally, a novel thiol reagent has been introduced in this work. CDDP, a selective sulfhydryl titrant was found to react irreversibly with protein thiols but reversibly with low molecular weight thiols. A large negative spectral change at 308 nm upon nucleophilic addition of a thiol allows the reaction to be continuously monitored spectrophotometrically.
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