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ISOLATION OF THE CALMODULIN N-METHYLTRANSFERASE AND CHARACTERIZATION OF ITS INTERACTION WITH CALMODULIN

A Dissertation
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ABSTRACT

The methyltransferase that catalyzes the trimethylation of lysine 115 in calmodulin has been purified from sheep brain. The enzyme is a monomer with an apparent molecular weight of 38,000 based on gel filtration chromatography and SDS-polyacrylamide electrophoresis. In the presence of calcium the methyltransferase exhibited a Km of 100 nM for unmethylated calmodulin and a k_{cat} of 0.0278 sec^{-1}. The enzyme was able to use calcium-depleted calmodulin as a substrate albeit with less efficiency. The methylation of calcium-depleted calmodulin was inhibited by increases in ionic strength whereas calcium-saturated calmodulin was not affected. Conversely, a calmodulin binding peptide based on the calmodulin-dependent protein kinase II sequence and the naphthalene sulfonamide W-7 inhibit the calmodulin methyltransferase/calmodulin interaction in the presence of calcium but not in the absence of calcium. This suggests a difference in the mode of interaction of calcium-saturated and calcium-depleted calmodulins with the enzyme.

The oxidation of the methionines of calmodulin by performic acid treatment decreases the ability of the methyltransferase to recognize and methylate calmodulin. This data, and the peptide and W-7 inhibitor data, suggest that the integrity of the hydrophobic core/cleft is essential for enzyme binding. Removal of the NH_2-terminal lobe (residues 1-77) does not affect the ability of the calmodulin methyl

transferase to recognize and methylate lysine-115. Thus, the determinants for calmodulin methyltransferase binding reside solely in the COOH-terminal lobe of calmodulin.

Previous work showed that mutant *Paramecium* with a calmodulin mutant with an isoleucine-to-threonine change at residue 136 showed reduced endogenous level of lysine-115 methylation (Lukas et al., 1989). In this study, a calmodulin with a val to thr-136 substitution (V136T CaM) has been generated and expressed in *E. coli* to study its regulatory properties and its kinetics as a substrate for the calmodulin-methyltransferase. The rate of V136T calmodulin methylation by the calmodulin methyltransferase was indistinguishable from wild type VU-1 calmodulin in saturating (1 mM) calcium. However, unlike wild type VU-1 calmodulin, V136T calmodulin was incapable of methylation in the absence of calcium (1 mM EGTA). Calcium binding measurements revealed a decreased calcium-binding affinity of V136T calmodulin compared to wild-type calmodulin. Lower calcium-binding affinities were also reflected by an approximate 10-fold shift in the $K_{0.5}$ values for calcium-dependent activation of NAD kinase (shifted from 1.1 to 9.1 μM Ca^{2+}) and calcium-dependence of methylation (from 0.71 to 7.2 μM Ca^{2+}).

Tyrosine-138 fluorescence spectra showed a different quantum yield between the mutant (q = 0.048) and wild-type (q = 0.037) calmodulins in the absence of calcium, suggesting a different environment of tyr-138 between the two calmodulins

in their calcium-depleted forms. This is supported by the electrophoresis profile of V136T calmodulin on nondenaturing gels. The tyrosine fluorescence spectra of the two calmodulins in the presence of saturating calmodulin were identical (q = 0.70 to 0.71), however a higher concentration of calcium was required to titrate the fluorescence spectrum of V136T calmodulin. Overall, the results suggest that the mutation in this highly conserved position in the COOH-terminal hydrophobic cleft results in a lower calcium binding affinity which results in a lower ability to activate calmodulin-dependent enzymes, and a lower ability to be recognized by the methyltransferase under physiological calcium concentrations.

To investigate further the structural requirements of the calmodulin/methyltransferase interaction, an attempt was made to engineer a methylation sequence within a symmetrical position of the amino terminal lobe. An EKL sequence was introduced at position 41-43 of the NH_2-terminal lobe (corresponding to position 114-116 of the COOH-terminal lobe) of VU-3 calmodulin which has an arginine residue at position 115. Although the NH_2-terminal lobe of the mutant calmodulin [QNP(41-43)EKL/K115R] possessed the sequence of the loop (residues 112-117, LGEKLT) that is methylated in the COOH-terminal lobe, it was not methylated by the methyltransferase. The finding suggests that other residues unique to the COOH-terminal lobe are necessary for binding and recognition by the

enzyme.

A calmodulin-DHFR sandwich fusion protein was generated by insertion of VU-1 calmodulin into the β-bulge region of DHFR to observe the effects of structurally constraining the calmodulin structure. The calcium binding properties of the sandwich protein were almost identical to VU-1 calmodulin. However, NAD kinase and PDE activator properties of the sandwich protein were lower than that of VU-1 calmodulin, and the sandwich protein was poorly methylated by the methyltransferase, particularly in the presence of trimethoprim. Overall, the data suggest that the insertion of calmodulin into the DHFR structure has little effect on calcium binding by the individual lobes of calmodulin, but may constrain the lobes in a manner that results in altered interaction with the calmodulin-dependent proteins, and severely influences recognition by the methyltransferase.

TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION....1

Properties of EF-hand proteins....2

Calmodulin: a multifunctional, ubiquitous, and highly conserved calcium effector protein....9

Structural and functional overview of calmodulin: calcium binding....13

Structural and functional overview of calmodulin: target protein binding....20

Calmodulin methylation....24

II. MATERIALS AND METHODS....28

Materials....28

Preparation of calmodulin and calmodulin fragments....29

Site-directed mutagenesis of calmodulin....32

Sandwich protein gene construction and expression....34

Purification of the calmodulin N-methyltransferase from sheep brain....38

Purification of calmodulin-dependent enzymes....40

Enzyme Assays....41

Calcium Binding....44

Spectroscopic methods....47

General methods....48

III. RESULTS....50

Purification of the calmodulin methyltransferase....50

Calcium-dependence of the interaction of calmodulin with the methyltransferase.....54
Effect of methionine oxidation of calmodulin on its interaction with the methyltransferase.....62
Inhibition of calmodulin methylation by calmodulin-binding drugs and peptides.....63
Interaction of calmodulin methyltransferase with the COOH-terminal lobe of calmodulin.....68
Production of V136T calmodulin.....77
Activator properties of V136T calmodulin.....83
Kinetics of V136T calmodulin methylation.....83
Calcium-binding properties of VU-1 and V136T calmodulin.....89
Tyrosine 138 fluorescence spectroscopy.....93
Urea unfolding studies.....96
Design of a methylation site on the NH_2-terminal lobe of calmodulin.....99
VU-1 calmodulin-DHFR sandwich protein.....103
IV. DISCUSSION.....115
The calmodulin N-methyltransferase.....115
Recognition of calcium-bound and calcium-depleted calmodulin.....122
Methionine residues in calmodulin function and methylation.....130
The methylation site is located on the COOH-terminal lobe.....133

cam2 calmodulin.....137
Calmodulin-DHFR sandwich fusion protein.....142
Summary.....144
LIST OF REFERENCES.....145
VITA.....158

LIST OF TABLES

TABLE PAGE

I. Examples of calmodulin-target proteins and their cellular functions.....10

II. Recovery table for the purification of the calmodulin N-methyl transferase.....51

III. Catalytic parameters of Calmodulin N-methyltransferase.....60

IV. Calcium binding parameters for VU-1 and V136T.....92

V. Calcium binding parameters for sandwich protein.....108

VI. Comparison of calmodulin N-methyltransferases isolated from various sources.....117

VII. Comparison of properties of calcium-bound and calcium-free calmodulin as methyltransferase substrates.....123

LIST OF FIGURES

FIGURE PAGE

1. Structural organization of the EF-hand domain.....4
2. Amino acid sequence and structural domains of VU-1 calmodulin.....12
3. Structures of apo, calcium-bound, and calcium-peptide bound calmodulins.....17
4. Sandwich protein construction and sequence.....36
5. Molecular exclusion chromatography of purified calmodulin N-methyltransferase from sheep brain.....52
6. SDS-PAGE of purified calmodulin N-methyltransferase.....53
7. Calcium and ionic strength dependence of the calmodulin N-methyltransferase interaction with calmodulin-Sepharose..55
8. Calcium dependence of the methyltransferase activity as a function of ionic strength.....56
9. Pseudo-first order kinetics of calmodulin N-methyltransferase activities.....58
10. Double-reciprocal plot of initial velocities of VU-1 calmodulin methylation.....59
11. Effect of calcium on calmodulin N-methyltransferase activity.....61
12. SDS-PAGE of normal and performic acid oxidized VU-1 calmodulins.....63
13. Analysis of the NAD kinase activation by normal and performic acid oxidized VU-1 calmodulins.....65

14. Effect of methionine oxidation on the kinetics of lysine methylation of VU-1 calmodulin.....66
15. Properties of CaM kinase II synthetic peptides as antagonists of calmodulin methylation.....68
16. Calmodulin binding peptide selectively affects the calmodulin N-methyltransferase in the presence of calcium.....69
17. The calmodulin-antagonis W-7 selectively inhibits calmodulin methylation in the presence of calcium.....70
18. HPLC fractionation of the tryptic digested VU-1 calmodulin fragments.....72
19. SDS-PAGE analysis of HPLC fractionated VU-1 calmodulin tryptic digested fragments.....73
20. Methylation of the COOH-terminal lobe (residues 78-148) of calmodulin.....75
21. Inhibition of the methylation of intact VU-1 calmodulin or the COOH-terminal fragment of VU-1 calmodulin by W-7....76
22. Inhibition of the methylation of intact VU-1 calmodulin or the COOH-terminal fragment of VU-1 calmodulin by a calmodulin-binding peptide.....78
23. Amino acid sequence of VU-1 and mutant calmodulins.....79
24. SDS-PAGE of VU-1 and V136T calmodulins.....81
25. Nondenaturing gel electrophoresis of VU-1 and V136T calmodulins expressed and purified from *E. coli*.....82
26. Activation of calmodulin dependent enzymes by VU-1 and V136T calmodulins in the presence of 1 mM $CaCl_2$.....84

27. Calcium dependence of NAD kinase activation by VU-1 and V136T calmodulins.....85
28. Comparison of the calmodulin N-methyltransferase activities with VU-1 and V136T calmoduln substrates.. ..87
29. Calcium dependence of the methylation of VU-1 and V136T calmodulins.....88
30. Calcium binding isotherms of VU-1 and V136T.....90
31. Calcium dependent changes in the fluorescence spectra of VU-1 and V136T calmodulins.....94
32. Changes in the fluorescence quantum yield of VU-1 and V136T calmodulins as a function of calcium.....95
33. Far-UV CD spectra of VU-1 and V136T calmodulins in the absence of calcium.....97
34. Urea denaturation curves of VU-1 and V136T calmodulins in the presence of 1 mM EDTA.....98
35. Overlaid backbone structures of residues 40-44 and 113-117 of calmodulin.....100
36. NAD kinase activation by VU-1, VU-3, and [QNP(41-43)EKL/K115R] mutant calmodulin.....103
37. Constructed model structure of VU-1 calmodulin-DHFR sandwich protein.....104
38. Calcium binding isotherms of VU-1 calmodulin-DHFR sandwich protein.....106
39. NAD kinase activation by VU-1 calmodulin-DHFR sandwich protein.....109

40. PDE activation by Vu-1 calmodulin DHFR sandwich protein.....110
41. Calcium dependence of the methylation of VU-1 calmodulin-DHFR sandwich protein.....111
42. Kinetics of the methylation of VU-1 calmodulin-DHFR sandwich protein.....112
43. Calmodulin N-methyltransferase activities in the presence or absence of TMP.....114
44. Binding models of the various forms of calmodulin with the methyltransferase.....128

LIST OF ABBREVIATIONS

AdoMet, S-adenosyl methionine; **^{3}H-AdoMet**, [^{3}H-methyl]-S-adenosyl methionine; **BCA**, bicinchoninic acid; **cam2**, the pantophobiac A^2 behavioral mutant of *Paramecium tetraurelia* (Lukas et al., 1989); **CHAPS**, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; **DHFR**, dihydrofolate reductase; **DTT**, dithiothreitol; **EGTA**, ethylene glycol-bis (β-aminoethylether)N,N,N',N'-tetraacetic acid; **FPLC**, fast protein liquid chromatography; **HEPES**, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); **IPTG**, isopropyl β-D-thiogalactopyranoside; **MLCK**, myosin light chain kinase; **PMSF**, phenylmethylsulfonylfluoride; **[QNP(41-43)EKL/K115R]**, VU-1 calmodulin mutant with a substitution of a glutamate-41, lysine-42, and leucine-43, and arg-115; **SAH**, S-adenosylhomocysteine; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **Tris**, tris(hydroxymethyl)aminomethane; **VU-1 calmodulin**, calmodulin derived from a cloned synthetic gene (Roberts et al. 1985); **V136T calmodulin**, mutant calmodulin with a valine to threonine substitution at position 136 of VU-1 calmodulin; **W-7**, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

CHAPTER I

INTRODUCTION

Calcium's role as a second messenger is well established in eukaryotic cells, and it is involved in the regulation of a multitude of cellular process including fertilization and development, cell growth, cell transformation, secretion, muscle and non-muscle cell contraction, sensory perception, neuronal signalling and metabolic regulation. The basis of calcium-dependent regulation lies in a large concentration gradient that exists between the cytosolic compartment, and intracellular organelles or the extracellular space. Cytosolic calcium levels are buffered to a low concentration (ca. 1×10^{-7}M), and rapidly and transiently rise to levels of 10×10^{-7}M or more in response to extracellular signals due to the opening of calcium channels (reviewed in Carafoli, 1987; Berridge & Irvine, 1989; Berridge, 1993; Clapham & Sneyd, 1995).

Numerous calcium channels are present on cellular membrane including plasma membrane channels that respond to voltage (reviewed in Bean, 1989) or ligands (reviewed in Olivera et al., 1994), as well as internal channels that respond to the release of second messengers, such as inositol phosphate, at the plasma membrane (reviewed in Berridge & Irvine, 1989; Berridge, 1993; Clapham & Sneyd, 1995). Basal calcium levels are restored by the efflux of calcium through a variety of plasma membrane or organellar pumps (reviewed in Strehler,

1991; Clapham & Sneyd, 1995), or by the binding of calcium to cytosolic "calcium buffer" proteins (reviewed in da Silva & Reinach, 1991). The net effect is that the calcium signal is carefully controlled, and sensors of calcium action need to detect changes in cytosolic calcium concentration that range from 0.1 μM in the resting state, and increase to 1-5 μM in stimulated state, against a background of 0.1 M K^+ and mM Mg^{2+}.

Properties of EF-hand proteins

A large family of calcium-binding proteins, known as the "calcium modulated" proteins (Kretsinger, 1980), have evolved and possess the structural and functional properties to sense and transduce this calcium signal. Specifically, these proteins bind messenger calcium, and undergo a conformational change resulting in their activation.

Calcium-modulated proteins are distinguished by the presence of 2 to 8 copies of a specialized calcium structure known as the EF-hand (reviewed in Kretsinger, 1980; Van Eldik et al., 1982; Strynadka & James, 1989; Moncrief et al., 1990; Nakayama and Kretsinger, 1994; Kawasaki & Kretsinger, 1994). The EF-hand was first observed in the crystal structure of parvalbumin (Moews and Kretsinger, 1975) which contains three homologous helix-loop-helix domains, designated AB, CD, and EF (from N-terminus to C-terminus). The CD and EF domains are functional calcium binding domains while the AB domain serves as a cap to stabilize the folding and packing of the C,D,E,

and F helices (Moews and Kretsinger, 1975). Based on the structural features of the EF domain, Kretsinger (1975) proposed the " EF-hand hypothesis". This hypothesis contends that proteins that bind calcium selectively with micromolar affinity contain EF-hand structures and that calcium binding modulates their activity. This basic functional and evolutionarily conserved domain has 29 amino acids arranged in a helix-loop-helix (HLH) structure, consisting of an E-helix (residues 1-10), a twelve amino acid calcium-binding loop (residues 10-21), and a F-helix (residues 19-29) (Fig. 1). Kretsinger (1975) proposed a "hand-like" orientation of these structural elements with the E-helix representing an extended index finger, the F-helix representing an extended thumb, and the curled middle finger representing the calcium binding loop.

The calcium ion is coordinated by oxygen atoms at positions 10(X), 12(Y), 14(Z), 16(-Y), 18(-X), and 21(-Z) in the calcium binding loop (reviewed in Kretsinger, 1980; Strynadka & James, 1989). The residue at position 10(X) of the loop is occupied by an invariant aspartic acid which contributes a carboxylate oxygen as a calcium ligand. The ligand at position 12(Y) is most frequently contributed by the side chain of either an aspartic acid or asparagine residue. The ligand at position 14(Z) is usually contributed by the side chain of an aspartic acid, serine or asparagine. Position 16(-Y) ligates calcium via its peptide backbone

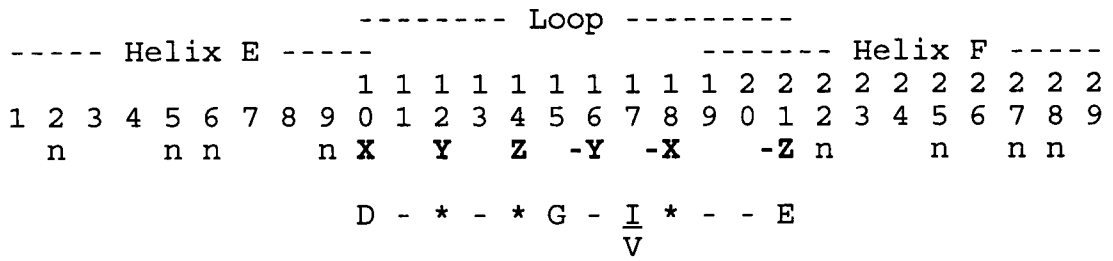


Figure 1. Structural organization of the EF-hand domain. The organization of the two amphipathic α-helices and 12 amino acid calcium binding loop of EF-hands is shown as discussed previously (Kretsinger, 1980; Kawasaki & Kretsinger, 1994). n indicates preference for a nonpolar residue which contributes to the hydrophobic faces of the E or F helix. * indicates any residue donating an oxygen atom as a calcium ligand. - denotes any amino acid. The X, Y, Z, -Y, -X, and -Z designations indicate the vertices of a pentagonal bipyramidal coordination complex that forms between the loop oxygen atoms and calcium. The -Z ligand contributes two oxygen atoms. Asp (D) at position 10, gly (G) at position 15, and glu (E) at position 21 are invariant residues. Position 17 is always ile or val.

carbonyl oxygen. Position 18(-X) is usually an aspartate, serine, or glutamate, and calcium is usually bound at this position via a bridging water molecule. Position 21(-Z) is an invariant glutamate that ligates calcium to both side-chain carboxylate oxygen atoms. In addition to these conserved ligand positions, the calcium binding loop possesses an invariant glycine at position 15 and a highly conserved aliphatic hydrophobic residue (usually isoleucine or valine) at position 17 (reviewed in Kretsinger, 1980; Strynadka and James, 1989; Kawasaki & Kretsinger, 1994). The invariant glycine is necessary to accommodate a 90° turn in the loop, whereas the hydrophobic ile or val packs into the core of the molecule, attaching the calcium binding loop. X-ray crystal structures show that the seven oxygen atoms of the calcium coordination complex form a pentagonal bipyramidal geometry with the oxygen atoms of Y, Z, -Y, and -Z vertices forming a pentagonal plane the axis of which is X and -X (reviewed in Strynadka & James, 1989; Kawasaki & Kretsinger, 1994).

The E and F helices are amphipathic, and possess hydrophobic residues on one helix face at positions 2, 5, 6 and 9 (E helix) and at positions 22, 25 and 26 (F helix) (Fig. 1). In most proteins, EF-hands exist in pairs (Kretsinger, 1980). The analysis of the X-ray crystal and NMR structures of several calcium modulated proteins, including parvalbumin (Moews and Kretsinger, 1975), troponin C (Herzberg & James, 1988; Satyshur et al., 1988), calmodulin (Babu et al., 1988;

Chattopadhyaya et al., 1992; Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995), sarcoplasm calcium-binding protein (Kumar & Cook, 1992), S100 (Sundaralingam et al., 1985), and recoverin (Flaherty et al., 1993) show that these hydrophobic residues pack against other E and F helices of a second EF-hand motif to form a hydrophobic core. In addition, a short anti-parallel β-sheet structure (three residues) exists between the two calcium-binding loops (Ikura, 1996; Babu et al., 1988; Dalgarno et al., 1984; Ikura et al., 1985). At the center of these two β-sheet segments is the invariant aliphatic hydrophobic ile or val at position 17 (Fig. 1). Over 150 EF-hand proteins/cDNAs have been sequenced and can be classified into 32 distinct subfamilies of proteins based on dendrograms constructed from sequence comparisons (Nakayama & Kretsinger, 1994; Kawasaki & Kretsinger, 1994). The complexity of the dendrograms among the subfamilies reflects a wide distribution and a broad range of functions of EF-hand proteins, some of which no longer bind calcium but have other functions (Moncrief et al., 1990; Nakayama & Kretsinger, 1994). Evolutionary relationships among EF-hand proteins do not suggest that a pattern in the gain or loss of calcium binding ability in the EF-hand domain. This suggests that EF hand mutations that produced or reduced calcium binding occurred numerous times during evolution (Moncrief et al., 1990; Nakayama & Kretsinger, 1994; Kawasaki & Kretsinger, 1994).

Another simple classification method was proposed by da Silva and Reinach (1991) based on functional characteristics of EF-hand proteins. EF-hand proteins which are responsible for the transmission of calcium signals by binding other target proteins can be classified as "calcium-sensor" proteins. Examples of these include calmodulin, troponin C, calcineurin B, myosin light chains, recoverin, S-modulin, visinin, neurocalcin, hippocalcin, frequenin, caltractin, calpain large subunit, S100 protein, and several others. Other EF-hand proteins are involved in the maintenance of a buffered intracellular calcium concentration, and can be classified as "calcium-buffer" proteins. Examples of these include parvalbumin, calbindin D_{9k}, calbindin D_{28k}, and calretinin (reviewed by da Silva and Reinach, 1991; Ikura, 1996).

Recent structural studies on calmodulin (Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995), TnC (Findlay & Sykes, 1993; Gagne et al., 1995; Slupsky & Sykes, 1995), and calbindin D_{9k} (Skelton, 1994) have revealed substantial differences in calcium-induced conformational changes among EF-hand proteins. Interestingly, most "calcium-sensors" show large conformational changes upon binding calcium, whereas most "calcium-buffers" do not (reviewed by Ikura, 1996). For example, the well known "calcium-sensor", calmodulin undergoes large structural rearrangements in its EF-hands upon binding calcium, including a substantial change in interhelical angles

exposing a hydrophobic cleft, which leads to its ability to activate enzymes (see discussion below). However, calbindin D_{9k} which is a well known "calcium-buffer" shows very small conformational changes upon binding calcium. The interhelical angles of EF-helices change less than 12° and the molecule retains the calcium-free "closed" conformation even after binding calcium (Skelton, 1994; reviewed in Ikura, 1996).

It is notable that "calcium-buffers" have generally higher calcium-binding affinity than "calcium-sensors" (reviewed in Ikura, 1996), and this can play an important role in the control of calcium signalling. Muscle relaxation, for example, occurs when calcium diffuses from the low-affinity site of troponin C to the high-affinity Ca^{2+} - Mg^{2+} sites of parvalbumin (Haiech et al., 1979). Parvalbumin has two high-affinity Ca^{2+} - Mg^{2+} sites which bind two Mg^{2+} ions in resting muscle cells (Potter et al., 1977). After stimulation, the substitution of Mg^{2+} by Ca^{2+} ions occurs more slowly than does the binding of calcium to the low-affinity, calcium-specific sites of troponin C. This could explain why calcium can trigger contraction in the presence of a high concentration of high-affinity Ca^{2+} - Mg^{2+} sites of parvalbumin in muscle cells (Haiech et al., 1979).

Even though it is hard to predict from amino acid sequence whether an EF-hand protein is a sensor or buffer, the functions are strongly coupled with the structural changes of the proteins in response to calcium-binding. Hence, calcium-

induced exposure of hydrophobic clefts, formed by a pair of EF-hands, might be critical for "calcium-sensor" functions, whereas only a small conformational change might be enough for "calcium-buffer" functions (Ikura, 1996).

Calmodulin: A multifunctional, ubiquitous, and highly conserved calcium effector protein

Calmodulin was first discovered in 1970 as a protein factor that activates cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970). This factor was found in several tissues (Cheung, 1971) and its action was shown to be calcium dependent (Kakiuchi & Yamazaki, 1970; Kakiuchi et al., 1972). These early observations are credited for the discovery of calmodulin, and the term "calmodulin" was named by Cheung (1980). Since this initial discovery, more than 30 calmodulin-stimulated enzymes and proteins have been found that regulate numerous and diverse calcium-dependent processes in eukaryotes (Table I).

Calmodulin has been found in every eukaryotic cell examined and has been isolated and sequenced from a wide variety of vertebrates, invertebrates, plants, and protozoa show a very high degree of sequence similarity (more than 90 % identity) including the presence of four EF hands (Toda et al., 1985; Roberts et al., 1986a; Cohen, 1988; Roberts & Harmon, 1992, Figure 2). The high sequence conservation of calmodulin and its ubiquitous distribution in eukaryotes suggests a fundamental role as a cellular regulator. The

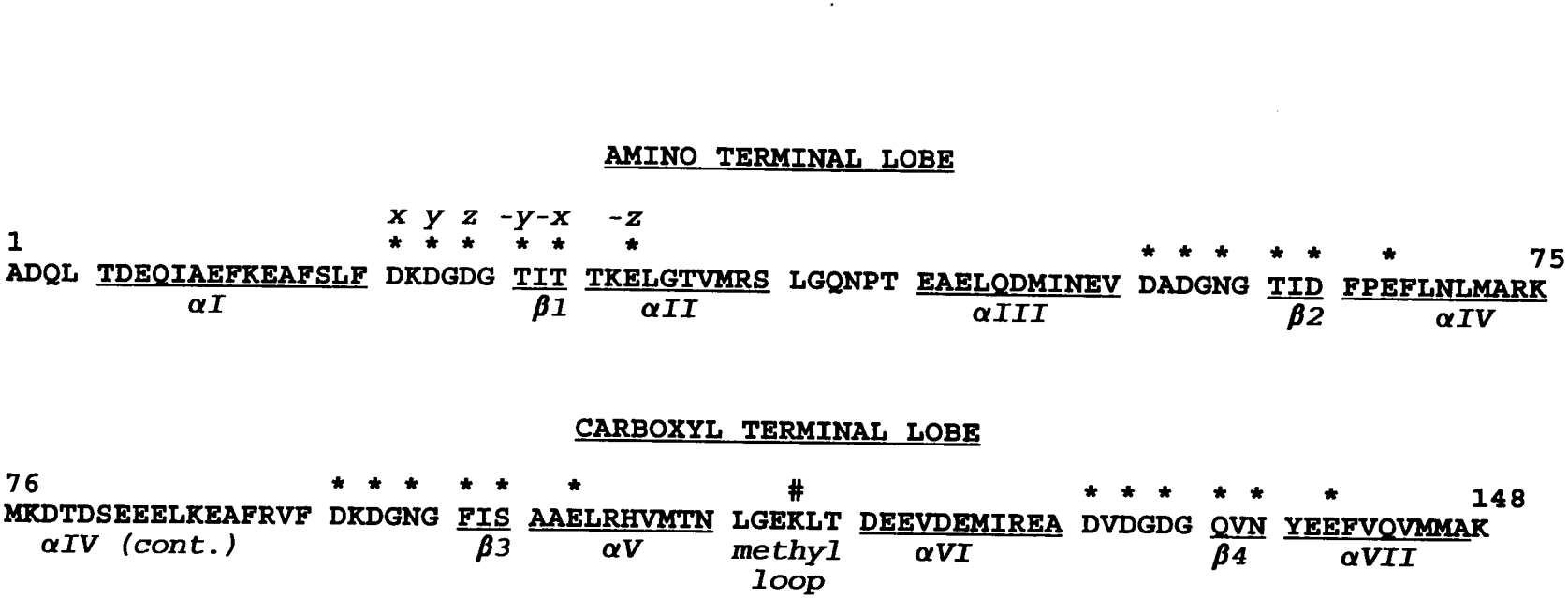
Table I. Examples of calmodulin-target proteins and their cellular functions.

Target proteins	Cellular/Biochemical Functions	Ref.
cyclic nucleotide phosphodiesterase	cyclic nucleotide hydrolysis and homeostasis	a
myosin light chain kinase (MLCK)	phosphorylation of regulatory myosin light chain/contractile regulation	b,l
CaM kinase I	multifunctional kinase, synapsin I/neuronal function	c
II	multifunctional kinase/learning and memory	d
III	elongation factor 2 phosphorylation	e
IV	multifunctional kinase/ transcriptional regulation	f
CaM kinase kinase	Activation of CaM kinase IV	g
Phosphorylase kinase	activation of phosphorylase b and glycogen breakdown	h
NO synthase	synthesis of NO signals	i
NAD kinase	conversion of NAD to NADP nicotinamide homeostasis	j
Adenylate cyclase	cAMP production from ATP cyclic nucleotide homeostasis	k
Calponin	F-actin binding, inhibition actomyosin crossbridge formation in smooth muscle	l

Table I. continued

Target proteins	Cellular/Biochemical Functions	Ref.
Caldesmon	F-actin binding, inhibition actomyosin crossbridge formation in smooth muscle	l
Calcineurin	serine/threonine phosphatase multifunctional	m
Ca^{2+}-ATPase	calcium export from cytosol calcium homeostasis	n
Neuromodulin	axonal growth	o
Neurogranin	neonatal synaptogenesis	p
Myosin I	non muscle contraction	q

a. Beavo, 1995; b. Kemp & Stull, 1990; c. Nairn & Greengard, 1987; d. Colbran & Soderling, 1990; e. Nairn et al., 1985; f. Enslen et al., 1994; g. Tokumitsu et al., 1995; h. Cohen, 1988; i. Masters, 1996; j. Roberts & Harmon, 1992; k. Shattuck & Storm, 1988; l. Allen & Walsh, 1994; m. Klee & Cohen, 1988; n. Grover & Khan, 1992; o. Apel & Storm, 1992; p. Baudier et al., 1991; q. Swanjung-Collins & Collins, 1994.



AMINO TERMINAL LOBE

```
                        x y z -y-x  -z
1                       * * *  * *   *                                  * * *  * *   *        75
ADQL TDEQIAEFKEAFSLF DKDGDG TIT TKELGTVMRS LGQNPT EAELQDMINEV DADGNG TID FPEFLNLMARK
          αI                 β1    αII                αIII              β2      αIV
```

CARBOXYL TERMINAL LOBE

```
76                  * * *  * *   *            #                  * * *  * *   *        148
MKDTDSEEELKEAFRVF DKDGNG FIS AAELRHVMTN LGEKLT DEEVDEMIREA DVDGDG QVN YEEFVQVMMAK
  αIV (cont.)             β3     αV     methyl    αVI                β4     αVII
                                         loop
```

FIGURE 2 Amino acid sequence and structural domains of VU-1 calmodulin. The amino acid sequence of VU-1 calmodulin (Roberts et al., 1985) is shown. Positions involved in calcium binding are marked with asterisks, and the *x*, *y*, *z*, *-x*, *-y*, and *-z* vertices of the calcium co-ordination complex (Kretsinger, 1980) are indicated. Residues involved in α helix or β sheet structures based on the crystal structure of calcium-saturated calmodulin (Babu et al., 1988; Chattopadhyaya et al., 1992) are underlined. Helix VI is disordered in the middle in the solution structure of calmodulin (Ikura et al., 1991) The loop between helix V and VI which contains lysine 115 is indicated, and lysine 115 is marked with #.

essential nature of calmodulin is demonstrated by the observation that disruption or deletion of the calmodulin gene results in a recessive lethal mutation in yeast (Davis et al., 1986; Takeda & Yamamoto, 1987). Below an overview of the structure and function of calmodulin is given with an emphasis on calcium binding and the interaction with target proteins

Structural and functional overview of calmodulin: calcium binding

Consistent with the presence of four EF-hands, calmodulin has four calcium binding sites that bind calcium reversibly with dissociation constants in the range of 10^{-5} - 10^{-6} M (reviewed by Klee et al., 1988; Klee & Vanaman, 1982; Linse & Forsen, 1995). Analysis of various calcium binding isotherms of calmodulin by the Adair model reveals different classes of calcium binding sites (reviewed in Klee, 1988). It was proposed that calmodulin has a low affinity pair of sites as well as a high affinity pair of sites. Analysis of tyrosine fluorescence of calmodulin provided initial information on the location of the higher affinity calcium binding sites (Kilhoffer et al., 1981). Binding of the first two equivalents of calcium results in a large increase in the fluorescence yield of tyr 138 of calmodulin (Kilhoffer et al., 1981). No further increase in the quantum yield was observed upon the binding of the next two equivalents of calcium, suggesting that the higher calcium affinity sites reside in the COOH-terminal half of calmodulin (Kilhoffer et al., 1981).

This was further confirmed by direct binding measurements to isolated tryptic fragments constituting the intact amino and carboxyl terminal lobes of calmodulin. The experiments revealed the existence of higher calcium affinity sites in the COOH-terminal lobe (residues 78-148) and lower calcium affinity sites in the NH_2-terminal lobe (residues 1-78) of calmodulin (Minowa & Yagi, 1984; Yazawa et al., 1987; Linse et al., 1991).

The two calcium binding sites within each lobe of calmodulin interact and the binding of calcium ions to each globular domain of calmodulin show positive cooperativity, (Linse et al., 1991; Minowa & Yagi, 1984; Yazawa et al., 1987; Crouch & Klee, 1980; Haiech et al., 1981, 1991; reviewed in Linse & Forsen, 1995). In addition, the calcium affinity of isolated fragments containing a single EF-hand is much lower than that of intact calmodulin (Klee, 1988). Overall, the results show that the interaction between two calcium binding loops in each domain is important for both the stabilization of calmodulin structure (Ikura et al., 1984) and for cooperative calcium binding affinity (Klee, 1988).

Previous studies showed that the calcium affinity of calmodulin is governed by several factors, including ionic strength and target protein binding. Calcium binding measurements at higher KCl and/or Mg^{2+} concentrations show a decreased affinity of calmodulin for calcium (Haiech et al., 1981, 1991; Svensson et al., 1993). In addition, under these

ionic conditions, a disappearance of the positive cooperativity between the first two sites was observed (Haiech et al., 1981, 1991). Therefore, under conditions of physiological ionic strength, both K^+ and Mg^{2+} might compete with calcium at all calcium binding sites of calmodulin, and that these ions need to be displaced in order for calcium to activate calmodulin.

Binding of a target peptides results in a higher calcium affinity and a high cooperativity of calcium-binding among all four binding sites (Olwin & Storm, 1984; Yazawa et al., 1987; Ikura et al., 1989; Haiech et al., 1991), and can even restore calcium binding activity of mutant calmodulins (Haiech et al., 1991; Findlay et al., 1995). The higher affinities of calmodulin and TnC when complexed with target proteins might be derived from energetically favorable interactions with hydrophobic residues. In calmodulin and TnC, the exposure of the hydrophobic cleft contributes to a reduced calcium affinity (reviewed in Linse and Forsen, 1995). The free energy penalty for this exposure becomes smaller if the exposed hydrophobic side chains are either replaced by polar ones (Pearlstone et al., 1986; da Silva et al., 1992) or retracted from water due to formation of a complex with target protein (Linse and Forsen, 1995).

The X-ray crystal structure of calcium-bound calmodulin has a dumbbell shape, with a central helix connecting two globular lobes (Babu et al., 1985, 1988; Kretsinger and

Weissman, 1986; Chattopadhyaya et al., 1992). Each domain has two EF-hand calcium binding motifs that form classical EF hand interactions as described above, but the two lobes do not appear to interact with each other (Fig. 3). Solution structures of calmodulin suggest that the central helix is very flexible and disordered near its midpoint (Ikura et al., 1991; Barbato et al., 1992), and it is proposed that the central linker/"helix" region of calmodulin is a flexible tether (Persechini and Kretsinger, 1988) that enables the two globular domains to come together when binding to calmodulin target proteins (Ikura et al., 1992; Meador et al., 1992, 1993) (see Fig. 3).

Several differences are apparent from a comparison of calcium-saturated and calcium-depleted calmodulin that provide insight into the nature of the conformational change and calmodulin function. In the absence of calcium, the calcium-binding loops (residues 20-31, 56-67, 93-104, and 129-140) are more flexible with the first six residues of each calcium-binding loop show poor definition by NMR measurements (Kuboniwa et al., 1995; Zhang et al., 1995; Finn et al., 1995). Repulsions between negative charges on asp and glu side chains, and a lack of hydrogen bonds, especially in the first six residues in the calcium-binding loop, might result in the high flexibility of this region (Zhang et al., 1995). Both the N- and C-terminal lobe of calmodulin have the

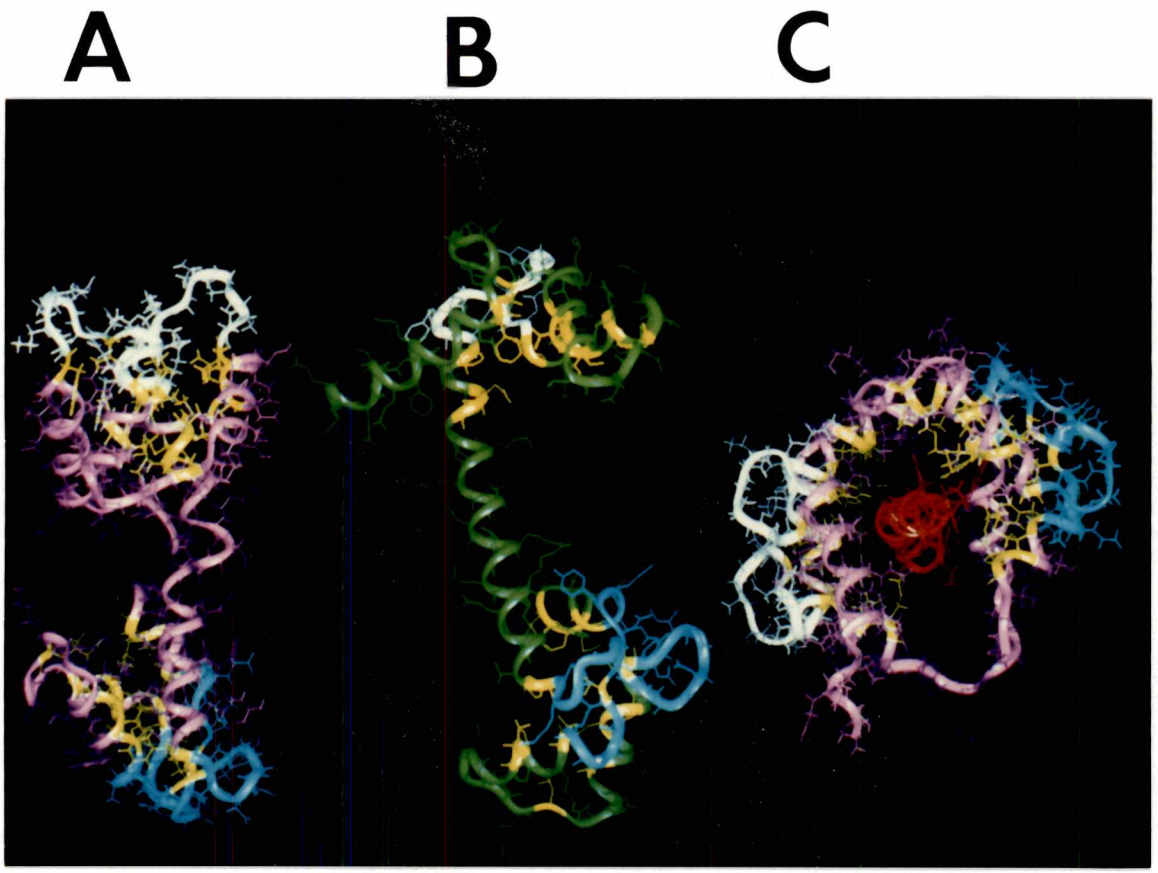


Figure 3. Structures of apo, calcium-bound, and calcium-peptide bound calmodulins. In each structure, the calcium binding loops are shown in white (N-terminal lobe) or in blue (the C-terminal lobe). Residues forming the hydrophobic core/cleft in both lobes are shown in yellow. **(A)** NMR structure of apo (calcium-depleted) calmodulin (Kuboniwa et al., 1995); **(B)** X-ray crystal structure of calcium-saturated calmodulin (Babu et al., 1988); **(C)** NMR structure of peptide bound form of calcium-saturated calmodulin (Ikura et al., 1992). The bound peptide is shown with red.

characteristic, short antiparallel β-sheet between adjacent calcium-binding loops as discussed above. In the calcium bound structure, the sheets comprise residues 26 to 28 and 62 to 64 in the NH_2-terminal lobe and residues 99 to 101 and 135 to 137 in the COOH-terminal lobe (Babu et al., 1988). The β-sheet units are preformed in the apo-structure (Dalgarno et al., 1984; Ikura et al., 1985; Finn et al., 1993), but the COOH-terminal sheet is more twisted than the NH_2-terminal sheet (Kuboniwa et al., 1995; Zhang et al., 1995).

While the calcium-free form of calmodulin possesses essentially the same secondary structure as the calcium-saturated form, the helix-packing of the calcium-free structure is quite different (Finn et al., 1993, 1995; Kuboniwa et al., 1995; Zhang et al., 1995). In the calcium-free state, the two helices in each EF-hand are more antiparallel (interhelical angles of 128° - 137°) (Kuboniwa et al., 1995; Zhang et al., 1995; Finn et al.,1995), whereas the calcium binding induces an opening of the EF-hand helices resulting in an almost perpendicular orientation (interhelical angles of 86° - 101°) (Babu et al., 1988).

One possible driving force of this helix movement is the neutralization of clustered negative charges on Asp and Glu side chains of the calcium-binding loops by the formation of electrostatic interactions with calcium ions (Linse and Forsen, 1995). The binding of calcium to the flexible loops presumably initiates structural rearrangements around the

calcium-binding sites, which allow negatively charged side chains and backbone carbonyl groups to form the bipyramidal calcium coordination complex geometry (Babu et al., 1988). This facilitates helix movement and enables calmodulin to lock into an "open state" (Zhang et al., 1995).

These concerted conformational changes through the helix pairs between adjacent EF-hand motifs should allow one EF-hand to transfer calcium-binding information to the other EF-hand. Therefore, these inter-motif helix pairings were proposed to be one of the key structural features of the cooperativity in calcium binding to the adjacent calcium binding site (Zhang et al., 1995). Comparison of the calcium-bound and calcium-depleted structures (Fig. 3A and B) show that these changes result in a transition from the "closed" to the "open" form with an exposed hydrophobic surface. This is the basis for calcium-dependent activation of calmodulin. Each exposed hydrophobic cleft is approximately 10.0 Å x 12.5 Å and has a depth of about 9.5 Å (Babu et al., 1988; Meador et al., 1992; Ikura et al., 1992), (Fig. 3B). These hydrophobic regions are formed by the R groups of 14 residues in each half of the molecule. Eleven of these R-groups are derived from the 4 EF-hand helices, two R groups are contributed by the hydrophobic amino acid residues in the calcium binding loop, and one side-chain is provided by a residue from the short linker region connecting two calcium-binding lobes (Babu et al., 1988). The hydrophobic clefts possess a large number of

methionine residues, which are unusually abundant in calmodulin. Met has a relatively long, unbranched side-chain which is more flexible than that of other hydrophobic residues and may allow calmodulin to accommodate divergent target sequences (O'Neil and DeGrado, 1990; Vogel, 1994).

Structural and functional overview of calmodulin: target protein binding

Insight into the general mechanism of how calmodulin binding regulates enzyme activity came from the elucidation of the sequence of the calmodulin-binding domains of target proteins. The first study was done by Blumenthal et al. (1985) who identified the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase (skMLCK) by limited proteolysis. A synthetic 26-residue peptide analogue of this sequence inhibited the calmodulin-dependent activation of skeletal muscle MLCK and bound calmodulin in a calcium-dependent manner with a molar stoichiometry of 1:1 (Klevit et al., 1985). Similar studies have been carried out using a variety of calmodulin-dependent enzymes and in general these structures form linear basic amphipathic α-helices that interact strongly (K_d = nM) and in a calcium-dependent manner with calmodulin (reviewed by O'Niel and Degrado, 1990; Clore et al., 1993). Calmodulin-binding domains on calmodulin-regulated enzymes are generally located within or near "autoinhibitor" or "pseudosubstrate" sequences (reviewed by Soderling, 1990; Kemp and Pearson, 1991). Calmodulin binding

to this region relieves autoinhibition and allows the activation of the enzyme activity.

The three-dimensional structure of the complex between calcium-bound calmodulin and target peptides from smooth muscle myosin light chain kinase (Meador et al., 1992) and CaM kinase II (Meador et al., 1993) had been determined by x-ray crystallography, and the structure of calmodulin complexed with the target peptide from skeletal muscle MLCK has been determined by multidimensional NMR (Ikura et al., 1992). In general both methods show the same trend (reviewed in Clore et al., 1993). Both the NH_2- and the COOH-terminal lobe of the calmodulin (residues 6-73 and 83-146) remain essentially unchanged upon complex formation, the long central helix (residues 65-93), however, is disrupted into two helices connected by a long flexible loop (residues 74-82), thereby enabling the two domains to clamp residues 3-21 of the bound peptide, which adopts a helical conformation (Meador et al., 1992; Ikura et al., 1992), (Fig. 3C). The overall structure of the complex is globular, approximating an ellipsoid of dimensions 47 by 32 by 30 Å. The complex is mainly stabilized by hydrophobic interactions which involve an unusually large number of methionines in calmodulin (Meador et al., 1992; Ikura et al., 1992) (Fig. 3C). In addition to hydrophobic interactions, electrostatic interactions between the negatively charged residues in polar rim around the hydrophobic cleft and positively charged residues of the

target proteins contribute to the binding energy (Ikura et al., 1992; Meador et al., 1992).

A comparison of calmodulin bound to two different target peptides (from calmodulin dependent protein kinase II and smooth muscle myosin light chain kinase) shows how the central helix acts in order to position the two domains optimally in the recognition of different target sequences (Meador et al., 1992; Meador et al., 1993). The central helix of calmodulin unwinds from residues 73 to 83 upon binding to the CaM kinase II peptide, whereas it unwinds from residue 73 to 77 upon binding to smooth muscle MLCK peptide (Meador et al., 1992). Therefore, the central helix of calmodulin plays a key role by acting as a "variable expansion joint", allowing different relative positioning of the lobes as different target proteins are recognized (Meador et al., 1993). This characteristic of central helix, along with the flexibility of the methionine residues in the hydrophobic clefts, could account for the ability of calmodulin to recognize several target proteins of varied sequences.

In addition, to basic amphipathic α-helices, there are other types of calmodulin-target protein interactions. Some proteins, such as the neuromodulin, neurogranin, and myosin I, can interact with calcium-free calmodulin but not calcium-bound calmodulin (Apel and Storm, 1992; Swanljung-Collins, 1994). Interestingly, these molecules share an IQ motif with a consensus sequence of IQxxxRGxxxR (Mercer et al., 1991;

Cheney & Mooseker, 1992), and it has been proposed that ionic interactions are important for the recognition of these targets (Chapman et al., 1991).

In addition to protein and peptide ligands, several biopharmacological agents such as phenothiazine derivatives (Levin & Weiss, 1977, 1979) and naphthalene-sulfonamide derivatives (e.g. W-7) (Hidaka et al., 1980) selectively bind to calmodulin in a calcium-dependent manner, and to inhibit the activation of calmodulin-dependent enzymes *in vitro*. The essential features of the structures of these calmodulin-binding drugs are a large hydrophobic region, consisting of two or three aromatic rings, and a positively charged side-chain amino group that is at least four atoms away from the aromatic ring structure (Weiss et al., 1982).

A difference Fourier map for the N-terminal half of calmodulin showed that the aromatic portion of phenothiazine fits into the hydrophobic cleft of calmodulin, and the positively charged side-chain amino group of the drug extends toward the central helix, which has negatively charged R groups in the middle (Babu et al., 1988). By using the calmodulin fragment 107-148, Dalgarno et al. (1984) showed that trifluoperazine (TFP) binding shifts the NMR resonances of methionine 109, 124, 144, and 145, as well as the resonance of phenylalanine 141. X-ray crystal structure of a calmodulin-TFP complex showed a remarkable similarity to the structure of calmodulin-peptide complexes (Cook et al., 1994).

TFP bind to calmodulin with 1:1 stoichiometry which is sufficient to bend the central helix and to juxtapose the N- and C-terminal lobe of calmodulin which is similar to that observed in calmodulin-peptide complex (Cook et al., 1994). TFP makes extensive contacts with 13 residues (10 of them are hydrophobic residues, and the other three are glu residues) in the C-terminal lobe of calmodulin but only a few contacts with glu 11 in the N-terminal lobe (Cook et al., 1994). The structure suggests that the drug binding to the C-terminal lobe is sufficient to cause the conformational changes in calmodulin and to cause the blocking calmodulin interactions with its target proteins (Cook et al., 1994).

Calmodulin methylation

Calmodulin is found to be trimethylated at lysine 115 in most eukaryotic cells (reviewed in Siegel et al., 1990; Roberts & Harmon, 1992). Based on the crystal structure of calmodulin (Babu et al., 1988; Chattopadhyaya et al., 1992) as well as NMR analyses (Zhang et al., 1994), lysine 115 is a solvent accessible residue that is found on a highly conserved six amino acid loop (LGEKLT) located between the helix V in the third calcium binding site and helix VI in the fourth calcium binding site (Fig. 1).

Even though this posttranslational modification is common among eukaryotic cells, it is not found in some organisms including *Dictyostelium discoideum* (Bazari & Clarke, 1981), *Neurospora crasa* (Cox et al., 1982), *Chlamydomonas reinhardtii*

(Van Eldik et al., 1980a; Schleicher et al., 1984), trypanosomes (Ruben et al., 1984), and crayfish (Hergenhan et al., 1984).

The biological role of lysine 115 remains a subject for debate. Studies with cell cultures show that calmodulin methylation is modulated depending upon the cell growth state (Oh & Roberts 1990; Oh et al., 1992). Further the introduction of genes encoding nonmethylatable calmodulin derivatives into transgenic plants results in severe growth phenotypes (Roberts et al., 1992). Thus, the disruption of calmodulin methylation *in vivo* leads to altered growth and development by mechanisms that have yet to be fully elucidated.

In addition, lysine 115 methylation of calmodulin affects certain *in vitro* activities. For example, unmethylated calmodulins activate NAD kinase to substantially higher activity levels compared to methylated calmodulins (Roberts et al., 1986; 1992). In addition, it has been proposed that unmethylated lysine 115 of calmodulin is a site for ubiquitin conjugation (Gregori et al., 1985, 1987). Based on this latter observation, it was proposed that both the post-translational methylation of lysine 115 and the N-terminal acetylation of calmodulin are important for protecting calmodulin from ubiquitination and ubiquitin-dependent proteolytic pathway. However, Ziegenhagen et al. (1990) observed that a mono-ubiquitination product of calmodulin

still possess 1 mole of trimethyllysine per mole of calmodulin, and proposed that ubiquitination does not necessarily occur at lysine 115, and it is not clear whether trimethylation affects calmodulin ubiquitination or not, and its *in vivo* relevance remains unknown.

The enzyme that catalyzes calmodulin methylation is a lysine N-methyltransferase that utilizes S-adenosyl methionine as a co-substrate (reviewed in Siegel et al., 1990). The *in vitro* N-methylation of calmodulin was first demonstrated by Sitaramayya et al. (1980). The enzyme has been partially purified from several sources and characterized (Oh & Roberts, 1990; Rowe et al., 1986; Morino et al., 1987; Pech & Nelson, 1994). Several observations suggest that this enzyme exclusively methylates lysine 115 of calmodulin and that it has a specific recognition site that is dictated by residues adjacent and/or distal to position 115. First, the only documented substrate for the enzyme is calmodulin, and other commonly methylated protein substrates are not recognized by the enzyme (Morino et al., 1987). Consistent with this, amino acid sequence surrounding N-methylation site of calmodulin is quite different from that of other proteins containing trimethyllysine residues (Siegel et al., 1990). Second, the levels of the enzyme and endogenous calmodulin methylation appear to be coordinately regulated in vivo (Rowe et al., 1986). Third, site-directed mutants of calmodulin in which arginine and isoleucine replace lysine-115 are no longer

methylated but are potent competitive inhibitors of the enzyme suggesting that the recognition site for the enzyme remains intact and that other lysines in calmodulin are not methylated (Oh and Roberts, 1990; Roberts et al., 1990). Overall, this suggests that this is a dedicated and highly specific enzyme that co-evolved with calmodulin with the sole function of methylating lysine-115. However, information about the structural determinants on calmodulin for recognition by the calmodulin methyltransferase remain undefined.

To increase our understanding of how the calmodulin methyltransferase recognizes and interacts with calmodulin with high specificity, we have purified the enzyme from sheep brain, and have investigated its steady state kinetic properties under conditions that affect calmodulin structure. In addition, we have produced several recombinant calmodulin derivatives from a cloned synthetic gene (Roberts et al., 1985) and have investigated their properties as substrates for the calmodulin methyltransferase, as well as its abilities to bind calcium and activate calmodulin-dependent enzymes.

CHAPTER II

MATERIALS AND METHODS

Materials

^{3}H-AdoMet, and $^{45}CaCl_2$ were purchased from Du Pont-New England Nuclear. Phenyl-Sepharose CL-4B, cyanogen bromide-activated Sepharose 4B, and Superose HR 12 were purchased from Pharmacia Fine Chemicals. Phosphocellulose P-11 was obtained from Whatman Biochemical. Chelex 100 resin and Bradford protein assay reagent were purchased from Bio-Rad. Synthetic oligonucleotides were purchased from Oligos Etc. Restriction enzymes were purchased from New England Biolabs. The sequenase version 2.0 DNA Sequencing kit was purchased from US Biochemicals. Calmodulin-dependent protein kinase II synthetic peptides were purchased from Bachem California. W-7 was purchased from Fluka Biochemicals. Fura-2 was purchased from Molecular Probes. Atomic absorption calcium and magnesium standards were purchased from J.T. Baker Inc. BCA protein assay reagents and bovine serum albumin standard were purchased from Pierce. Mono-Q HR 5/5 column was purchased from Phamacia LKB Biotechnology. A MacroDialyzer and dialysis membranes were purchased from Spectrum Medical Industries Inc. For all solutions or buffers, NANO pure (SYBRON/Barnstead) treated water was used. Sheep brains were obtained from the Food Technology Department of the University of Tennessee. Other reagents were purchased from commercial sources and were of reagent grade or better.

Preparation of calmodulin and calmodulin fragments

VU-1 calmodulin derived from a recombinant synthetic gene was expressed in *E. coli* and purified by a modification of the procedure of Roberts et al. (1985). Cells were grown at 37°C in LB media (2 L) to an A_{550} of 0.8. IPTG was added to a final concentration of 1 mM and cells were grown with shaking at 37°C overnight. Cells were lysed by lysozyme treatment as previously described (Roberts et al., 1985) and calmodulin was purified by calcium-dependent hydrophobic chromatography on phenyl-Sepharose (1.5 cm x 5 cm column) (Gopalakrishna and Anderson, 1982). Fractions containing calmodulin were pooled, were dialyzed overnight at 4°C against 4 liters of 20 mM NH_4HCO_3, and then were dialyzed extensively against deionized water. The sample was lyophilized, resuspended in 1 ml of 10 mM NH_4HCO_3, and stored at -80°C.

Preparation of performic acid-oxidized VU-1 calmodulin was done essentially as described by Van Eldik and Watterson (1981). Performic acid reagent was prepared by adding 0.5 ml of 30 % (v/v) hydrogen peroxide to 9.5 ml of formic acid and incubating the resulting solution at room temperature for 2 hrs. The reagent was chilled on ice for 30 min prior to use. VU-1 calmodulin (220 μg) was dissolved in 1 ml of formic acid and incubated on ice for 30 min. Three ml of the performic acid reagent was added and the reaction was allowed to proceed for 150 min on ice. 500 μl of 10 mM NH_4HCO_3 was added, and the mixture was dialyzed against 10 mM NH_4HCO_3 at 4° C

overnight. The sample was lyophilized and redissolved in 50 μl of 10 mM NH_4HCO_3. The final product was stored at -80° C.

A tryptic fragment (amino acid residues 78-148) that contains the COOH-terminal lobe of VU-1 calmodulin was prepared by a modification of the method of Newton et al. (1984). VU-1 calmodulin (2 mg) was incubated with 32 μg of bovine pancreas trypsin (type III, Sigma) at 30°C for 40 min in 1 ml of 50 mM NH_4HCO_3, 50 mM NaCl, and 2 mM $CaCl_2$. The digestion reaction was terminated by adding 100 μg of soybean trypsin inhibitor (Sigma). The COOH-terminal fragment of VU-1 calmodulin was purified by HPLC on a Vydac 219TP phenyl reverse phase column (0.46 cm x 15 cm) as described by Newton et al. (1984). The tryptic digest (200 μg of protein) was injected onto the column, and the column was eluted with stepwise increases in CH_3CN concentrations as follows: 15 % (v/v) CH_3CN, 85 % (v/v) buffer A (10 mM KPO_4, pH 6.25, 2 mM EGTA, 5 % CH_3CN) for 2 min, followed by a linear gradient from 15 % to 20 % CH_3CN over 20 min, followed by a second linear gradient from 20 % to 23 % CH_3CN over 10 min. The tyrosine-containing COOH-terminal fragment (residues 78-148) was distinguished from the NH_2-terminal fragment (residues 1-77) by their differential absorbance at 280 nm. Peak fractions were collected, lyophilized and resuspended in 500 μl of deionized water. The COOH-terminal fragment was purified further by FPLC on a Mono-Q column (0.5 x 5 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.2 mM EGTA, and 0.05

% (w/v) CHAPS. The COOH-terminal fragment was eluted with a linear gradient of 0- 0.5 M NaCl in equilibration buffer. The column was run at a flow rate of 0.5 ml/min and the column effluent was monitored at 280 nm. The peak fractions were collected and the purity of the protein was verified by SDS-PAGE on a 17 % (w/v) polyacrylamide gel. The fractions were pooled and dialyzed against nanopure water by using a dialysis membrane with a MW cut off of 3,500. The final product was stored at -80° C until assay.

Bovine or porcine brain calmodulin was purified via DEAE-cellulose and phenyl-Sepharose chromatography. Brains (782 g) were homogenized with 3.5 volumes of cold 50 mM Tris-HCl, pH 7.2, 1 mM β-mercaptoethanol, 2 mM EGTA, 0.3 mM PMSF. The homogenate was centrifuged at 11,000 x g for 20 minutes. The supernatant fraction was removed and stored on ice while pellets were reextracted in 1 volume of homogenization buffer and centrifuged as described above. The supernatant fraction was combined with 300 ml of DEAE-cellulose resin which was equilibrated in 20 mM Tris-HCl, pH 7.2, 0.1 mM EGTA, 35 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM imidazole (DEAE-cellulose column buffer). The suspension was mixed for 60 minutes at 4°C and the unadsorbed fraction was collected by vacuum filtration. The resin was washed with 4 L of DEAE-cellulose column buffer on a sintered glass funnel and was repacked into a column (4.5 x 20 cm). The column was washed with DEAE-cellulose column buffer containing 0.1 M NaCl until

the A_{280} of the effluent was zero. The sample was eluted with DEAE-cellulose column buffer containing 0.5 M NaCl, and $CaCl_2$ was added to final concentration of 5 mM. Calmodulin was purified from the sample by calcium-dependent hydrophobic chromatography on a phenyl-Sepharose column (2.5 cm x 6 cm) (Gopalakrishna and Anderson, 1982).

Bovine brain calmodulin-Sepharose 4B was prepared by a modification of the procedure of Klee and Krinks (1978). CNBr-activated Sepharose 4B (1 g) was swelled in 10 ml of 1 mM HCl and was washed on a sintered glass funnel with at least 50 ml of same solution. Bovine brain calmodulin (20 mg) was dissolved in 5 ml of, 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl (coupling buffer), and was mixed with the activated resin by rotating in a stoppered vessel end-over-end for 1 hr at room temperature. Excess calmodulin was washed away with 50 ml of coupling buffer. Remaining active groups were blocked by reaction with 1 M ethanolamine, pH 8.0 for 2 hrs at room temperature. The resin was washed with 50 ml of 0.1 M sodium acetate buffer pH 4.0, 0.5 M NaCl followed by washing with 50 ml of 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl for at least three cycles. The final product was stored in 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1 mM DTT, and 0.05 % (w/v) sodium azide at 4°C until use.

Site-directed mutagenesis of calmodulin

The V136T calmodulin gene was produced by cassette mutagenesis of the VU-1 calmodulin gene (Roberts et al., 1985,

1987). The calmodulin expression plasmid pVUCH-1 (Lukas et al., 1987) was digested with *Hpa*I and *Nru*I to remove a 31 bp region including the codon for val 136. After separation by electrophoresis on a 1 % (w/v) agarose gel in 50 mM Tris-acetate/EDTA buffer, pH 8.0 (Sambrook et al., 1989), the larger *Hpa*I/*Nru*I pVUCH-1 vector fragment (3.1 Kb) was purified by using a DEAE-cellulose membrane and the procedure of Dretzen et al. (1981). A cassette was formed from synthetic oligonucleotides (Oligos Etc.) as shown below and was ligated into the purified *Hpa*I/*Nru*I-digested pVUCH-1 fragment. The modified codon that encodes threonine is underlined.

5' C GAA GCT GAC GTC GAT GGT GAC GGC CAG ACT 3'

3' G CTT CGA CTG CAG CTA CCA CTG CCG GTC TGA 5'

The ligation mixture was used to transform competent *E. coli* JM101 cells. Transformants were selected from LB plates in 50 μg/ml ampicillin, and plasmids were isolated from 2 ml cultures of transformants as described by Sambrook et al. (1989). Since the V136T mutation results in the loss of *Hpa*I restriction site, mutants were identified by restriction mapping by *BamHI*/*Hpa*I or *EcoRI*/*Hpa*I digestion, followed by electrophoresis on a 8 % (w/v) polyacrylamide gel in Tris-borate/EDTA buffer (Sambrook et al., 1989). The mutant calmodulin gene was cleaved with *EcoRI* and *BamHI*, and subcloned into the same sites of the replicative form of M13mp19. Single-stranded M13mp19 templates were prepared by using the method of Schreier and Cortese (1979), and the

mutant sequences were confirmed by dideoxynucleotide DNA sequencing by using a Sequenase kit (US Biochemicals) and the manufacturer's protocol.

The [QNP(41-43)EKL/K115R] mutant calmodulin gene was produced by cassette mutagenesis of the cloned synthetic calmodulin gene, VU-3, which expresses the K115R mutant calmodulin (Roberts et al., 1986). The pVUCH-3 plasmid was digested with *Msc*I and *Pst*I to remove a 27 bp region including the coding region for residues 41-43. A cassette was formed from synthetic oligonucleotides shown below and was ligated into *Msc*I/*Pst*I-digested pVUCH-3. The modified codons that encode EKL 41-43 are underlined.

5' C GAA AAG CTT ACT GAA GCT GAA CTG CA 3'

3' G CTT TTC GAA TGA CTT CGA CTT G 5'

Transformation of E. coli JM101 and plasmid preparation were performed as described above. Restriction mapping was done by *BamHI*/*Msc*I digestion, to test for the loss of the *Msc*I site that accompanies the introduction of the mutant sequence.

Sandwich protein gene construction and expression

To insert the calmodulin gene into the β-bulge region of the chromosomal DHFR gene of *E. coli*, the VU-1 calmodulin construct was mutagenized to: 1. remove the stop codon ; 2. introduce an eight amino acid hydrophilic linker region at the COOH-terminal end; and 3. introduce *Bgl*II restriction sites at the ends of the gene to facilitate cloning into the DHFR gene.

Two steps of cassette mutagenesis were performed to

achieve these goals. First, a 73 bp cassette I was constructed with a blunt end corresponding to the *Hpa*I site of the VU-1 calmodulin gene (Roberts et al., 1985) and an extension at the 3' end which contained *Bgl*II, *Sma*I, and *BamHI* restriction sites (Fig. 4). The cassette was inserted into *Hpa*I and *BamHI* cleaved VU-1 calmodulin/pUC18 vector and was cloned into *E.coli* strain UT481 by using similar approaches to those previously described (Roberts et al., 1987). The resulting plasmid was cleaved with *EcoR*I and *Bcl*I and the cassette II was inserted into the vector to introduce a *Bgl*II site (Fig. 4) in the 5' end of the calmodulin gene. The calmodulin gene was removed as a single fragment by digestion with *Bgl*II, and was inserted into the *Bgl*II site of DHFR gene in pD136 plasmid (Ogden, unpublished). Transformation of E. coli JM109 and plasmid preparation were performed as described above. The correct orientation of calmodulin gene was determined by *Hind*III digestion.

The sandwich protein was prepared by the following procedure. Cells were grown at 37°C in 6 L of modified version of terrific broth (TB) (Tartof & Hobbs, 1987) containing 200 μg/ml of ampicillin and 10 μg/ml of TMP for 48-52 hrs. Cells were centrifuged at 6,000 x g for 10 min, were resuspended in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA (1 x TE buffer), and were lysed by sonication. The cell lysate was centrifuged at 10,000 x g for 30 min. Supernatants were adjusted to 40 % saturation by the slow addition of ammonium

A.

Cassette I:

*Hpa*I

5' AAC TAC GAA GAG TTC GTT CAG GTT ATG ATG GCT AAG GCT ACC GAT
3' TTG ATG CTT CTC AAG CAA GTC CAA TAC TAC CGA TTC CGA TGG CTA
N Y E E F V N V M M A K A T D

*Bgl*II *Sma*I *BamH*I

GAT AAG GAT AAG AAG ATC TTC CCG G 3'
CTA TTC CTA TTC TTC TAG AAG GGC CCTAG 5'
D K D K K I

Cassette II: 5' AATT**AGATCT**CT 3'
3' **TCTAGA**GACTAG 5'

B.

1 70
MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWSIGRPLPGRKNIILSSQPGTDD
71 140
RVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWEI*SDQL*
141 *210*
TDEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLNLMAR
211 *280*
KMKDTDSEEELKEAFRVFDKDGNGFISAAELRHVMTNLGEKLTDEEVDEMIREADVDGDGQVNYEEFVQV
281 *319*
MMAK-ATDDKDKK-ILFSEFHDADAQNSHSYSFEILERR
linker

Figure 4 Sandwich protein construction and sequence. A. Cassette formation. Cassette I was inserted into *Hpa*I and *BamH*I cleaved VU-1 calmodulin/pUC18 vector (Roberts et al., 1987). The resulting plasmid was cleaved with *EcoR*I and *Bcl*I and the cassette II was inserted into the vector to introduce a *Bgl*II site (shown in bold) in the 5' end of the gene. Both the 5' and 3' ends of the calmodulin gene were digested with *Bgl*II, and inserted into the *Bgl*II site of DHFR gene in the pD136 plasmid. **B. Amino acid sequence of sandwich protein.** The sequence of calmodulin is shown in italics and bold. The calcium-binding domains of calmodulin are underlined. The eight residue linker region separating the C-terminus of calmodulin and the C-terminus of DHFR is indicated.

sulfate with stirring. After an additional 40 min of stirring, the sample was centrifuged at 10,000 x g for 20 min. The resulting supernatant was adjusted to 90 % saturation with solid ammonium sulfate, was stirred for 40 min, and was centrifuged at 10,000 x g for 20 min. The pellet obtained from the 40 to 90 % saturation precipitation step was resuspended in 10 ml of 50 mM potassium phosphate, pH 6.0, 1 mM EDTA (1 x PE buffer), was dialyzed against the same buffer overnight at 4°C, and was applied to a methotrexate column equilibrated with 1 x PE buffer. The column was washed with 50 ml of 1 x PE, pH 6.0, 1 M KCl, with 50 ml of 1 x PE, pH 8.0, 1 M KCl, and then with 50 ml of 1 M sodium borate, pH 9.0, 1 M KCl. The sandwich protein was eluted with 1 M sodium borate, pH 9.0, 1 M KCl, 1 mM folate. Fractions with the greatest DHFR activity were pooled and dialyzed overnight against 1 x TE buffer. The dialyzed sample was applied to a DEAE Sephacel column. The column was washed with 200 ml of 1 x TE buffer and was eluted with a linear salt gradient from 0 to 0.5 M KCl in 1 x TE buffer. Sandwich protein was purified from the fractions which have DHFR activity by phenyl-Sepharose as described above. Fractions containing high activity of DHFR were pooled, and were precipitated and stored as a slurry in 40 % ammonium sulfate at 4°C until use. Before use, the sample was centrifused, was resuspended in 1 ml of 1 x TE buffer, and was dialyzed against the same buffer for 4 hrs.

Purification of the calmodulin N-methyltransferase from sheep brain

Ten sheep brains (856 g) were homogenized with 3.5 volumes of cold 25 mM Hepes-NaOH, pH 7.4, 4 mM β-mercaptoethanol, 0.25 M sucrose, 5 mM EGTA, 0.1 mM PMSF. The homogenate was centrifuged at 11,000 x g for 30 minutes. The supernatant fraction was removed and stored on ice while pellets were reextracted in 1 volume of homogenization buffer and centrifuged as described above. The supernatant fractions were combined and were subjected to differential ammonium sulfate fractionation (Rowe et al., 1986). Supernatants were brought to 35 % saturation with ammonium sulfate added slowly with stirring, stirred an additional 45 min, and centrifuged at 15,000 x g for 30 min. This supernatant was brought to 70 % saturation with ammonium sulfate, stirred for 45 min, and centrifuged at 15,000 x g for 30 min. The pellet obtained from the 35 to 70 % saturation precipitation step was resuspended in 25 mM Hepes-NaOH, pH 6.8, 0.1 mM EGTA, 4 mM β-mercaptoethanol, 0.1 mM PMSF, and was dialyzed against the same buffer overnight at 4°C. The dialyzed sample was centrifuged at 15,000 x g for 30 min at 4°C. The supernatant fraction was combined with 600 ml of phosphocellulose resin which was equilibrated in 25 mM Hepes-NaOH, pH 6.8, 4 mM β-mercaptoethanol, 0.01 % [w/v] Triton X-100, 0.1 mM PMSF (phosphocellulose column buffer). The suspension was mixed for 90 minutes at 4°C and the unadsorbed fraction was

collected by vacuum filtration. The resin was washed with 4 L of phosphocellulose column buffer on a sintered glass funnel and was repacked into a column (6 x 20 cm). The column was washed with phosphocellulose column buffer until the A_{280} of the effluent was zero. The enzyme was eluted with phosphocellulose column buffer containing 0.5 M NaCl. The eluent was concentrated by ultrafiltration in an Amicon stirred cell with a YM-10 membrane. The sample was dialyzed overnight against 4 L of 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.1 mM $CaCl_2$, 0.05 % (w/v) CHAPS, 4 mM β-mercaptoethanol, 0.1 mM PMSF (calmodulin-Sepharose buffer). CHAPS was included in all buffers at this point since it was found to stabilize and increase the recovery of enzyme activity during these later purification steps. The sample was applied to a bovine calmodulin-Sepharose column (1.5 x 4 cm), and the column was washed with 100 ml of calmodulin-Sepharose buffer containing 2 mM DTT instead of β-mercaptoethanol. The column was washed with 100 ml of calmodulin-Sepharose buffer containing 0.6 M NaCl, and was eluted with 20 mM Tris-HCl, pH 7.4, 2 mM DTT, 1 mM EGTA, 1 M NaCl, 0.05 % (w/v) CHAPS. The fractions with calmodulin methyltransferase activity were pooled and concentrated by ultrafiltration on a Centricon-30 unit (Amicon).

The calmodulin-Sepharose purified-enzyme was purified further by molecular exclusion FPLC on a Superose HR 12 column (1 x 30 cm) in 25 mM Hepes-NaOH (pH 7.5), 2 mM DTT, 0.2 M

NaCl, 0.05 % (w/v) CHAPS. Fractions that tested positive for calmodulin methyltransferase activity were pooled and were stored at -80°C. The enzyme activity was stable for several months stored in this manner.

Purification of calmodulin-dependent enzymes

Pea NAD kinase was purified through the polyethyleneglycol (PEG) step by using the procedure of Roberts et al. (1985). After this step, calmodulin was removed by a modified DEAE-ion exchange chromatography step. After PEG precipitation, the enzyme was resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, and was centrifuged for 15 min at 27,000 x g at 4°C to remove insoluble material. The supernatant was applied to a DEAE-Sephadex A-50 column (2.5 x 6 cm), and the column was washed with 50 mM Tris-HCl, pH 7.0, 100 mM KCl, 3 mM $MgCl_2$, 10 % (v/v) glycerol, 0.1 mM $CaCl_2$, until the A_{280} was less than 0.02. Calmodulin-dependent NAD kinase was eluted with 50 mM Tris-HCl (pH 7.0), 100 mM KCl, 3 mM $MgCl_2$, 10 % (v/v) glycerol, 1 mM EGTA. Active fractions were pooled and stored at -80°C.

Calmodulin-dependent cyclic nucleotide phosphodiesterase was prepared from sheep brain by a modification of the protocol of Sharma et al.(1980). After being partially thawed at room temperature, sheep brains (200 g) were homogenized with 2 volumes of cold 100 mM Tris-HCl, pH 7.5, 2 mM EDTA (homogenation buffer). The homogenate was squeezed through

cheesecloth and the filtrate was centrifuged at 10,000 x g for 20 minutes at 4°C. Pellets were reextracted in 1 volume of homogenization buffer and the filtrate was centrifuged as described above. The supernatant fractions were combined, and β-mercaptoethanol and EGTA were added to a final concentration of 10 mM and 0.1 mM respectively. The sample was applied to a DEAE-cellulose column (3 x 30 cm) which was pre-equilibrated with 20 mM Tris-HCl, pH 7.0, 1 mM imidazole, 1 mM Mg Acetate, 0.1 mM EGTA, 10 mM β-mercaptoethanol, and 0.05 M NaCl (DEAE column buffer). After application, the column was washed with 200 ml of DEAE column buffer, and then was eluted with a linear salt gradient from 0.05 to 0.5 M NaCl. Fractions containing high activities of calmodulin-dependent cyclic nucleotide phosphodiesterase were pooled and were dialyzed at 4°C against 10 mM Tris-HCl, pH 7.5, 0.1 mM β-mercaptoethanol, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ overnight. The dialyzed sample was lyophilized, and stored at -80°C. Lyophilized powder was reconstituted in nanopure water prior to assay.

Enzyme Assays

Methyltransferase activity was assayed by a modification of the general protocol of Rowe et al. (1983) as described by Oh and Roberts (1990) and Han et al. (1993). The standard reaction mixture (100 μl) contained VU-1 calmodulin (1.67 μg) and 12.0 μM [^{3}H]AdoMet (1.5 μCi) in 0.1 M glycylglycine-NaOH, pH 8.0, 0.15 M KCl, 2 mM $MgCl_2$, 5 mM DTT, 0.01% (w/v) Triton X-100, 0.1 mM $CaCl_2$. The reaction was initiated by the

addition of enzyme and incubation was typically done for 20 min at 37°C. The reactions were terminated by heating at 90°C for 3 min. The samples were microfuged for 6 min and the supernatants were combined with 200 μl of a 1:1 phenyl-Sepharose slurry equilibrated in 50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 0.1 mM $CaCl_2$. The mixture was vortexed and microfuged. The collected resin was washed with 2 ml of same buffer. [^{3}H]methyl-labeled calmodulin was eluted with 0.1 M NH_4HCO_3, pH 8.0, 2 mM EDTA and radioactivity was measured by liquid scintillation counting in a Beckman LS 3801 scintillation counter.

A modified protocol was used for the assay of the calmodulin methyltransferase in the presence of calmodulin-binding peptides. Assays were performed under the standard calmodulin methyltransferase assay conditions in the presence of 0.1 mM $CaCl_2$ and 1 μM VU-1 calmodulin. After termination of the reaction, 10 μl of 10 mM EGTA was added and the peptide was removed by adsorption to 40 μl of phosphocellulose. The unadsorbed fraction, which contains calmodulin, was then analyzed by the standard phenyl-Sepharose method as described above.

The analysis of kinetic data and determination of kinetic parameters for the methyltransferase were done by using the form of the Michaelis-Menten equation for sequential bireactant systems as described previously (Oh, 1992).

$$V = \frac{V_{max}\ [A]\ [B]}{[A]\ K_{mB} + [B]\ K_{mA} + [A]\ [B]} \qquad \text{[Eq. 1]}$$

Where A and B are the varied substrates, K_{mB} and K_{mA} are the Michaelis-Menten constants for substrates A and B respectively.

For determinations of the dependence of the enzyme activity on calcium, the assay buffer was modified to contain chelex-treated 0.1 M glycylglycine, pH 8.0, 0.15 M KCl, and varying concentrations of EGTA, $CaCl_2$ and $MgCl_2$. A computer program (Perrin and Sayce, 1967) was used to determine the amounts of EGTA, $CaCl_2$, and $MgCl_2$ required to yield 2 mM free Mg^{2+} and a free Ca^{2+} concentration that varied from 10^{-8} to 10^{-3} M. Free calcium concentrations (from 10^{-8} to 10^{-6} M) in reactions were verified and quantified with fura-2 (Molecular Probes) as described by Tsien et al. (1985). The fluorescence intensities at an emission wavelength 510 nm were measured from excitation wavelengths ranging from 280 to 450 nm. These excitation scans could be used to calculate free calcium in each solution with the standard calibrating equation (Eq. 2).

$$[Ca^{2+}] = \frac{K_d * B * (R - R_{min})}{(R_{max} - R)} \qquad \text{[Eq. 2]}$$

where R indicates the ratio of intensities measured at excitation wavelength of 340 and 380 nm. R_{max} and R_{min} are the 340/380 ratios obtained for the same solutions where fura-2 is fully Ca^{2+}-saturated and Ca^{2+}-free. K_d is the dissociation

constant of fura-2 for calcium (224 nM). B is the ratio of fluorescence for 380 nm excitation of fura-2/Ca^{2+}-fura-2. All buffer and reagent stocks were decalcified by treatment with Chelex-100 resin (Crouch & Klee, 1980).

Pea NAD kinase activity was assayed as described by Roberts et al. (1985). Assays for the calcium dependence of NAD kinase activation were done in 50 mM Hepes-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM EGTA, 3 mM ATP, 2 mM NAD, 0.1 μM calmodulin, and various $CaCl_2$ concentrations that yielded a free Ca^{2+} ranging from 10^{-8} to 10^{-3} M as determined by the approach described above. Phosphodiesterase assays were done as described previously (Sharma et al., 1979).

Calcium Binding

All buffers were decalcified by treatment with Chelex 100 resin (Biorad) as described previously (Crouch and Klee, 1980). Free calcium concentrations in solution was quantified with a Variant AA-1475 atomic absorption spectrophotometer by using a hollow calcium lamp (Fisher Scientific Co.). Thermal settings were: dry at 120°C for 30 s, char at 1100°C for 20 s, and atomize at 2500°C for 5 s. Standard curves were made by reading serial dilutions of an atomic absorption calcium standard. Both samples and standards were diluted with decalcified deionized water prepared as described above.

Prior to calcium binding analyses, calcium was removed from calmodulin by precipitation with trichloroacetic acid as described by Haiech et al. (1981). After the last

precipitation step in this protocol, the pellet was briefly washed with Chelex-treated water, and was resuspended in 1 ml of 100 mM Hepes-NaOH, pH 7.5, 0.1 M KCl. Calcium stock solutions were standardized by atomic absorption spectrophotometry as described above.

Calcium-binding studies were done by the flow dialysis approach of Colowick and Womack (1969) as adapted for calmodulin (Haiech et al., 1980; Porumb, 1994). All experiments were performed with a Macrodialyzer apparatus (Spectra), with a dialysis membrane (Mr cutoff = 3,500, Spectra/Por* 6) separating the sample (upper) and perfusion (lower) chambers. The upper chamber (1 ml) contained 30 μM calmodulin, 50 mM Hepes-NaOH (pH 7.5), 0.1 M KCl, 2 mM $MgCl_2$. The lower chamber was perfused with 50 mM Hepes-NaOH, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$ at a flow rate of 3 ml/min. With this flow rate it was determined that the release of ligand into the lower chamber reached a steady state after 2 min. The titration was initiated by the addition 5 μCi $^{45}CaCl_2$ (370 Mbq/ml) to the calmodulin sample in the upper chamber. Titration was performed by addition of 4 μl aliquots of standardized $CaCl_2$ stock solutions to the upper chamber followed by the collection of four 1.5 ml fractions. The radioactivity in each fraction was determined by liquid scintillation counting. The remaining $^{45}Ca^{2+}$ in the upper chamber was chased out with 10 μl of 1 M $CaCl_2$ after the 24th cycle of titration. By the end of the titration, less than 10

% of the total starting $^{45}Ca^{2+}$ was lost from the upper chamber. Calcium binding calculations were corrected for this loss as described by Porumb (1994).

Calculation of free and bound ligand concentrations was done as described by Colowick and Womack (1969). When excess unlabeled calcium is added at the end of titration, the radioactivity in the effluent is taken to be that corresponding to 100 % of free calcium. Therefore, dividing the cpm value of each calcium addition by this maximum cpm value of final calcium addition gives the fraction of the free calcium at each titration step. Each data set was fit to the Adair equation (Eq. 3) as described by Haiech et al. (1981).

$$r = \frac{(K_1x + 2K_1K_2x^2 + 3K_1K_2K_3x^3 + 4K_1K_2K_3K_4x^4)}{(1 + K_1x + K_1K_2x^2 + K_1K_2K_3x^3 + K_1K_2K_3K_4x^4)} \qquad \text{[Eq. 3]}$$

r is the molar ratio of Ca^{2+} bound to calmodulin at a free Ca^{2+} concentration of x, and K_1, K_2, K_3, and K_4 are the apparent macroscopic binding constants calculated from the equation by using the Scientist program (Micromath Scientific Software). The Hill constants were obtained by fitting the calcium binding curves of calmodulin to the Hill equation (Dahlquist, 1979):

$$r = \frac{(4[Ca^{2+}]^{\alpha})}{(K^{\alpha} + [Ca^{2+}]^{\alpha})} \qquad \text{[Eq. 4]}$$

α is the Hill constant, and K is the overall dissociation constant (mol/liter).

Spectroscopic methods

Fluorescence emission spectra were measured on a Perkin-Elmer LS 50B Luminescence spectrometer. The excitation wavelength was set at 275 nm to excite the single tyrosine 138 residue, and the emission spectrum between 285 and 450 nm was recorded. All measurements of fluorescence emission were made at 22°C. Prior to analysis, calmodulin was depleted of calcium by TCA precipitation by the method of Haiech et al. (1981) as described above. All fluorescence measurements were made in Chelex-treated, 25 mM Hepes-NaOH, pH 7.5. Calcium titrations were made by addition of standardized $CaCl_2$ directly to samples in the fluorescence cuvette. Quantum yields (q) were determined as previously described (Gerard et al., 1975) by taking L-tyrosine as a reference (q = 0.14 at 20°C).

CD spectra of calmodulin samples were recorded from 260 to 200 nm with a JASCO J-720 spectropolarimeter with a cuvette pathlength of 0.2 cm. Protein samples (0.166 mg/ml) were prepared in 20 mM Hepes-NaOH, pH 7.5 in the presence of 1 mM EGTA or 1 mM $CaCl_2$. The CD spectral output (millidegrees) was converted to mean residue ellipticity ($[\theta]_{MRE}$) based on a mean residue weight of 112.7 for calmodulin (Martin and Bayley, 1986).

CD spectroscopy was also used to monitor urea-induced unfolding of calmodulin as described by Martin and Bayley (1986). Urea stocks (10 M) for denaturation were prepared

fresh in 10 mM Tris HCl, pH 7.0 and 1 mM EDTA, and were standardized by refractometry (Pace et al., 1990). Various standard amounts of urea were added to calmodulin (5 μM) in 10 mM Tris HCl, pH 7.0, 1 mM EDTA, and samples were incubated at 22°C for an hour prior to recording the CD spectra. Protein unfolding was monitored by plotting the urea concentration against the change in the intensity of the CD signal at 222 nm. The Gibbs free energy change between folded and unfolded calmodulin (ΔG_{H2O}) was calculated by fitting the resulting data to the following equation (Santoro and Bolen, 1988):

$$\theta_{obs} = \{(\theta^{\circ}_{N} + M_{N}[urea]) + (\theta^{\circ}_{U} + M_{U}[urea]) \times \exp[-1(\Delta G_{H2O} + M_{G}[urea])/RT]\}/\{1 + \exp[-1(\Delta G_{H2O} + M_{G}[urea])/RT]\} \quad \text{[Eq. 5]}$$

θ_{obs} is the CD signal at a given urea concentration, θ°_{U} is the CD signal of completely unfolded calmodulin, θ°_{N} is the CD signal of native calmodulin, M_G is the slope describing the primary change in the CD signal as a function of denaturant, M_U and M_N are the slopes of the post and pretransition parts of the curve, R is the gas constant, T is the temperature.

General methods

For estimations of the native molecular weight of the calmodulin methyltransferase, the purified enzyme was mixed with gel filtration protein standards (Bio-Rad), and was applied to a Superose HR 12 column (1.0 x 30 cm) using a flow rate of 0.5 ml/min. Fractions (0.25 ml) were collected and assayed under standard assay conditions as described above.

To determine molecular weight, a plot of K_{av} vs log MW of gel filtration protein standards was generated by using eq. 6.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \qquad \text{[Eq. 6]}$$

V_o is the void volume, V_e is the elution volume of the highest enzyme activity fraction, and V_t is the total volume of the column.

SDS-polyacrylamide gel electrophoresis was done by using the method of Laemmli (1970). Nondenaturing polyacrylamide gel electrophoresis of calmodulin was performed on 15 % (w/v) polyacrylamide gels in 50 mM Tris-boric acid, pH 7.5 in the presence of either 1 mM $CaCl_2$ or 1 mM EGTA (Burgess-Cassler et al., 1987). Proteins were stained with Coomassie Brilliant Blue G-250. Protein concentrations were determined by the method of Bradford (1976) or by the bicinchoninic acid method of Smith et al. (1985) using bovine serum albumin as the standard. Amino acid composition analysis of purified calmodulins was done on acid hydrolysates by Pico-tag analysis (Bidlingmeyer et al., 1984) as described previously (Roberts et al., 1985).

CHAPTER III

RESULTS

Purification of the calmodulin methyltransferase

To initiate our studies of the calmodulin methyltransferase, we first devised a modified protocol to purify the sheep brain calmodulin methyltransferase to homogeneity. The enzyme was purified by homogenation, ammonium sulfate fractionation (35 -70 % saturation), and anion-exchange chromatography on phosphocellulose. At this point, calcium-dependent calmodulin-Sepharose chromatography was employed as an affinity step. As shown in Table II, this resulted in a high fold purification of the enzyme. Final purification was achieved by FPLC molecular exclusion chromatography on Superose HR 12.

By this protocol, the enzyme was purified over 12,000-fold and possessed a final specific activity of 13,700 pmoles methyl groups incorporated/min/mg protein (Table II). The purified enzyme elutes from the Superose column with an apparent molecular weight of 38,000 (Fig. 5), and exhibits a single major band with an apparent mol. wt. of 38,000 based on SDS-polyacrylamide electrophoresis (Fig. 6). The data suggest that the sheep brain calmodulin methyltransferase is a monomer of 38,000 mol.wt. This preparation was used for the experiments pursued in the present study.

Table II. Recovery table for the purification of the calmodulin N-methyl transferase

Step	Total[a] Protein	Total Activity	% Recovery	Specific Activity	Fold Purification
crude extract	77,800	86.3	100	1.11	1
$(NH_4)_2SO_4$ ppt.	14,200	26.1	30.3	1.84	1.72
phospho-cellulose	2,810	24.2	28.1	8.63	7.78
calmodulin Sepharose	0.75	4.11	4.7	5,480	4,950
Superose HR 12	0.038	0.52	0.6	13,700	12,400

[a] Units are: total protein, mg; total activity, nmole/min; and specific activity, pmole/min/mg.

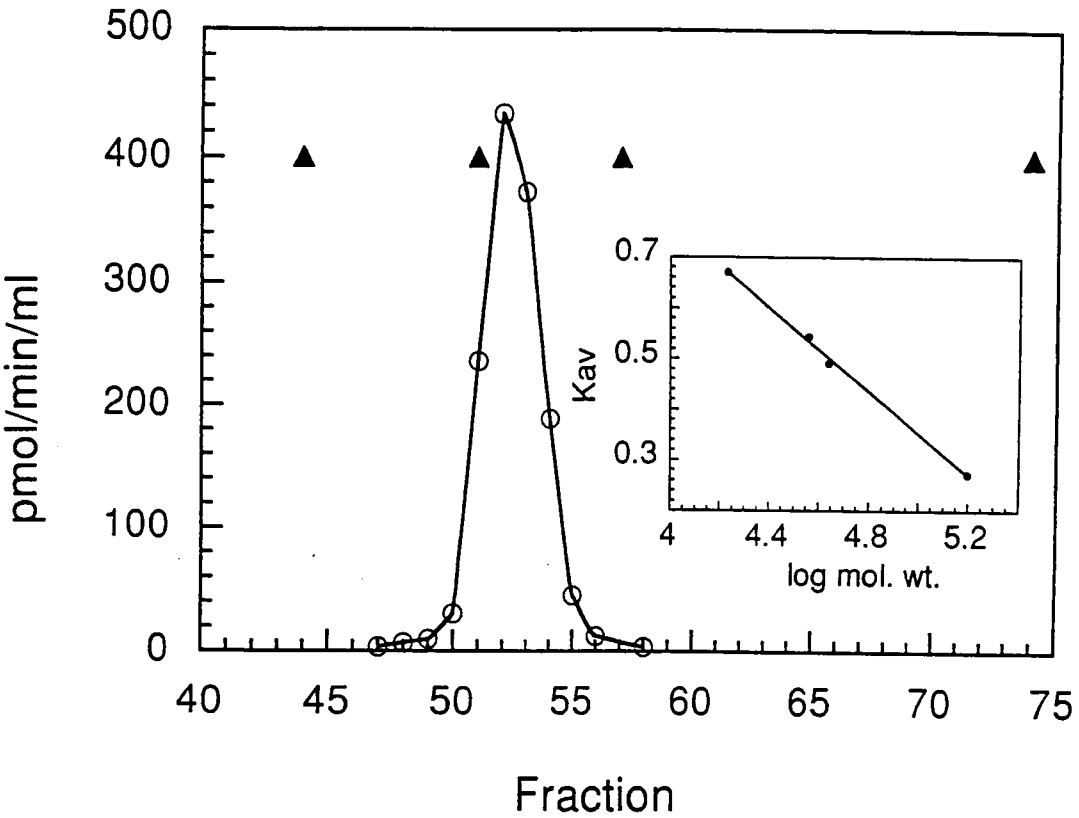


Figure 5. Molecular exclusion chromatography of purified calmodulin N-methyltransferase from sheep brain. Purified calmodulin N-methyltransferase was subjected to FPLC chromatography on a Superose HR 12 column (1.0 x 30 cm). Fractions (0.25 ml) were collected at a flow rate of 0.2 ml/min. Filled triangles represent the peak elution positions of gel filtration standards: 158,000 γ-globin, 44,000 ovalbumin, 17,000 myoglobin, 1,350 cyanocobalamin. Unfilled circles represent the calmodulin methyltransferase activity (pmole/min/ml) of each fraction. The inset shows the plot of K_{av} vs. log MW.

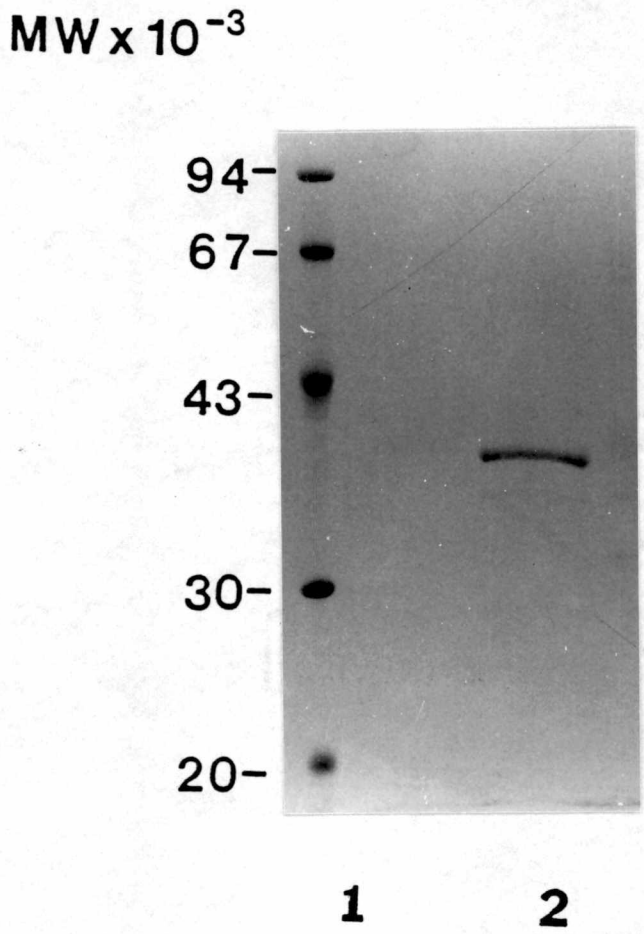


Figure 6. SDS-PAGE of purified calmodulin N-methyltransferase. Calmodulin N-methyltransferase purified through the Superose step was analyzed by SDS-PAGE on a 12.5% (w/v) polyacrylamide gel. Proteins were visualized by staining with Coomassie Blue. **Lane 1**, molecular weight standards; **Lane 2**, purified calmodulin N-methyltransferase.

Calcium-dependence of the interaction of calmodulin with the methyltransferase

To investigate the influence of calcium on the interaction of calmodulin with the methyltransferase, affinity chromatography of the enzyme on calmodulin-Sepharose was done under various conditions (Fig. 7). In a low ionic strength buffer (Fig. 7A), the enzyme was bound to the resin in both the presence of calcium or EGTA suggesting a calcium-independent interaction. However, when chromatography was conducted at a higher salt concentration (Fig. 7B), the enzyme was bound by calmodulin-Sepharose in a calcium-dependent fashion, and could be quantitatively eluted from the resin by the addition of EGTA. The calcium-dependent interaction of the calmodulin N- methyltransferase with calmodulin as a function of ionic strength is also manifested in activity measurements. In the presence of 100 μM $CaCl_2$ the enzyme showed little sensitivity to the change of KCl concentration (Fig. 8). However, enzyme activity was decreased in an ionic strength-dependent manner in the presence of 1 mM EGTA (Fig. 8). These data support the affinity chromatography results and suggest that the interaction of the enzyme with calmodulin shows a calcium-dependence that is a function of ionic strength. For kinetic analyses we adopted a standard concentration of ions (0.15 M KCl and 5 mM $MgCl_2$) that approximated physiological conditions.

Pseudo first order plots of calmodulin concentration vs.

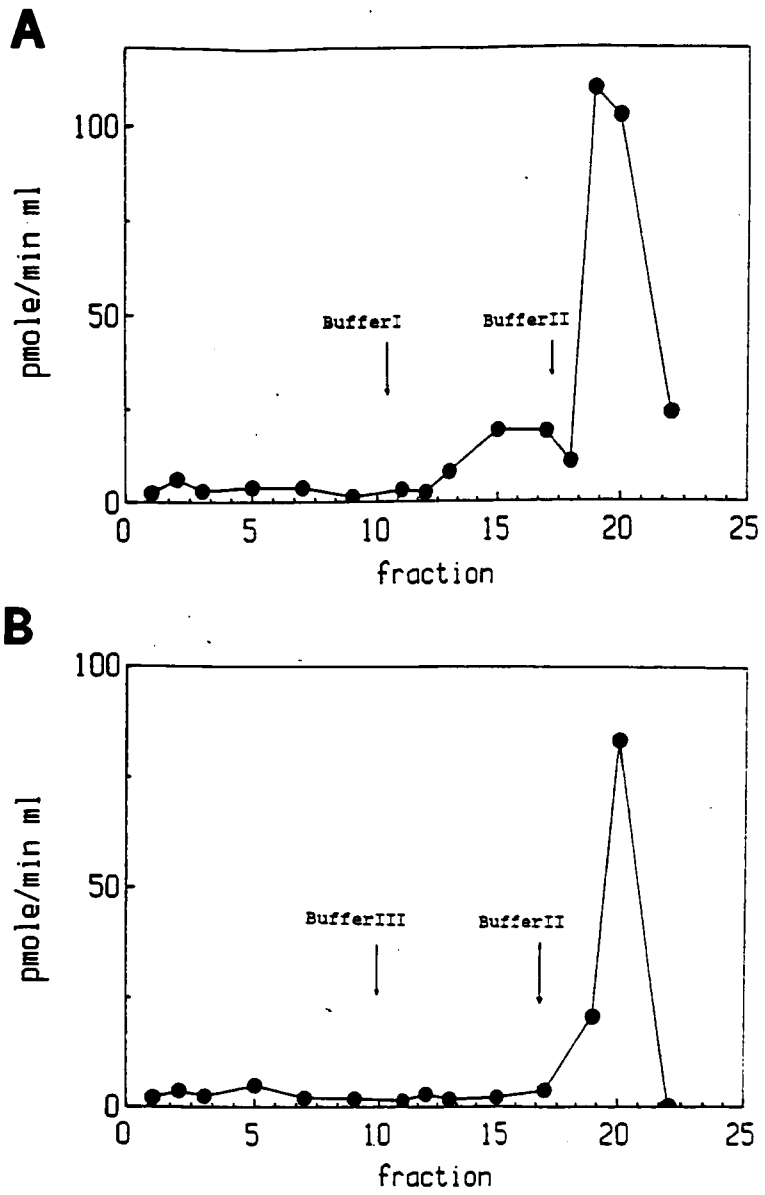


Figure 7. Calcium and ionic strength dependence of the calmodulin N-methyltransferase interaction with calmodulin-Sepharose. Calmodulin-Sepharose columns (0.74 x 2.3 cm) were equilibrated with 20 mM Tris-HCl, 0.1 mM $CaCl_2$ (pH 7.4). Samples (225 μg of protein, specific activity of 5,480 pmol/min/mg) were applied. The columns were washed with 10 ml of equilibration buffer and were eluted under the following conditions. One ml fractions were collected. **A. Buffer I**, 20 mM Tris-HCl, 1 mM EGTA (pH 7.4); **Buffer II**, 20 mM Tris-HCl, 1 mM EGTA, and 0.6 M NaCl (pH 7.4). **B. Buffer III**, 20 mM Tris-HCl, 0.6 M NaCl, and 0.1 mM $CaCl_2$ (pH 7.4); **Buffer II**, 20 mM Tris-HCl, 1 mM EGTA, and 0.6 M NaCl (pH 7.4).

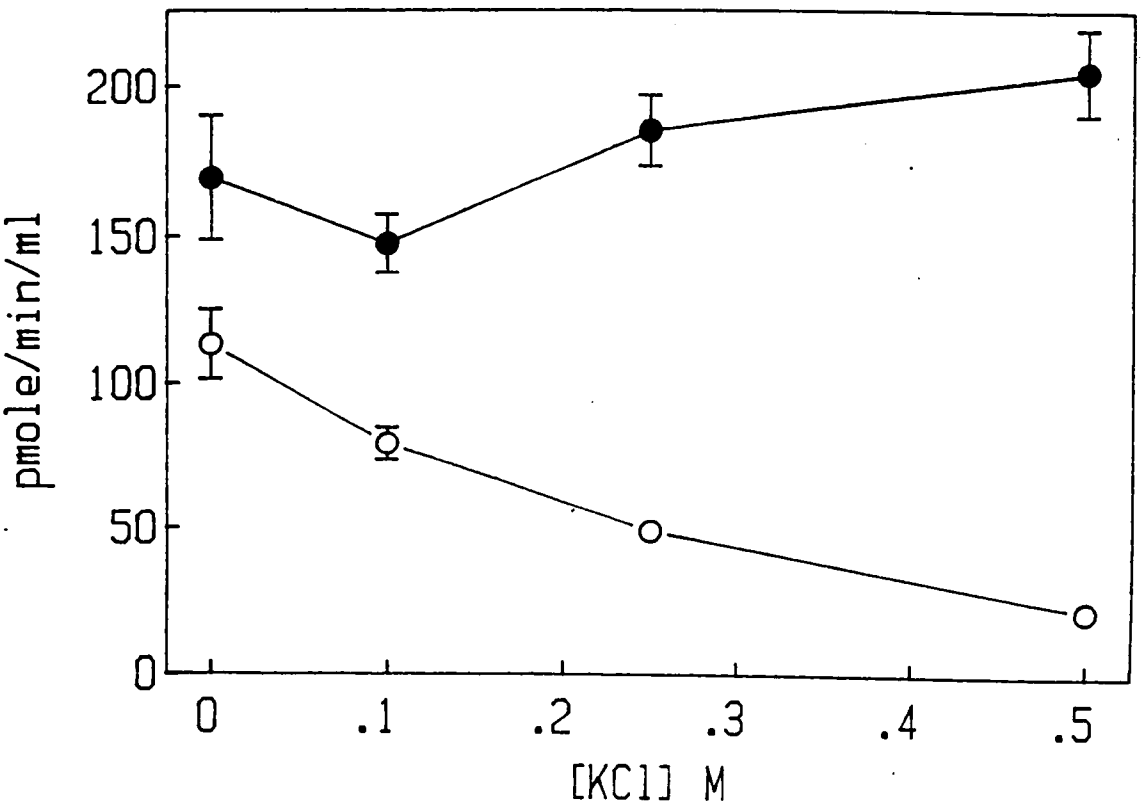


Figure 8. Calcium dependence of the methyltransferase activity as a function of ionic strength. Assays were performed under the standard assay conditions in the presence of 0, 100, 250, and 500 mM of KCl with 100 μM $CaCl_2$ (●) or 1 mM EGTA (o). The data points represent the mean of four determinations with error bars showing the standard error of the mean.

methyltransferase rate at a fixed AdoMet concentration indicates an enhanced reaction rate in the presence of calcium (Fig. 9). Kinetic parameters were calculated under saturating calcium or in the absence of calcium (1 mM EGTA) by constructing double reciprocal plots at various fixed concentrations of AdoMet using calmodulin as the varied substrate (Fig. 10). In the presence of calcium, the Km for calmodulin was 100 nM and the k_{cat} value was 0.0278 sec^{-1} (Table III). However, in the presence of EGTA the methyltransferase showed a lower k_{cat} (0.0106 sec^{-1}) and a higher Km value (220 nM), reflected by a k_{cat}/Km ratio that was 6-fold lower than that observed in the presence of calcium (Table III). In contrast, the Km for AdoMet was essentially the same in the presence of either EGTA or calcium. Overall, the data suggest that higher enzyme activity in the presence of calcium mostly results from a higher kcat and a lower Km value for calcium-bound calmodulin.

Under standard conditions, calcium stimulates the methyltransferase activity at least 3-fold and shows a $K_{0.5}$ of 0.71 μM (Fig. 11). This value is within the range of K_d values reported for calcium binding to calmodulin (reviewed in Klee and Vanaman, 1982; Cohen, 1988). Further, calcium-binding experiments show no interaction between the calmodulin methyltransferase and calcium. Thus, the effect of calcium on the methyltransferase activity appears to be through the binding of calcium to calmodulin rather then through a direct

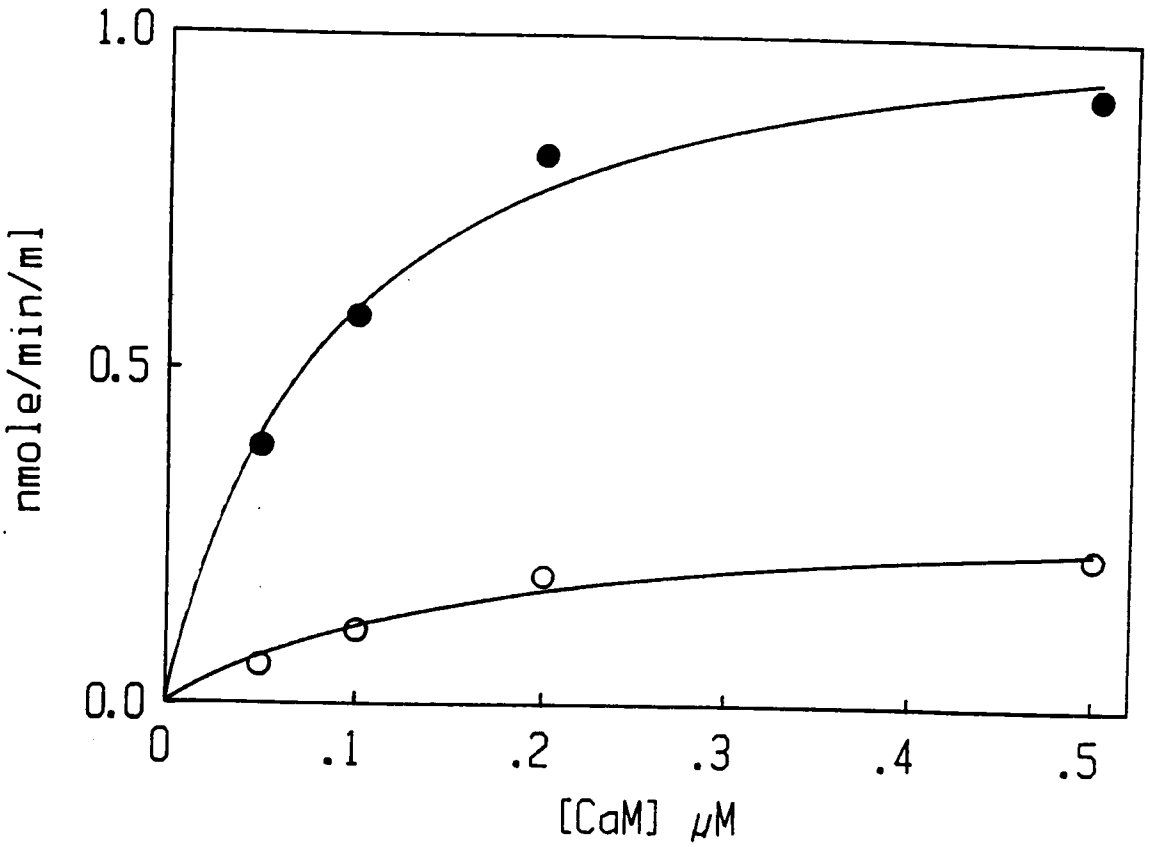


Figure 9. Pseudo-first order of calmodulin N-methyltransferase activities. Methyltransferase activities were measured with a saturated concentration (12.1 μM) of AdoMet in the presence of 0.1 mM of $CaCl_2$ (●) or 1 mM of EGTA (○).

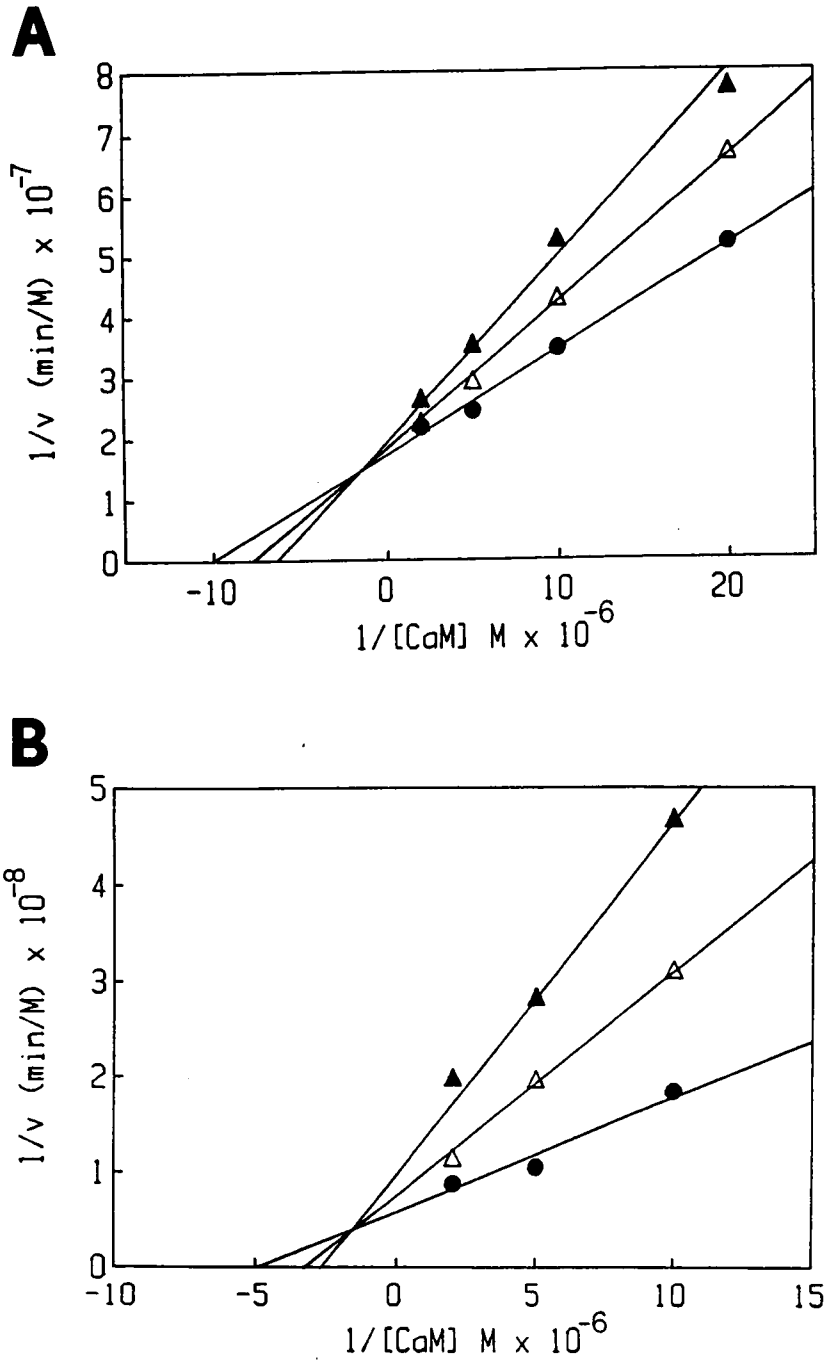


Figure 10. Double-reciprocal plot of initial velocities of VU-1 calmodulin methylation. Calmodulin methylation was assayed at 2.4 (▲), 4.8 (△),and 12.2 (●) μM of AdoMet with VU-1 calmodulin as the variable substrate in the presence of 0.1 mM of $CaCl_2$ under standard conditions **(A)** or 1 mM of EGTA **(B)**.

Table III. Catalytic parameters of Calmodulin-N-methyl transferase[a]

Parameters[b]	In the presence of 100 μM $CaCl_2$	In the presence of 1 mM EGTA
Km for calmodulin (nM)	100	220
Km for AdoMet (μM)	2.22	2.86
Vmax (nmole/min mg protein)	44.1	16.7
k_{cat} (sec^{-1})	0.0278	0.0106
k_{cat}/Km (sec^{-1} M^{-1})	27.8×10^4	4.82×10^4

[a] Assays were performed under standard conditions and in the presence of 0.15 M KCl.

[b] Data for the calculation of Km and Vmax were derived from double reciprocal plots of three assay sets using calmodulin as the variable substrate and maintaining AdoMet at constant concentrations of 2.4 μM, 4.8 μM and 12.1 μM. Km for AdoMet and Vmax were obtained from replots of 1/[AdoMet] vs. $1/Vmax_{app}$. Km for CaM was calculated from replots of 1/[AdoMet] vs. $slope_{1/[CaM]}$. k_{cat} was calculated using 38,000 as the molecular weight of the enzyme.

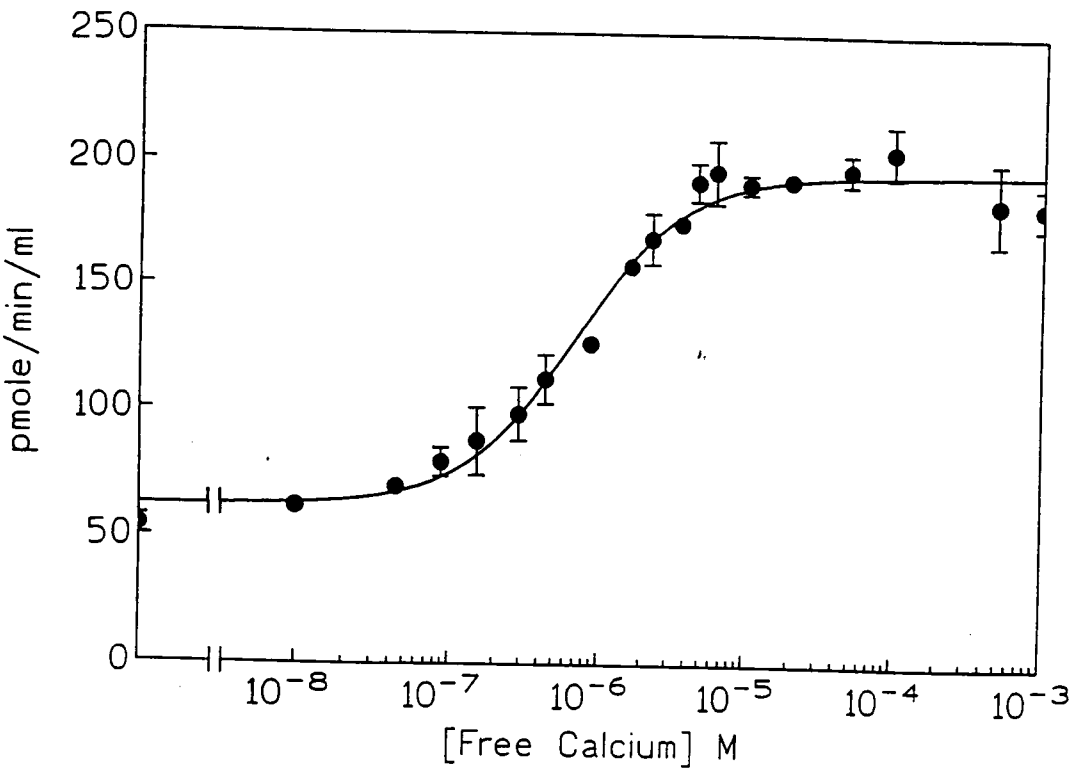


Figure 11. Effect of calcium on calmodulin N-methyltransferase activity. Enzyme activity was measured over a range of calcium concentrations by using an EGTA/Ca^{2+} buffering system as described in Materials and Methods. Error bars show the standard error of the mean of duplicate determinants.

effect of calcium on the enzyme. Overall, the results suggest that the enzyme prefers the calcium-bound form of calmodulinas a substrate.

Effect of methionine oxidation of calmodulin on its interaction with the methyltransferase

Based on the crystal structure of calcium-saturated calmodulin (Babu et al., 1988), nearly all methionine residues reside in the two hydrophobic clefts formed in the NH_2- and COOH-terminal lobes, where they constitute 46 % of solvent accessible surface area of hydrophobic cleft (O'Neil & DeGrado, 1990). The importance of methionine residues within these regions for the interaction of calmodulin with calmodulin-dependent enzymes is reflected by the extreme sensitivity of calmodulin activity to oxidizing agents that modify methionine residues (Walsh & Stevens, 1978; Guerini et al., 1984; Yao et al., 1996).

The finding that the calmodulin methyltransferase interacts with calmodulin in a calcium-dependent fashion raises the question whether similarity exists between the methyltransferase and other calmodulin-dependent enzymes in their mode of interaction with calmodulin. To test this, we oxidized the methionine residues of VU-1 calmodulin with performic acid and used it as a substrate for the calmodulin methyltransferase. Performic acid oxidizes cysteine (or cystine) to cysteic acid, and methionine to methionine sulfone (Hirs, 1967). In addition, it changes tryptophan to N-

formylkynurenine (Hirs, 1967). However, VU-1 calmodulin has neither cysteine nor tryptophan, and methionine sulfone is the only oxidized residue obtained upon calmodulin oxidation (Van Eldik and Watterson, 1981).

Performic oxidized VU-1 calmodulin showed a decreased electrophoretic mobility upon SDS-PAGE (Fig. 12), and showed a reduced ability to activate NAD kinase activation (26 % of maximal activity) (Fig. 13). Performic acid oxidization of calmodulin also dramatically decreased its ability to serve as a substrate for the methyltransferase, even in the presence of calcium (Fig. 14). Thus, similar to calmodulin-regulated enzymes, the integrity of the methionine residues in the hydrophobic clefts of calmodulin is essential for its interaction with the methyltransferase.

Inhibition of calmodulin methylation by calmodulin-binding drugs and peptides

To test the potential involvement of hydrophobic clefts in the interaction of calmodulin with the methyltransferase, reagents that bind to these regions of calmodulin were tested for their ability to block methylation. A synthetic peptide (LKKFNARRKLKGAILTTMLA, residues 290-309) corresponding to the calmodulin-binding domain peptide of calmodulin-dependent protein kinase II and the naphthalene sulfonamide derivative W-7 were tested for their ability to inhibit calmodulin methylation. In the presence of 1 μM VU-1 calmodulin and 100 μM Ca^{2+}, the calmodulin-binding peptide inhibited calmodulin

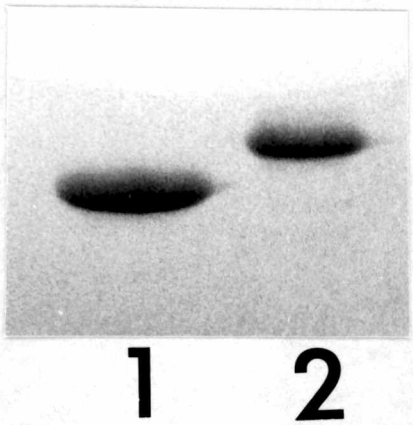


Figure 12. SDS-PAGE of normal and performic acid oxidized VU-1 calmodulins. The calmodulins were subjected to SDS-PAGE on a 15 % (w/v) polyacrylamide gel in the presence of 1 mM EGTA. Proteins were visualized by staining with Coomassie blue. **Lane 1**; normal VU-1 calmodulin, **lane 2**; oxidized VU-1 calmodulin.

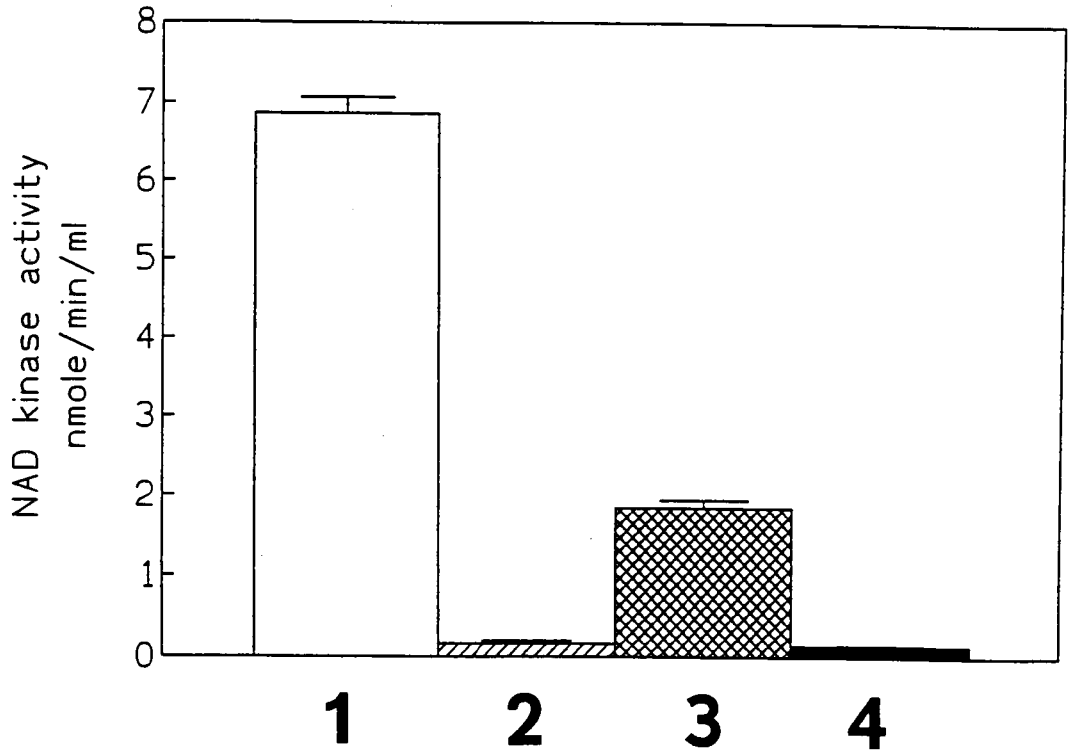


Figure 13. Analysis of the NAD kinase activation by normal and performic acid oxidized VU-1 calmodulins. The histograms show NAD kinase activation by a saturating dose (100 nM) of each calmodulin in the presence or absence of calcium. NAD kinase activation by: **bar 1**,VU-1 calmodulin in the presence of 1 mM $CaCl_2$; **bar 2**, VU-1 calmodulin in the presence of 1 mM EGTA; **bar 3**, oxidized VU-1 calmodulin in the presence of 1 mM $CaCl_2$; **bar 4**, oxidized VU-1 calmodulin in the presence of 1 mM EGTA. Error bars show the standard error of the mean.

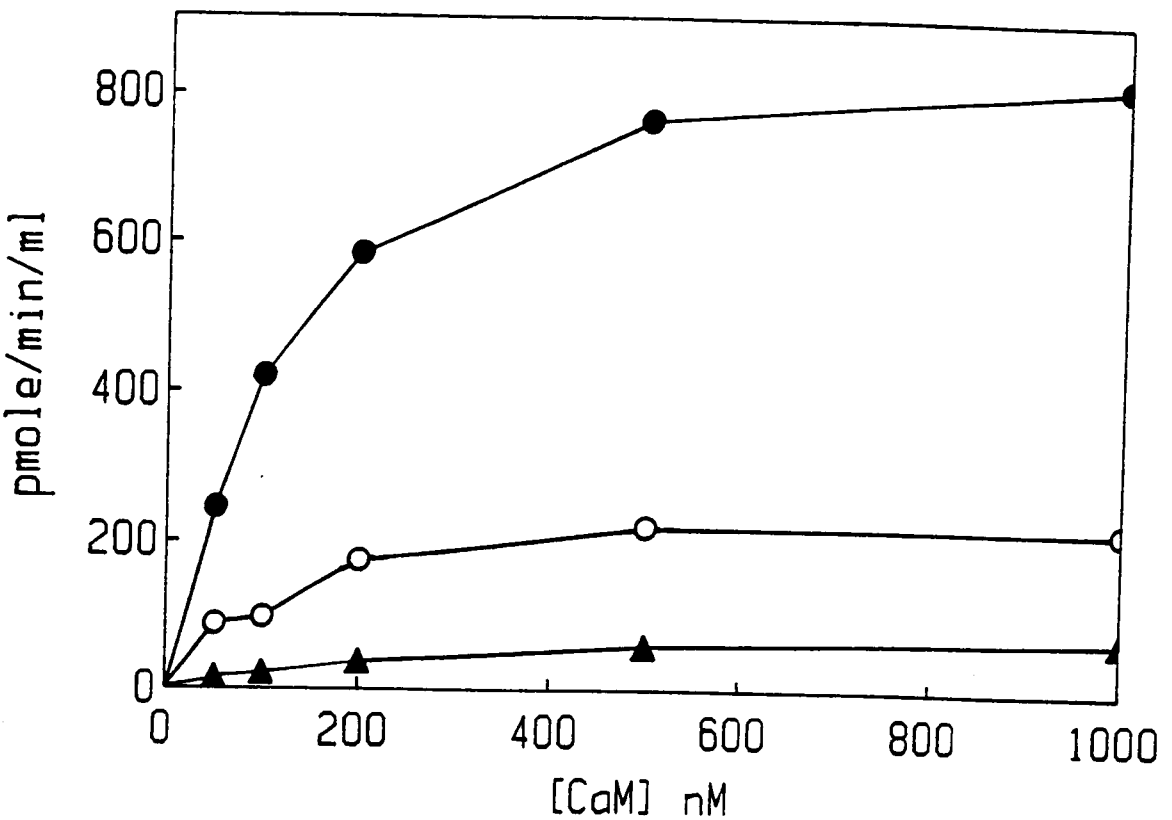


Figure 14. Effect of methionine oxidation on the kinetics of lysine methylation of VU-1 calmodulin. Calmodulin methylation was assayed under standard conditions with a fixed concentration of 12 μM AdoMet and with calmodulin as the varied substrate. Substrates: unoxidized VU-1 calmodulin in the presence of 100 μM $CaCl_2$ (●); unoxidized VU-1 calmodulin in the presence of 1 mM EGTA (○); performic acid-oxidized VU-1 calmodulin in the presence of 100 μM $CaCl_2$ (▲).

N-methyltransferase activity with an IC_{50} of 570 nM (Fig. 15). This is consistent with a one-to-one titration of peptide and calmodulin that is expected based on the concentration of calmodulin (1 μM) and the low Kd (45 nM) for the interaction of the peptide with calmodulin (Meyer et al., 1992). The peptide did not affect the rate of calmodulin methylation in the absence of calcium (Fig. 16). Thus, the inhibition appears to be due to the calcium-dependent interaction of the peptide with calmodulin rather than a direct influence on the methyltransferase. No inhibition was observed with a negative control peptide, the autophosphorylation domain (residues 281-289) of CaM kinase II, which does not bind calmodulin (Fig. 15).

Similar to the results with the calmodulin-binding peptide, the calmodulin antagonist W-7 also inhibits the methylation of calmodulin in the presence of calcium but not in the presence of EGTA (Fig. 17). Overall, the data suggest that compounds that antagonize the binding of calmodulin-regulated enzymes by binding to the hydrophobic clefts of calcium-bound calmodulin, also disrupt calmodulin interaction with the methyltransferase.

Interaction of calmodulin methyltransferase with the COOH-terminal lobe of calmodulin

The site of posttranslational methylation of calmodulin resides in the loop between the F-helix of the third EF-hand calcium binding site and the E-helix of the fourth EF-hand

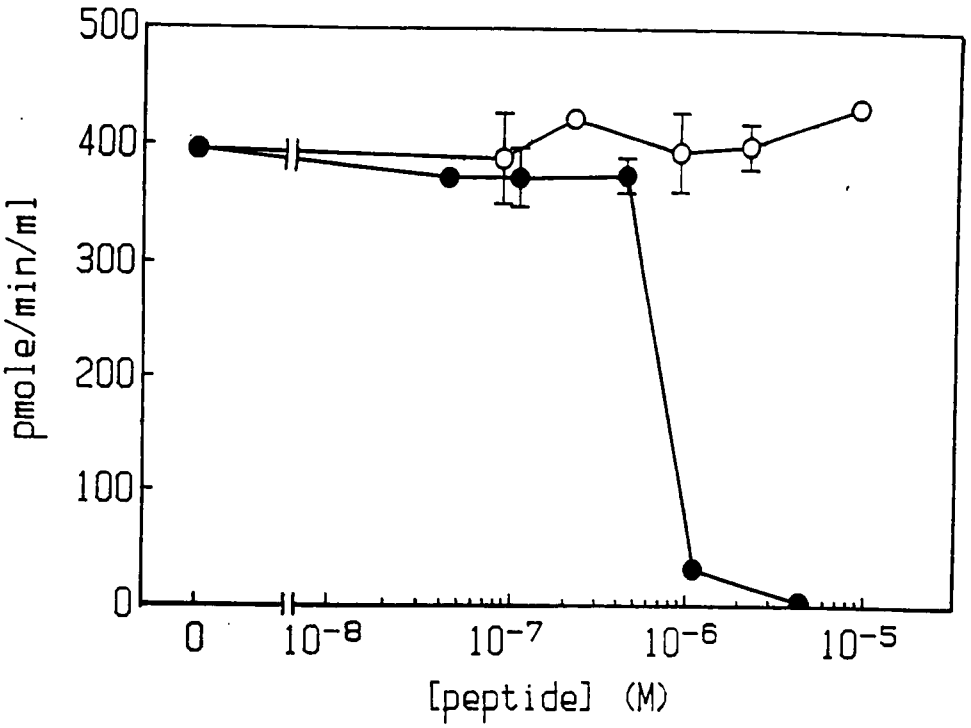


Figure 15. Properties of CaM kinase II synthetic peptides as antagonists of calmodulin methylation. Calmodulin N-methyltransferase was assayed under standard conditions as described in Materials and Methods. Assays were performed with VU-1 calmodulin (1 μM) as a substrate at the indicated concentration of peptides: calmodulin binding domain (residues 290-309, ●) peptide, and autophosphorylation domain (residues 281-289, ○) peptide of CaM kinase II. Error bars show the standard error of the mean.

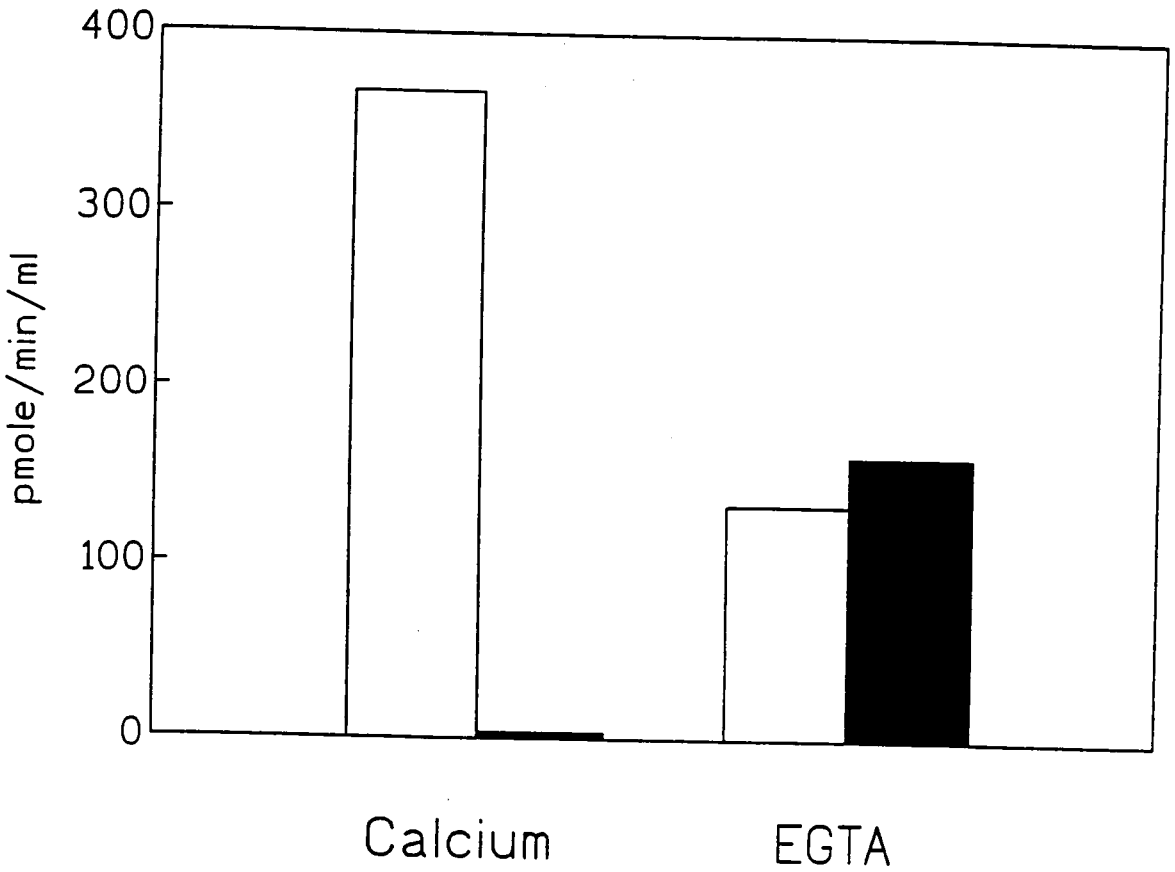


Figure 16. Calmodulin binding peptide selectively affects the calmodulin N-methyltransferase in the presence of calcium. The enzyme was assayed under standard conditions as described in the Materials and Methods. Assays were performed in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA with (**solid bars**) or without (**open bars**) 4.4 μM of the calmodulin binding domain (290-309) peptide of CaM kinase II.

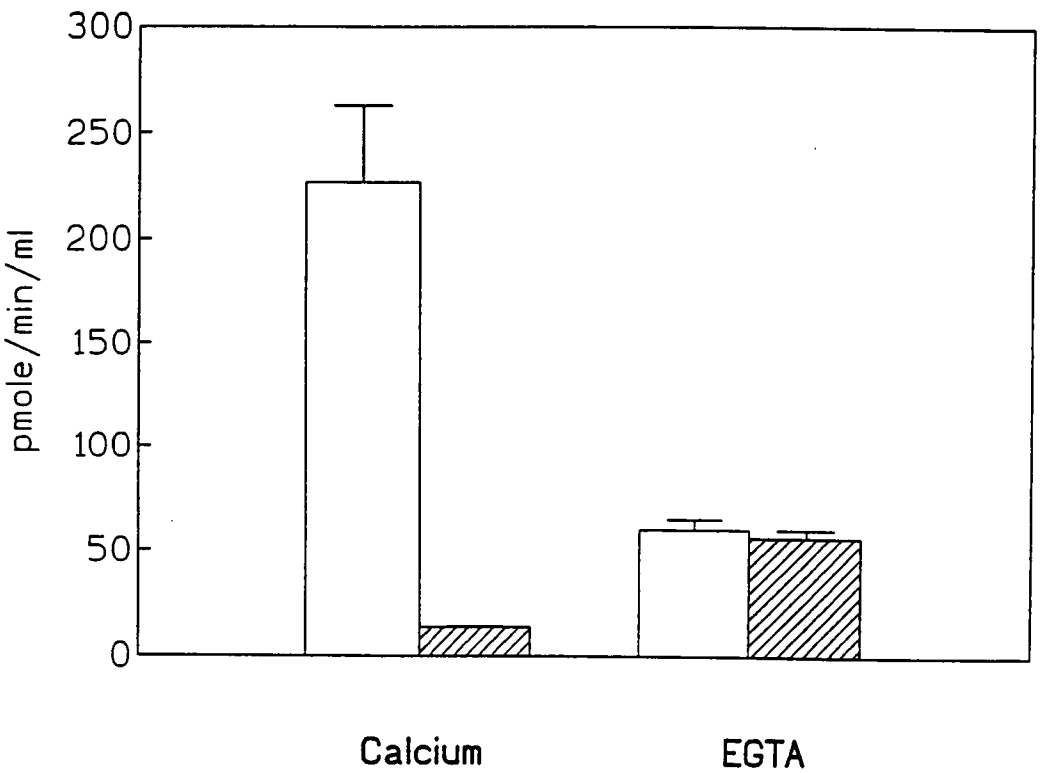


Figure 17. The calmodulin-antagonistic drug W-7 selectively inhibits calmodulin methylation in the presence of calcium. The enzyme was assayed under standard conditions as described in Materials and Methods. Assays were performed in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA with (**cross hatched bars**) or without (**open bars**) 100 μM of W-7. Error bars show the standard error of the mean.

calcium binding site within the COOH-terminal lobe (Babu et al., 1988, see Fig. 1). To test whether both lobes of calmodulin are required for methyltransferase recognition, the isolated COOH-terminal lobe of VU-1 calmodulin was tested.

Previous studies show that partial tryptic digestion of calmodulin in the presence of calcium cleaves the molecule preferentially at lys 77 of the central helix, yielding two fragments (residues 1-77 and 78-148) that constitute the amino terminal and carboxyl terminal lobes of calmodulin (Drabikowski et al., 1977; Walsh et al., 1977; Newton et al., 1984). This approach was selected since NMR studies of the tryptic fragments indicate that both the NH_2-terminal (residues 1-77) and COOH-terminal half-fragments (residues 78-148) retain most of the tertiary structure observed in the intact protein (Dalgarno et al., 1984; Ikura et al., 1984; Finn et al., 1993). In addition, the calcium binding characteristics of intact calmodulin is largely retained by the tryptic fragments (Minowa & Yagi, 1984; Linse et al., 1991). Thus, the amino terminal region can be removed without altering the general conformation of the COOH-terminal lobe.

Digestion of VU-1 calmodulin with trypsin resulted in a profile (Fig. 18, 19) that is similar to that previously observed with other calmodulins (Newton et al., 1984). The fragments and undigested calmodulin were fractionated by HPLC on a reverse phase phenyl column. The identity of the COOH-terminal fragment was established by the absorbance at 280 nm

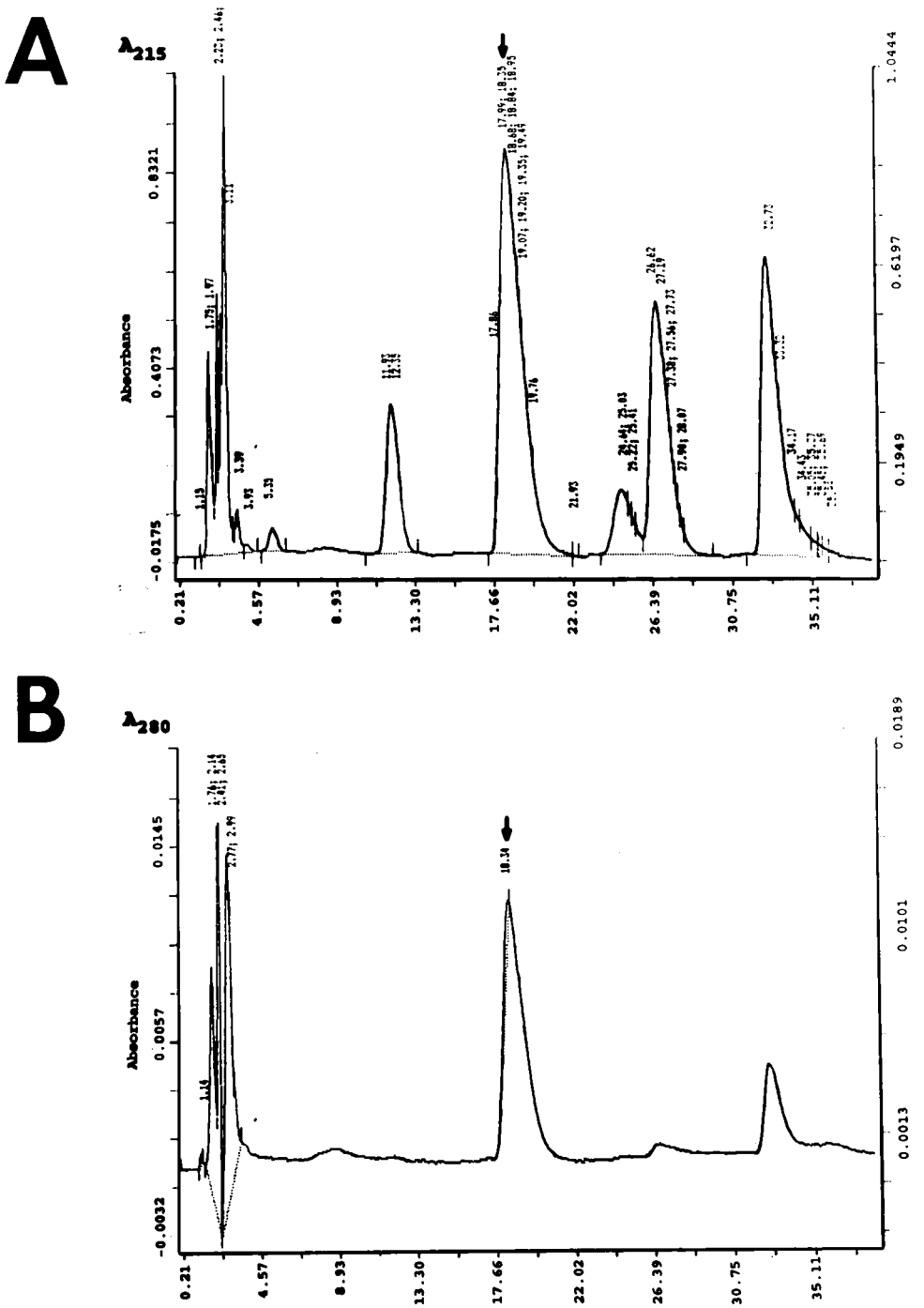


Figure 18. HPLC fractionation of the tryptic digested VU-1 calmodulin fragments. VU-1 calmodulin was partially digested with trypsin in the presence of $CaCl_2$, and the digest (200 μg protein) was subjected to HPLC separation on a phenyl reverse phase column as described in Materials and Methods. Fractions containing peptide fragments were monitored for absorbance both at 215 (**A**) and 280 nm (**B**). A arrow in each panel indicates the peak of the COOH-terminal fragment.

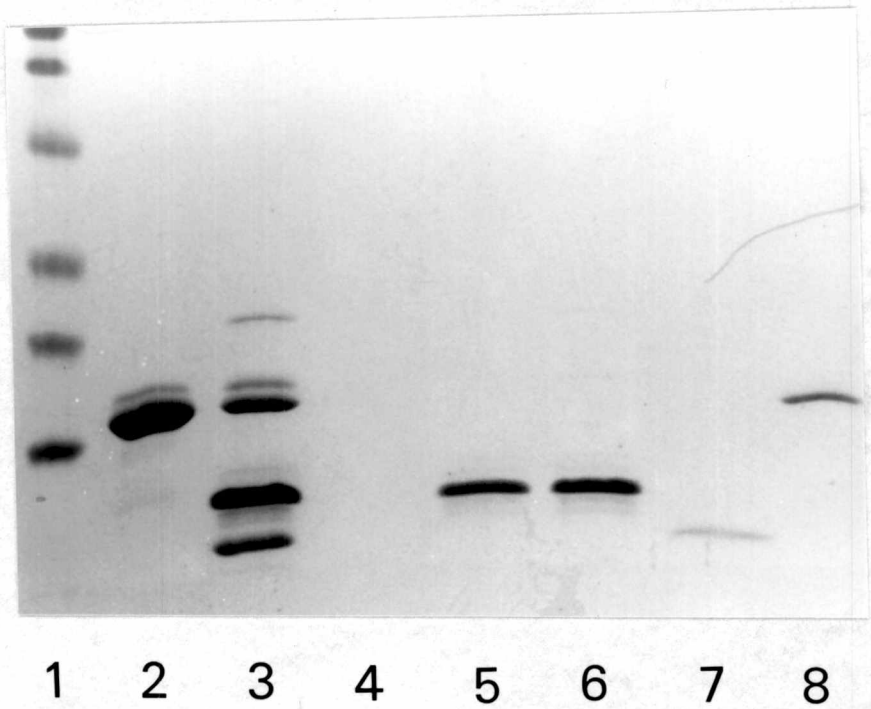


Figure 19. SDS-PAGE analysis of HPLC fractionated VU-1 calmodulin tryptic digested fragments. The samples were subjected to SDS-PAGE on a 15 % (w/v) polyacrylamide gel in the presence of 1 mM EGTA, and the gel was stained with Coomassie blue. **Lane 1**, low molecular weight protein standards (94,000; 67,000; 43,000; 30,000; 20,000; 14,000); **lane 2**, intact VU-1 calmodulin; **lane 3**, tryptic digest of VU-1 calmodulin; **lanes 4-8**, HPLC fractions at 12 min, 18 min, 25 min, 27 min and 32 min.

of the unique tyrosine at 138 (Fig. 18). The purity of the isolated product was verified by SDS-PAGE (Fig. 19).

The purified COOH-terminal fragment (residues 78-148) was tested as a methyltransferase substrate. As shown in Fig. 20, the rate of methylation obtained with the COOH-terminal lobe is indistinguishable from that of intact VU-1 calmodulin. Thus, the NH_2-terminal lobe of calmodulin (residues 1-77) is not necessary for binding and recognition by the methyltransferase.

We also expanded our studies of the effect of calmodulin antagonists (CaM kinase II binding peptides and W-7) on methylation to include analyses of the COOH-terminal lobe. These two calmodulin antagonists have a different binding mode for calmodulin. As stated above, both NH_2- and COOH-terminal lobes of calmodulin engage in the formation of calmodulin-peptide complexes (Ikura et al., 1992; Meador et al., 1992, 1993). However, the aromatic ring of W-7 can fit into individual hydrophobic cleft in either NH_2-terminal (residues 1-77) or COOH-terminal (residues 78-148) lobe of calmodulin based on affinity column studies (Vogel et al., 1983; Brzeska et al., 1983; Head et al., 1982). Further, the crystal structure of drug-calmodulin adducts show that the interaction of calmodulin with these ligands is stabilized largely by binding contacts within a single hydrophobic cleft (Cook et al., 1994).

As shown in Fig. 21, W-7 inhibits the methylation of the

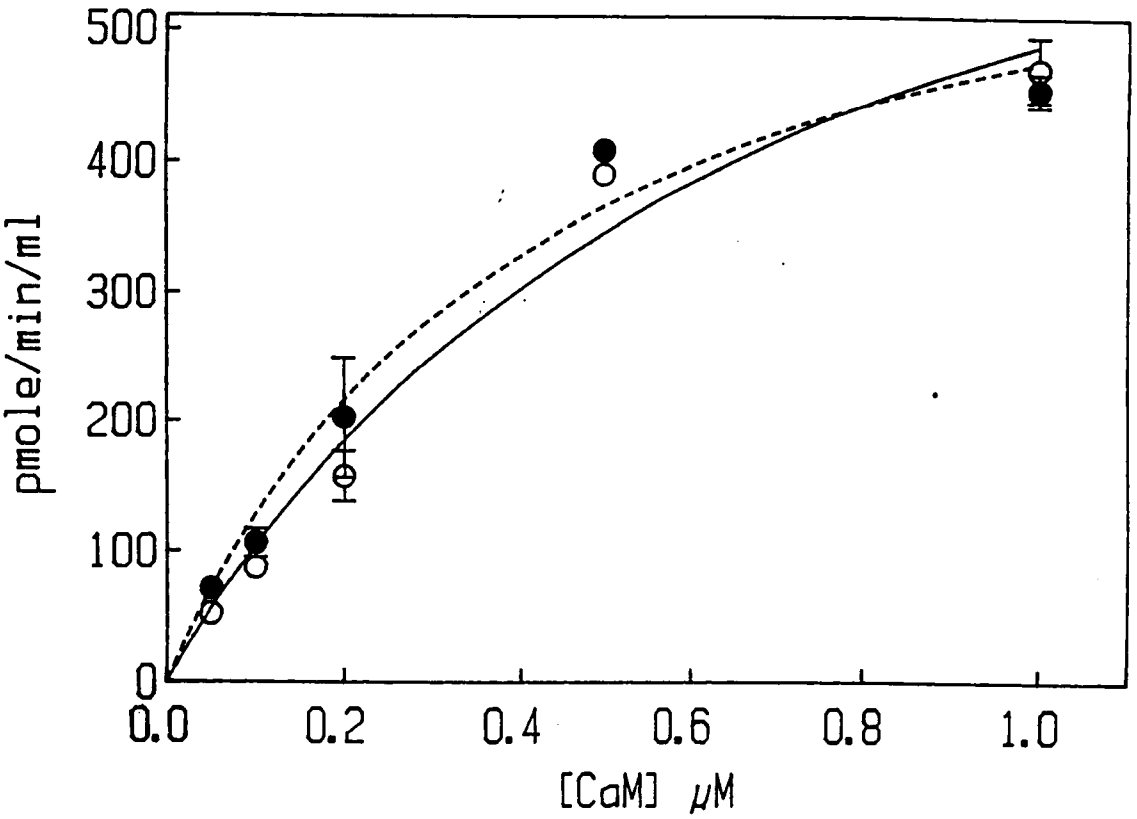


Figure 20. Methylation of the COOH-terminal lobe (residues 78-148) of calmodulin. Assays were done under standard conditions as described in Materials and Methods. Substrates: Normal, uncleaved VU-1 calmodulin (●, solid line); the COOH-terminal fragment (residues 78-148) generated from partial trypsin cleavage (o, dashed line). The error bars show the standard error of the mean. methylation of the COOH-terminal lobe (Fig. 22). Overall, the results suggest that the recognition site of the calmodulin methyltransferase resides solely in the COOH-terminal lobe of calmodulin, and that in the presence of calcium, reagents that bind to the hydrophobic cleft formed within the COOH-terminal

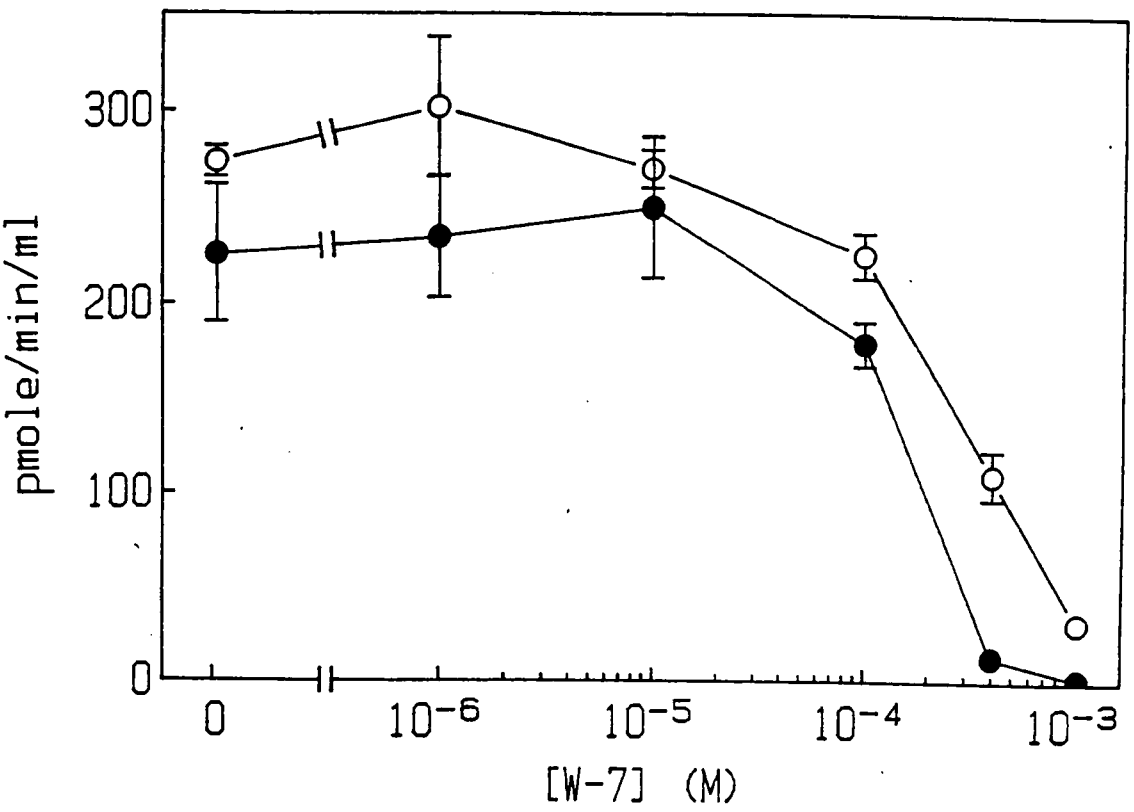


Figure 21. Inhibition of the methylation of intact VU-1 calmodulin or the COOH-terminal fragment of VU-1 calmodulin by W-7. Calmodulin N-methyl transferase was assayed under standard conditions in the presence of various concentrations of W-7 with 1 μM intact VU-1 calmodulin (●) or 1 μM of the COOH-terminal fragment (residues 78-148) of VU-1 (o). Error bars show the standard error of the mean.

COOH-terminal lobe in a manner similar to that observed with intact VU-1. This is not surprising since the COOH-terminal fragment still possesses one hydrophobic cleft and should retain the ability to bind W-7. However, the calmodulin binding peptide, which requires both lobes of calmodulin for high affinity binding did not inhibit the methylation of the COOH-terminal fragment of VU-1 calmodulin (Fig. 22).

Production of V136T calmodulin.

To investigate further the interaction between calmodulin and the calmodulin methyltransferase, several calmodulin mutants were generated by site-directed mutagenesis of the synthetic VU-1 calmodulin gene (Fig. 23).

The first mutant studied was V136T calmodulin. This mutant was based on a previous study that showed that a substitution of ile-136 with thr in the *Paramecium* mutant *cam2* decreases the methylation of lysine-115 *in vivo* (Lukas et al., 1989). The mutation site is at the eighth residue in the 4th calcium-binding loop of calmodulin. As discussed above, this residue in EF hands is generally occupied by an invariant ile or val (Fig. 1) which is packed into the hydrophobic core of the protein. The relative polarity of each of 20 amino acids has been determined experimentally by measuring the free-energy change of transferring a given residue from a hydrophobic solvent into water (Eisenberg, 1982). Based on these measurements, the free energies of transfer are very

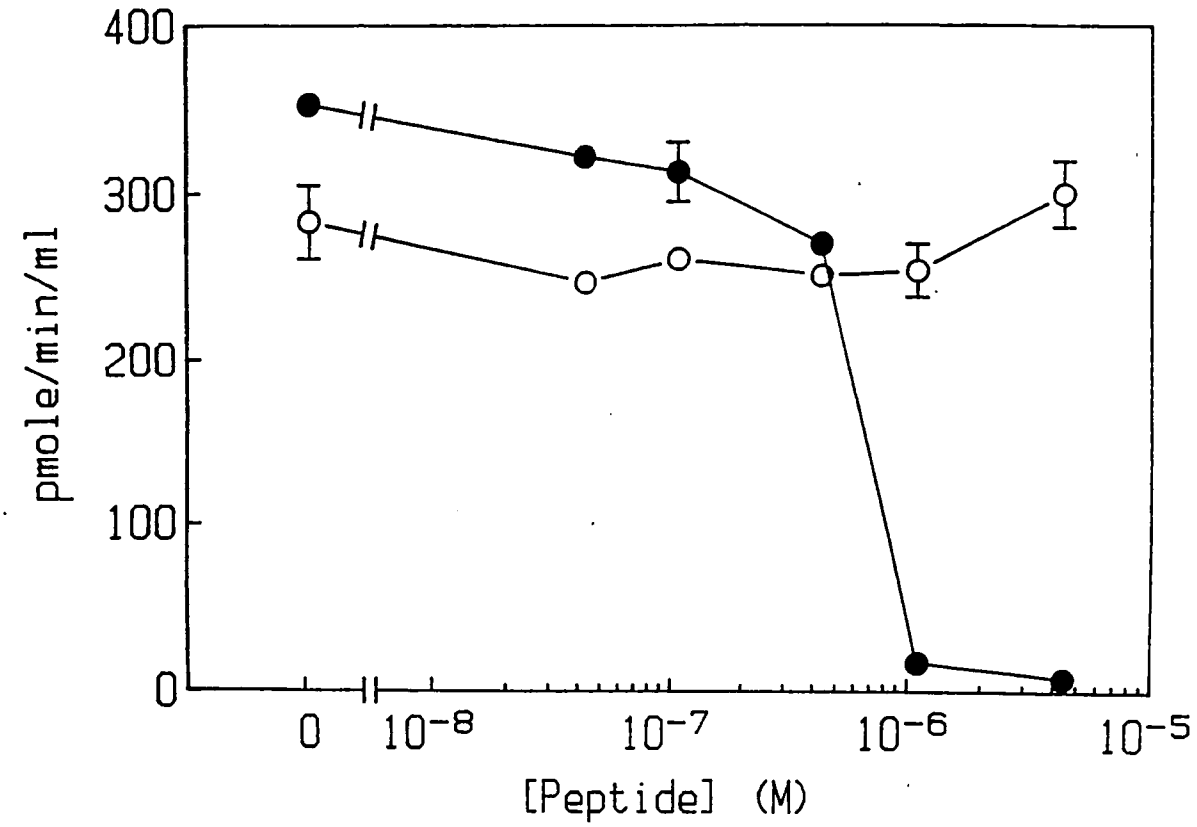


Figure 22. Inhibition of the methylation of intact VU-1 calmodulin or the COOH-terminal fragment of VU-1 calmodulin by a calmodulin-binding peptide. Calmodulin N-methyl transferase was assayed under standard conditions in the presence of 100 μM $CaCl_2$ and a constant concentration of AdoMet (12 μM), and 1 μM VU-1 calmodulin (●) or 1 μM of the COOH-terminal fragment (residues 78-148) of VU-1 (o), and various amounts of the calmodulin binding domain (290-309) of calmodulin-dependent protein kinase II. Error bars show the standard error of the mean.

```
                          1                    * * *   * *   *      39
VU-1                      ADQLTDEQIAEFKEAFSLF DKDGDG TIT TKELGTVMRSL
VU-3
V136T
[QNP(41-43)EKL/K115R]

                          40                  * * *   * *   *      75
VU-1                       GQNPT EAELQDMINEV DADGNG TID FPEFLNLMARK
VU-3
V136T
[QNP(41-43)EKL/K115R]      EKL

                          76                  * * *  * *   *      112
VU-1                         MKDTDSEEELKEAFRVF DKDGNG FIS AAELRHVMTN L
VU-3
V136T
[QNP(41-43)EKL/K115R]

                          113 #               * * *  * *   *      148
VU-1                         GEKLT DEEVDEMIREA DVDGDG QVN YEEFVQVMMAK
VU-3                           R
V136T                                                T
[QNP(41-43)EKL/K115R]        R
```

Figure 23. Amino acid sequence of VU-1 and mutant calmodulins. The amino acid sequence of VU-1 calmodulin (Roberts et al., 1985) is shown. Positions of substitutions in VU-3 (Roberts et al., 1986b), V136T, and [QNP(41-43)EKL/K115R] calmodulins are shown. Positions involved in calcium binding are marked with asterisks. Residues involved in α-helix or β-sheet structures based on the crystal structure of calcium-saturated calmodulin (Babu et al., 1988; Chattopadhyaya et al., 1992) are underlined. The methylation site, lysine 115, is marked with #.

endergonic for ile (3.1 KJ/mol) or val (2.3 KJ/mol), and exergonic for thr (-0.75 KJ/mol). Thus, the substitution of a threonine will result in increased hydrophilicity at this position, and could alter the packing of hydrophobic residues in the COOH-terminal lobe of calmodulin, disrupting methyltransferase recognition. To gain insight into how this mutation affects calmodulin methyltransferase recognition, a calmodulin with a threonine-136 substitution (V136T calmodulin) was generated from the VU-1 synthetic gene, and was expressed in *E. coli* in an unmethylated form for *in vitro* calcium binding, enzyme activation, and methylation analyses.

By SDS-PAGE analysis, purified V136T calmodulin showed a mobility identical to wild-type VU-1 calmodulin either in the presence (Fig. 24A) or absence of calcium (Fig. 24B). However, nondenaturing gel electrophoresis showed a quite different profile (Fig. 25). V136T calmodulin has a mobility identical to wild type VU-1 calmodulin in the presence of 1 mM calcium (Fig. 25A), but has a markedly reduced electrophoretic mobility compared to wild type calmodulin in the presence of 1 mM EGTA (Fig. 25B). *cam2* calmodulin shows a similar defect in electrophoretic mobility (Kink et al., 1990). Since a valine to threonine mutation should not affect the overall charge of calmodulin, the results suggest that this substitution causes a conformational change in the calcium-depleted state, that is manifested by an altered electrophoretic mobility on nondenaturing gels.

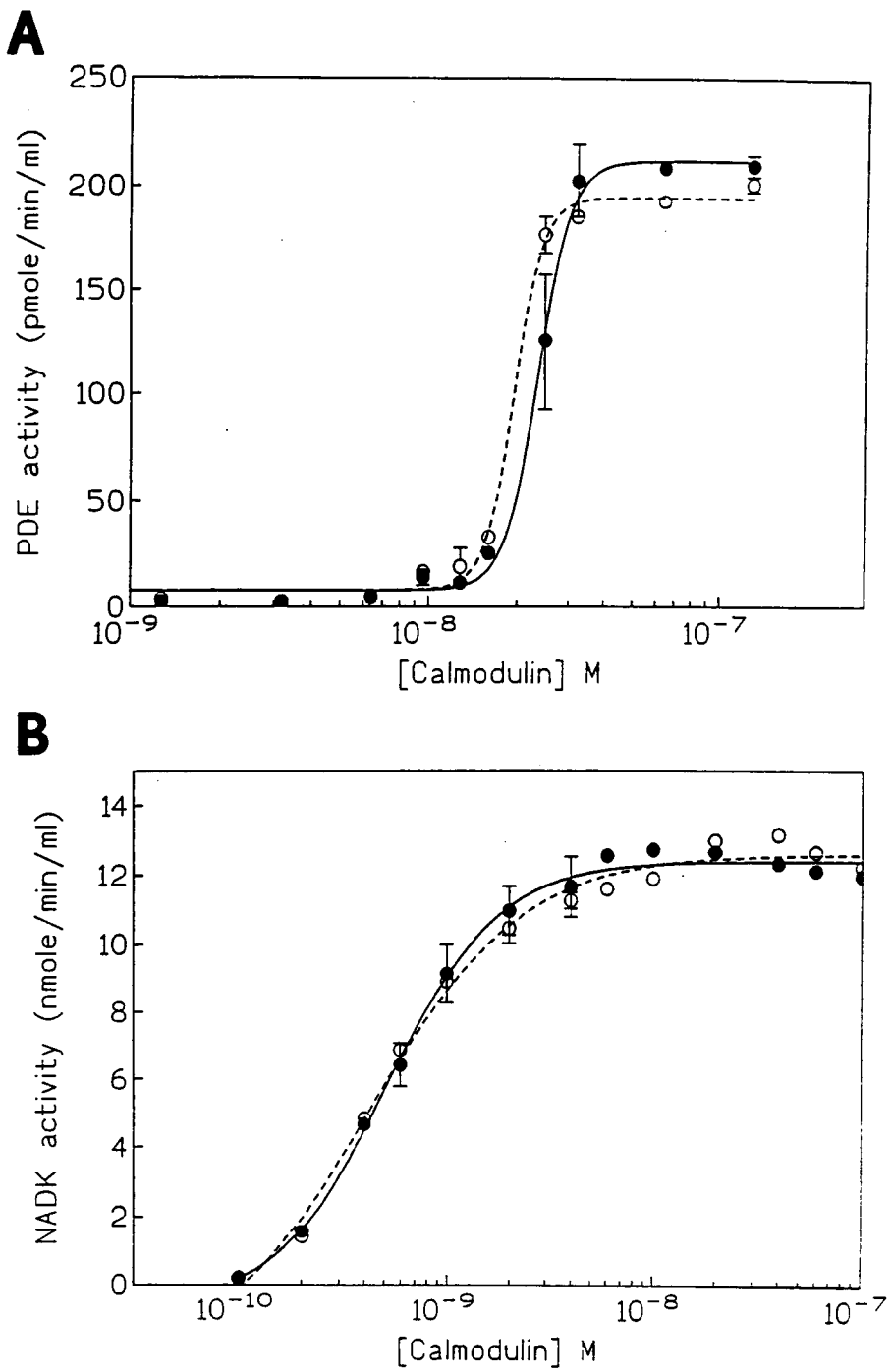


Figure 24. SDS-PAGE of VU-1 and V136T calmodulins. The calmodulins were subjected to SDS-PAGE on a 15 % (w/v) polyacrylamide gel in the presence of 1 mM $CaCl_2$ (**panel A**) or 1 mM EGTA (**panel B**). Gels were stained with Coomassie blue. **Lane 1**, molecular weight standards; **lane 2**, VU-1 calmodulin; **lane 3**, V136T calmodulin.

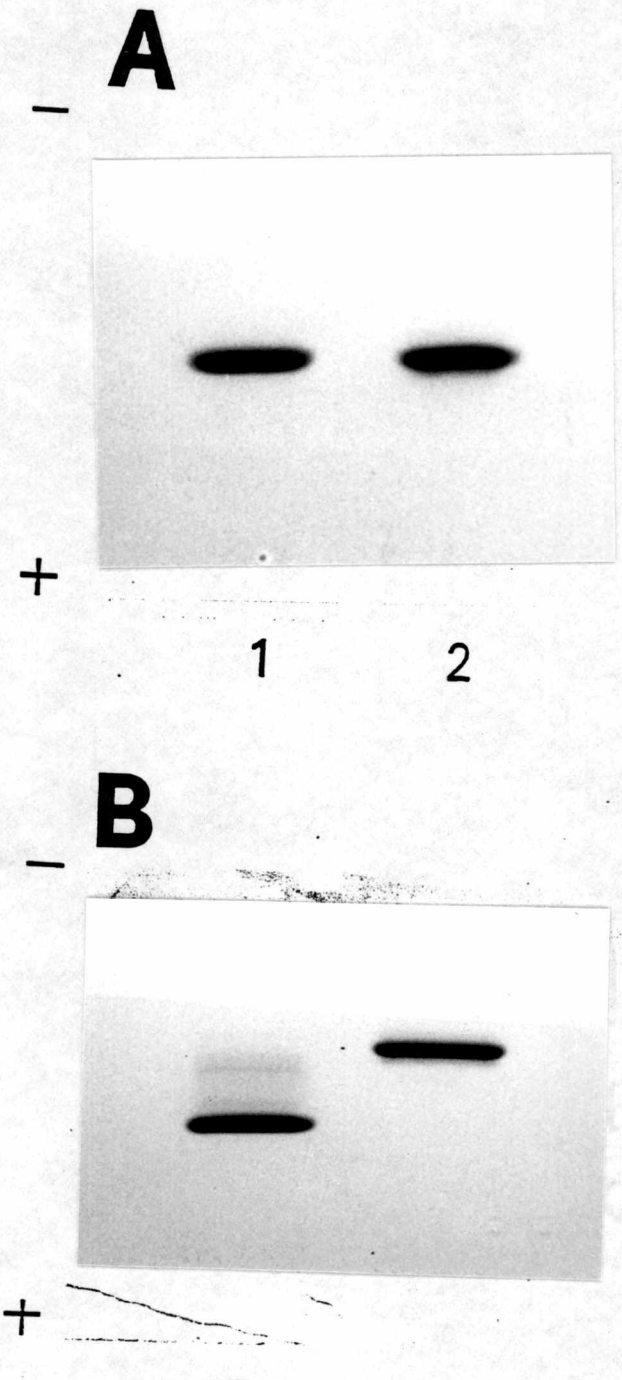


Figure 25. Nondenaturing gel electrophoresis of VU-1 and V136T calmodulins expressed and purified from *E. coli*. Calmodulins (2.5 μg/lane) were subjected to electrophoresis on 15 % (w/v) polyacrylamide gels in 50 mM Tris-borate (pH 7.5). Gels were stained with Coomassie blue. **Panel A**, electrophoresis in 1 mM $CaCl_2$; **Panel B**, electrophoresis in 1 mM EGTA in the sample buffer. **Lane 1**, VU-1 calmodulin; **lane 2**, V136T calmodulin.

Activator Properties of V136T Calmodulin

The ability of purified V136T calmodulin to activate calmodulin-dependent enzymes was compared to wild type VU-1 calmodulin. In the presence of saturating $CaCl_2$ (1 mM), V136T calmodulin was indistinguishable from wild type VU-1 calmodulin in its abilities to activate cyclic nucleotide phosphodiesterase and NAD kinase (Fig. 26). The results support previous findings with *cam2* activation of myosin light chain kinase (Lukas et al., 1989), and suggest that the substitution of a threonine residue at position 136 does not affect *in vitro* activation of calmodulin-dependent enzymes under saturating calcium conditions.

However, differences in the calcium-dependence of activation between the two calmodulins were readily apparent (Fig. 27). Half-maximal activation ($K_{0.5}$) of NAD kinase by V136T calmodulin was observed at a free calcium concentration of 9.1 μM, with full activation observed at 50 μM (Fig. 24). This was approximately 10-fold higher than the values obtained with VU-1 calmodulin ($K_{0.5}$ = 1 μM Ca^{2+}, full activation was observed at 4 μM), and could reflect a lower calcium-binding affinity by the mutant calmodulin.

Kinetics of V136T calmodulin methylation

To gain insight into why the substitution of a threonine at 136 in calmodulin leads to poor endogenous methylation, the steady state kinetic properties of calmodulin methyltransferase were investigated with mutant and wild-type

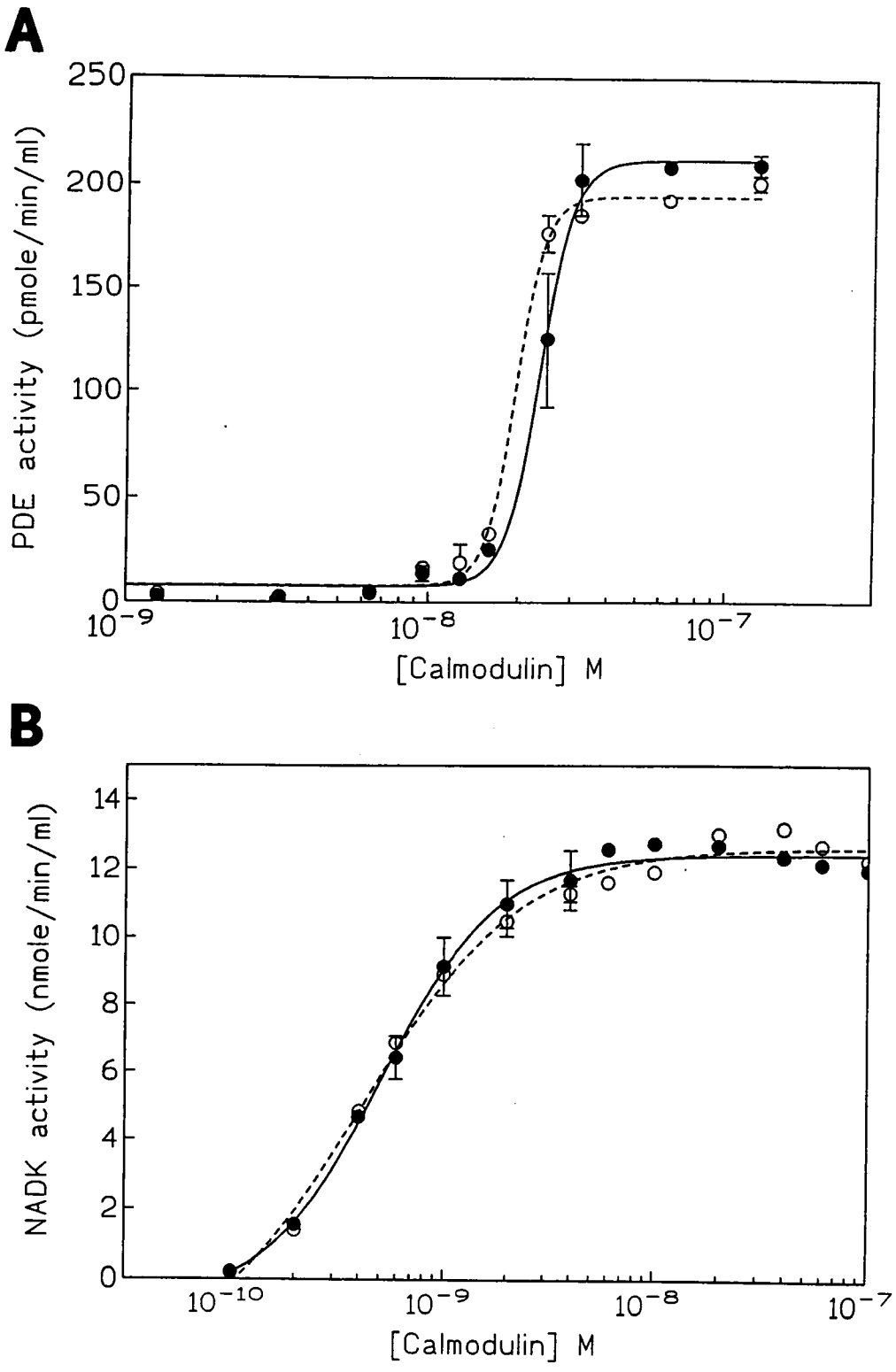


Figure 26. Activation of calmodulin dependent enzymes by VU-1 and V136T calmodulins in the presence of 1 mM $CaCl_2$. Calmodulin-dependent activation of cyclic nucleotide phosphodiesterase **(A)**, and NAD kinase **(B)** by varying amounts of VU-1 (●) and V136T (○) calmodulins in the presence of 1 mM $CaCl_2$. Error bars show the standard error of the mean.

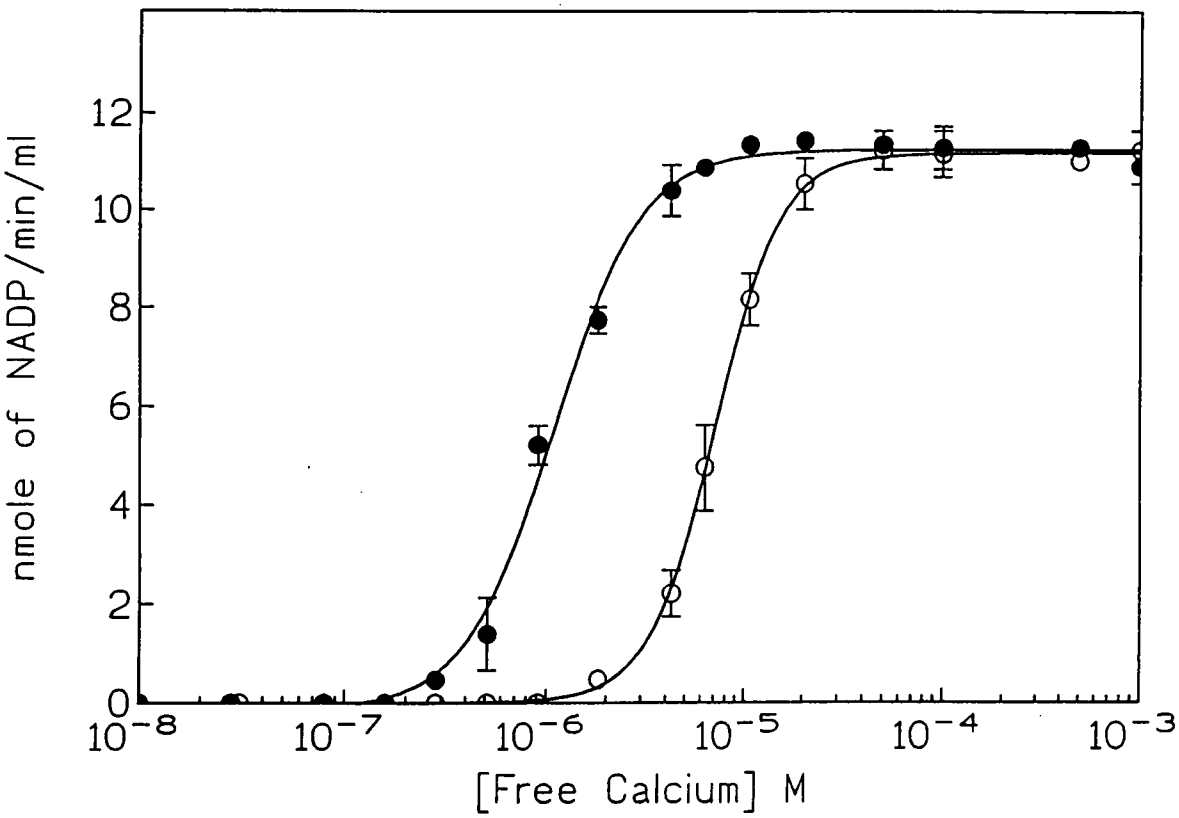


Figure 27. Calcium dependence of NAD kinase activation by VU-1 and V136T calmodulins. NAD kinase activation by 100 nM VU-1 (●) or 100 nM V136T (o) calmodulins were measured at varying concentrations of free calcium by using the EGTA/Ca^{+2} buffering system described in the Materials and Methods. Error bars show the standard error of the mean.

calmodulin substrates. As discussed above, it was observed that both the calcium-bound and calcium-depleted forms of VU-1 calmodulin can be methylated by the methyltransferase (Han et al., 1993). However, under conditions (0.15 M KCl and 3 mM Mg^{2+} concentrations) that approximate physiological conditions, the enzyme methylates calcium-depleted calmodulin with about 30 % of the V_{max} observed with calcium-bound calmodulin, and with a two-fold higher K_m (Table III, Han et al., 1993). Since cytosolic calcium concentrations could conceivably influence the methyltransferase activity, the enzyme activity with V136T calmodulin as a substrate was investigated as a function of calcium concentration.

Kinetic plots under conditions of saturating S-adenosyl methionine and varying calmodulin concentrations show that V136T calmodulin is indistinguishable from VU-1 calmodulin as a substrate for the methyltransferase in the presence of 1 mM $CaCl_2$ (Fig. 28A). However, the methylation kinetics of the two calmodulins differed strikingly in the absence of calcium (Fig. 28B), with VU-1 calmodulin showing methylation rates similar to those previously reported (Han et al., 1993, see Fig.9), whereas no detectable methylation of V136T calmodulin was observed.

Additional differences between the two calmodulins were observed upon investigation of the dependence of calmodulin methylation as a function of varied calcium concentration (Fig. 29). The methylation of VU-1 calmodulin is stimulated

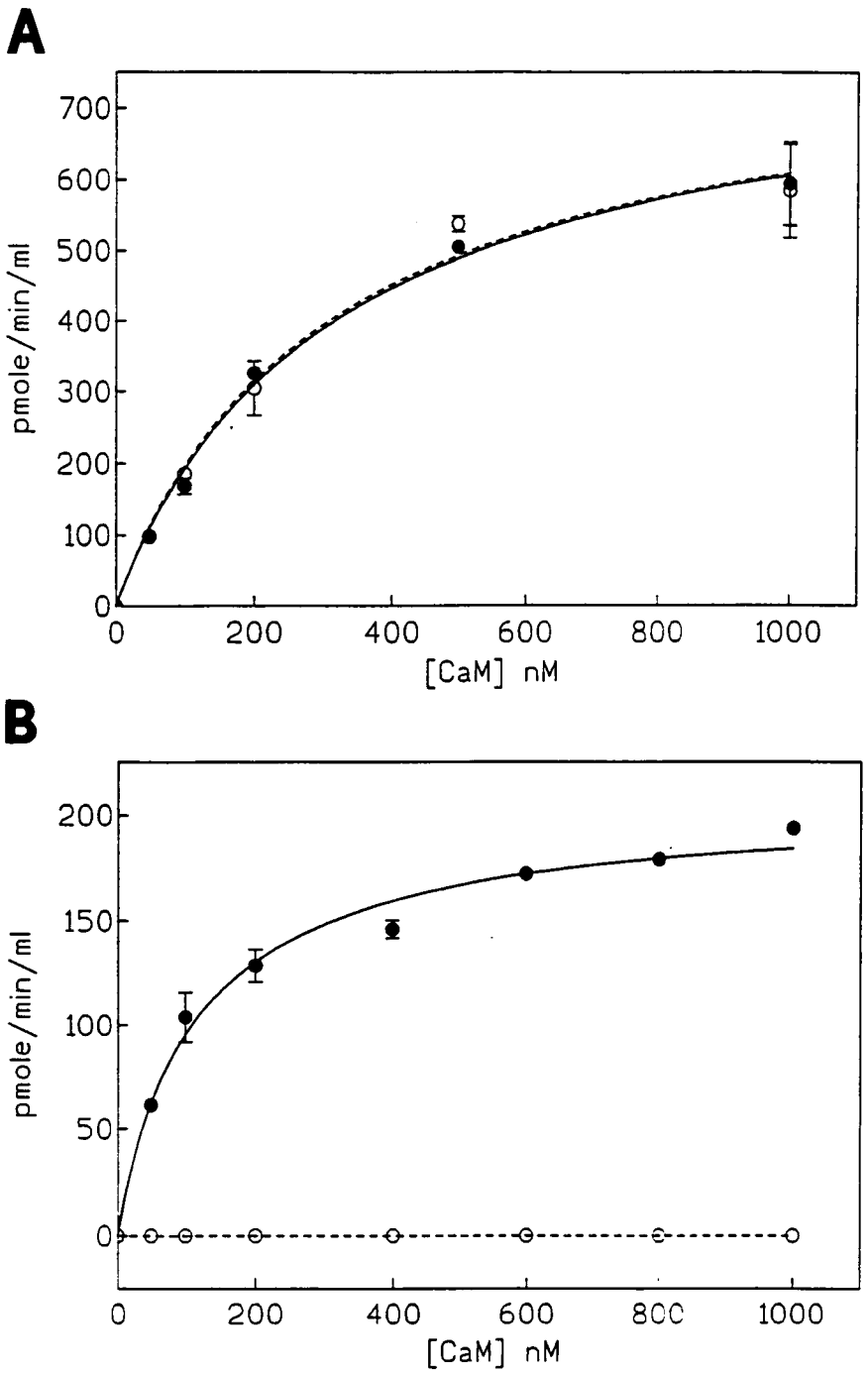


Figure 28. Comparison of the calmodulin methyltransferase activities with VU-1 and V136T calmodulin substrates. Pseudofirst order kinetic plots (v_i vs. [CaM]) with a constant saturating concentration of S-adenosylmethionine (12 μM) and with VU-1 (●) or V136T (o) calmodulins as the varied substrates. Assays were done in the presence of: **(A)** 1 mM $CaCl_2$ or **(B)** 1 mM EGTA. The error bars show the standard error of the mean.

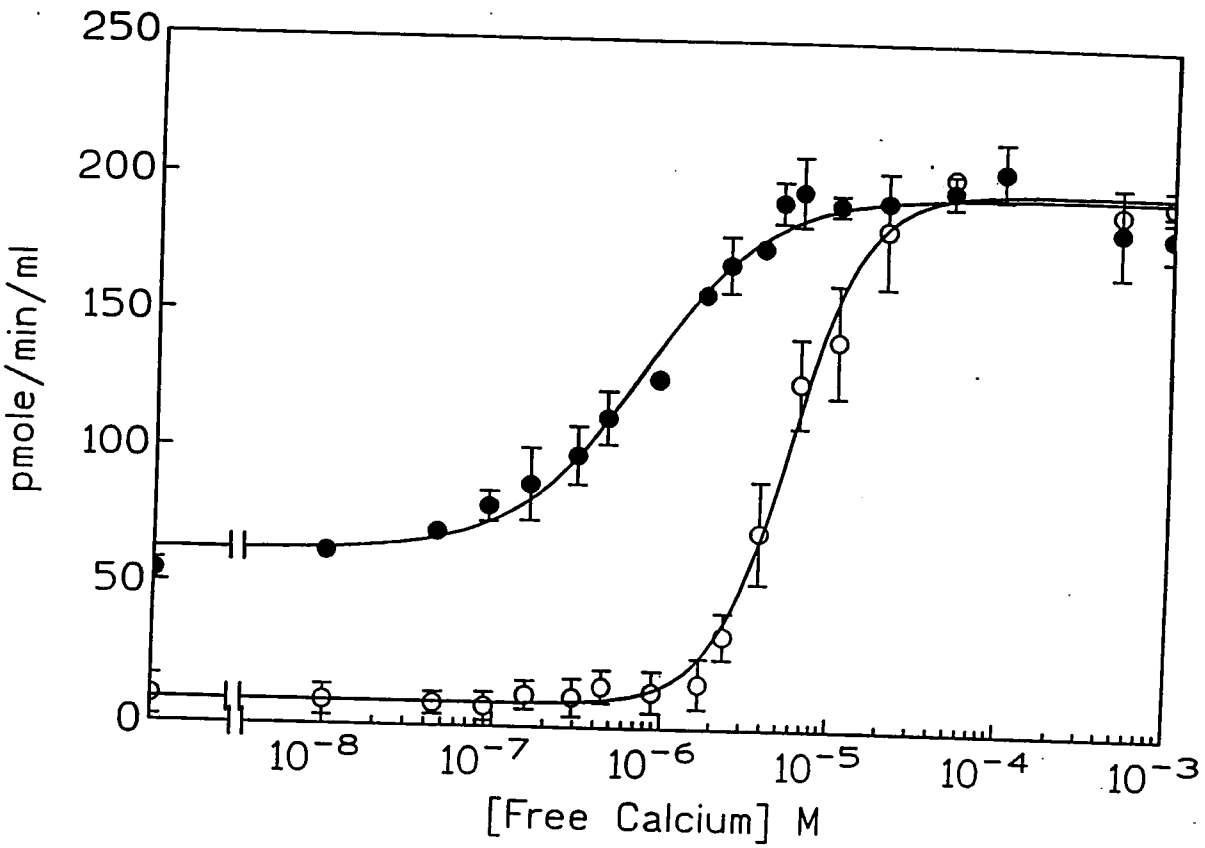


Figure 29. Calcium dependence of the methylation of VU-1 and V136T calmodulins. Methyltransferase activity was measured over the range of calcium concentrations with 1 μM VU-1 (●) or 1 μM V136T (o) calmodulins. Free calcium concentrations were controlled by using a EGTA/Ca^{+2} buffering system as described in the Materials and Methods. The other assay components were: 12 μM S-adenosylmethionine, 2 mM $MgCl_2$, 0.15 M KCl, 0.1 M glycylglycine, pH 8.0. Error bars show the standard error of the mean.

by calcium concentrations within the physiological range of intracellular calcium fluxes with a $K_{0.5}$ observed at 0.71 μM Ca^{2+}. However, similar to the results with NAD kinase activation (Fig. 27), the methylation rate of V136T calmodulin as a function of calcium concentration shows a ten-fold shift in the $K_{0.5}$ to 7.2 μM Ca^{2+} (Fig. 29). Further, unlike VU-1 calmodulin, V136T calmodulin showed little or no methylation below 1 μM free Ca^{2+} (Fig. 29). Overall, the findings suggest that the substitution of thr-136 results in an increase in the amount of calcium required for enzyme activation and methylation, and also a lower ability of the methyltransferase to recognize the calcium-depleted form of the mutant calmodulin.

Calcium-binding properties of VU-1 and V136T calmodulins

The findings that V136T shows a shift in calcium-dependent enzyme activation and methylation suggest a defect in calcium-binding. To address this directly the calcium binding properties of VU-1 and V136T calmodulins were analyzed by flow dialysis in the presence of physiological concentrations of K^+ and Mg^{2+} (Fig. 30). VU-1 calmodulin bound four equivalents of calcium, with half saturation ($K_{0.5}$) observed at a [Ca^{2+}] of 10.7 μM. This is identical within experimental error to the value (14 μM) reported previously (Haiech et al., 1991). Similar to previous reports of various calmodulins (Crouch & Klee, 1980; Haiech et al., 1981; Minowa & Yagi, 1984; Yazawa et al., 1987; Haiech et al., 1991; Linse et al., 1991;

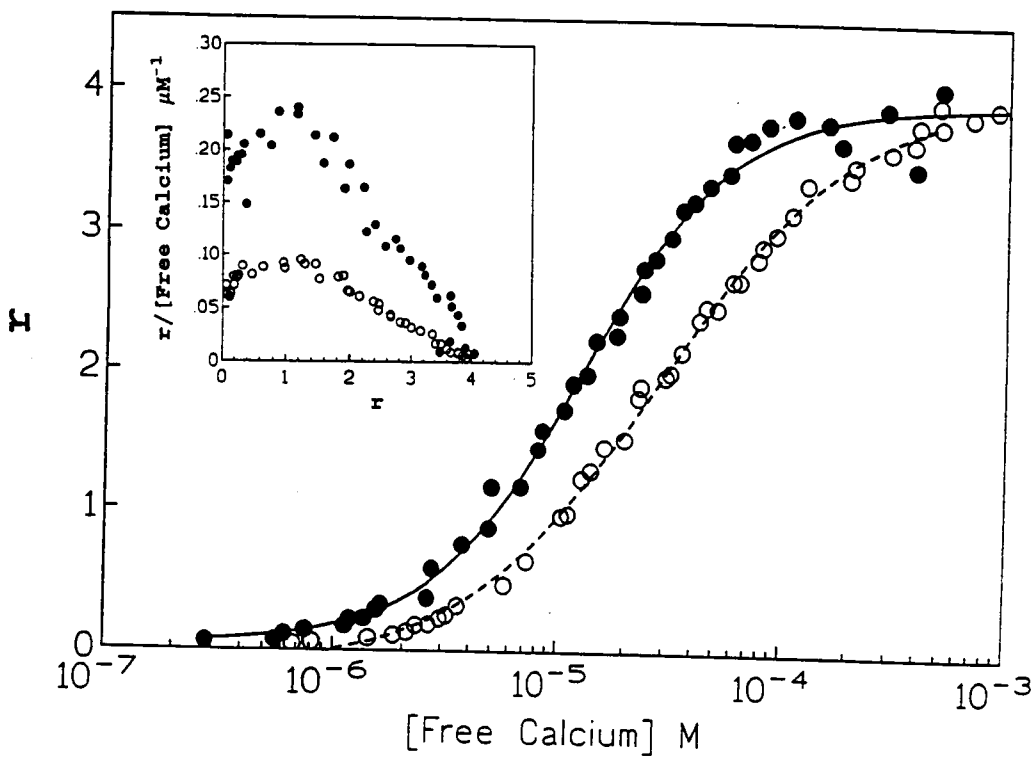


Figure 30. Calcium binding isotherms of VU-1 and V136T calmodulins. Calcium binding curves of VU-1 (●) and V136T (○) were generated by flow dialysis as described in the Materials and Methods. Calmodulin concentrations were 30 μM in 50 mM Hepes-NaOH buffer, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$. **r** denotes the mole ratio of calcium per calmodulin. The inset shows a Scatchard plot of the data.

Maune et al., 1992; reviewed in Klee, 1988), positive cooperativity was observed (Fig. 30, inset), with a calculated Hill co-efficient of 1.23 (Table IV). The apparent macroscopic binding constants calculated by fitting the data to the Adair equation (Table IV) were similar to those of several other calmodulin binding curves evaluated by this model (Crouch & Klee, 1980; Haiech et al., 1981; Porumb, 1994; Linse et al., 1991; Maune et al., 1992; reviewed in Klee, 1988), and can be divided into a high affinity pair (K_1 and K_2), and a low affinity pair (K_3 and K_4) of binding sites. Several previous studies (reviewed in Klee, 1988) indicate that the high affinity sites are found within the carboxyl terminal lobe.

V136T calmodulin also binds four equivalents of calcium with a $K_{0.5}$ that is approximately three-fold higher (28.8 μM) than that of VU-1 calmodulin. Positive cooperativity is still apparent in Scatchard plots (Fig. 30, inset), although the calculated Hill co-efficient (1.06) is lower than that of VU-1 calmodulin (Table IV). The calcium-binding data of V136T calmodulin was also well fitted to the Adair equation. Comparison of individual macroscopic constants to those of VU-1 calmodulin is difficult because of the uncertainty of the relationship of each binding event to a particular site of the wild type and mutant proteins. However, the binding constants calculated for the V136T calmodulin are lower than the corresponding constants for VU-1 calmodulin. Only one

Table IV. Calcium binding parameters for VU-1 and V136T

CaM	K_1[a]	K_2	K_3	K_4 (μM^{-1})	α[b]
VU-1	0.152 (0.049)	0.187 (0.084)	0.022 (0.013)	0.065 (0.031)	1.23
V136T	0.048 (0.009)	0.136 (0.029)	0.012 (0.002)	0.011 (0.001)	1.06

[a] K_1, K_2, K_3 and K_4 are the apparent macroscopic binding constants calculated by fitting the data in Fig. 30 to the Adair-Klotz equation as described in Materials and Methods. The R^2 for the fit was 0.9995 for V136T calmodulin and 0.9979 for VU-1 calmodulin. The numbers in parentheses indicate standard deviations of values obtained from fitted binding data.

[b] α is the Hill co-efficient obtained by fitting the data in Figure 30 to the Hill equation as described in Materials and Methods.

macroscopic constant (K_2) within the range of the high affinity calcium binding sites was calculated.

Tyrosine 138 fluorescence spectroscopy

VU-1 calmodulin has a single tyrosine at position 138 and has no tryptophan. Tyr-138 is found within the fourth calcium binding site and undergoes a large increase in the fluorescence yield upon calcium binding (Kilhoffer et al., 1981; Kilhoffer et al., 1989). This change occurs upon the first two calcium binding events, and thus is a good reporter for calcium binding to the high affinity sites in the carboxyl terminal lobe.

The steady state tyrosine fluorescence spectra of VU-1 and V136T calmodulins were virtually identical in the presence of calcium , q = 0.070 (S.E. = 0.0030) for VU-1 and 0.071 (S.E. = 0.0025) for V136T respectively (Fig. 31A). The calcium-depleted forms of both calmodulins also showed a decrease in the fluorescence yield (Fig. 31B). However, calcium-depleted V136T calmodulin showed a higher fluorescence yield q = 0.048 (S.E. = 0.0015) than that of VU-1 calmodulin (q = 0.037, S.E. = 0.0025). This suggests a difference in the environment of Tyr-138 between the two calmodulins in the absence of calcium. Calcium titration of the fluorescence signal shows that the $K_{0.5}$ (i.e., calcium concentration required for a half maximal increase in the fluorescence signal) was seven-fold higher for V136T calmodulin than for VU-1 calmodulin (Fig.32). This result, together with the

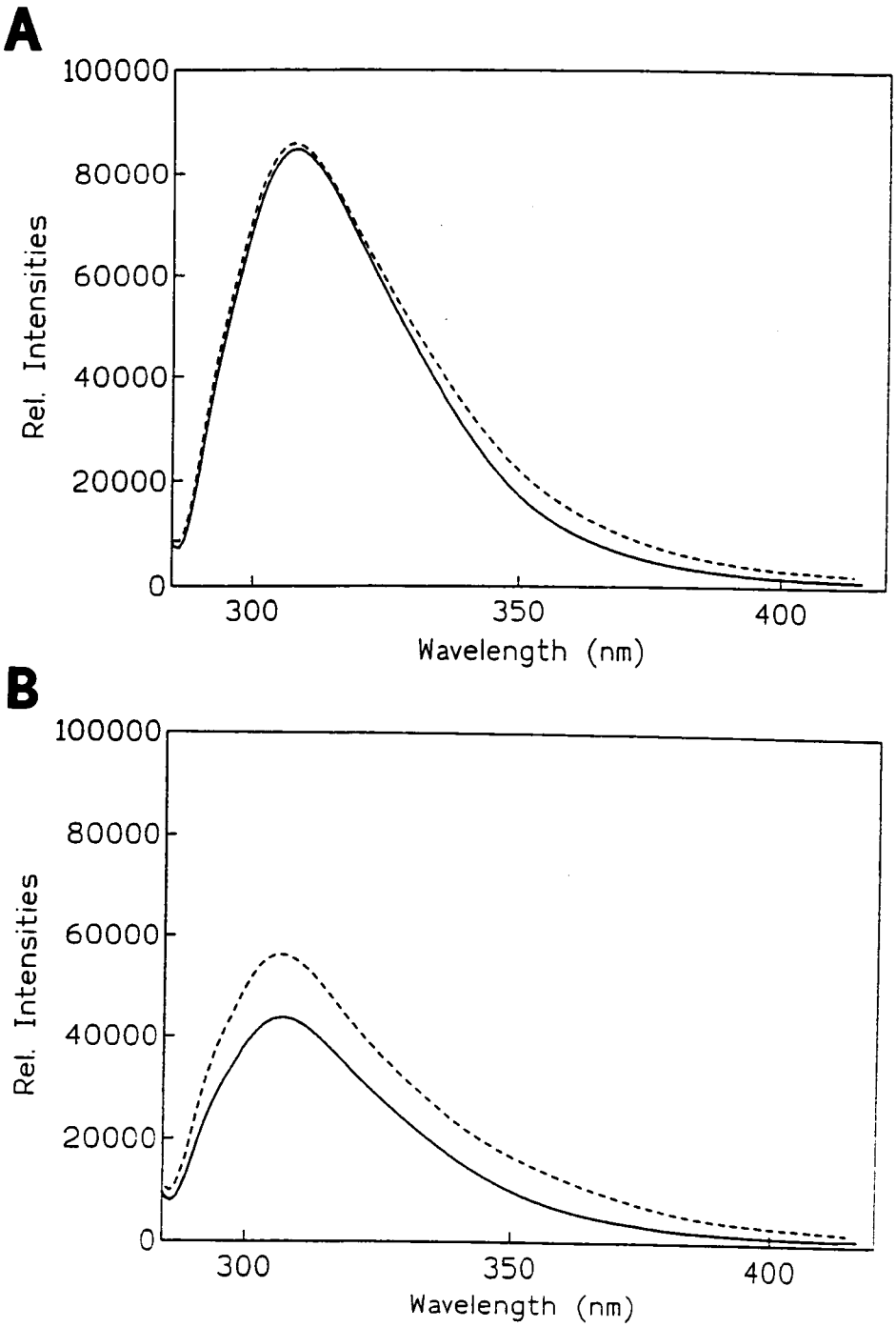


Figure 31. Calcium-dependent changes in the fluorescence spectra of VU-1 and V136T calmodulins. All calmodulin concentrations were 30 μM in 50 mM HEPES-NaOH buffer, pH 7.5, and 0.1 M KCl. Steady state fluorescence spectra of VU-1 calmodulin **(solid line)** and V136T calmodulin **(dashed line)** in 1 mM $CaCl_2$ **(Panel A)** or 1 mM EGTA **(Panel B)**. The excitation wavelength was 275 nm. Fluorescence intensity in arbitrary units is plotted against wavelength.

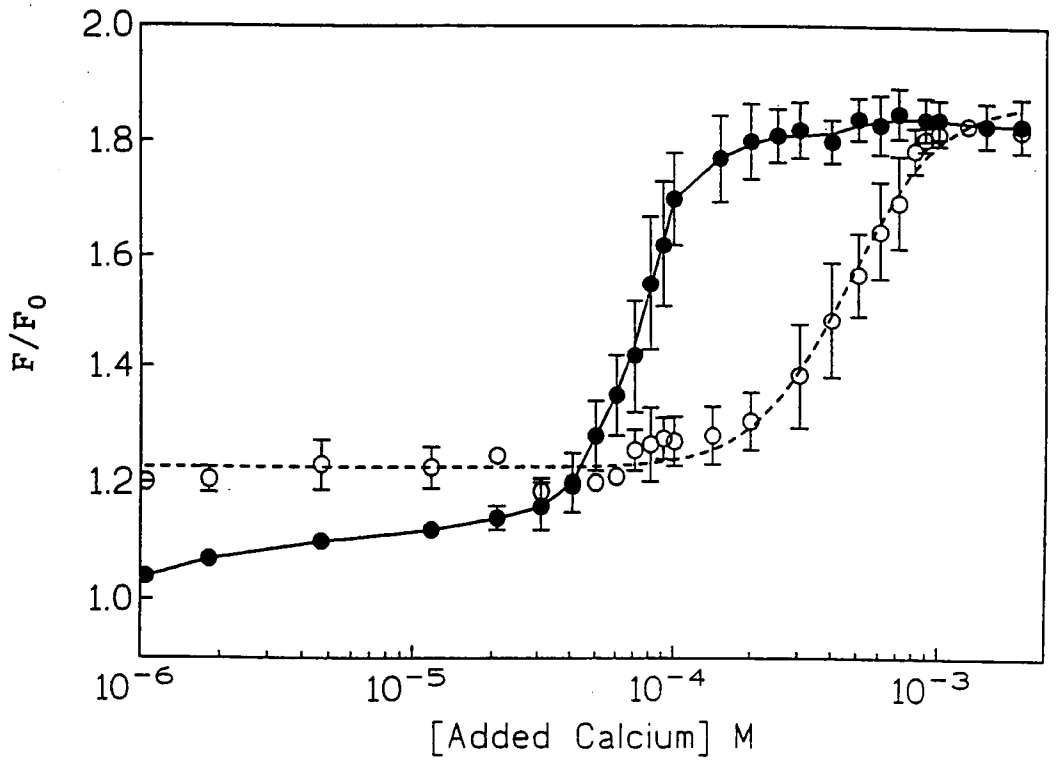


Figure 32. Changes in the fluorescence quantum yield of VU-1 (●) and V136T (○) calmodulins as a function of calcium. F/F_0 is the normalized fluorescence intensity obtained by dividing the observed fluorescence intensity of each sample **(F)** at 306 nm by the fluorescence intensity of VU-1 calmodulin **(F_0)** in the presence of 1 mM EGTA at 306 nm. The excitation wavelength was 275 nm. Error bars show the standard error of the mean.

calcium-binding and activator data, strongly suggests that the V136T mutation results in a lowered calcium binding affinity of sites in the carboxyl terminal domain.

Urea unfolding studies

As discussed above, the substitution of a threonine at position 136 could disrupt hydrophobic interactions leading to a reduced stability of the COOH-terminal lobe. Previous work has shown that the secondary structure of the calcium-depleted form of calmodulin is less stable than the calcium-saturated form of the molecule (Martin & Bayley, 1986; Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995). From these observations, and the findings here that the calcium-depleted form (but not the calcium-saturated form) of V136T calmodulin differs structurally and functionally from VU-1 calmodulin, it is possible that the thr-136 substitution may selectively affect the stability of the calcium-depleted structure. To address this possibility, the urea-induced unfolding of calcium-depleted VU-1 and V136T calmodulins were analyzed. Similar to previous studies with mammalian calmodulin (Martin and Bailey, 1986), circular dichroism spectra were used to monitor unfolding.

In the absence of calcium, the far UV circular dichroism spectra of V136T and VU-1 calmodulins were essentially identical (Fig. 33). In addition, the urea-induced unfolding curves (Fig. 34) for the two calmodulins were highly similar with the ΔG_{H2O} calculated to be 1.92 kcal/mol (S.E. = 0.25)

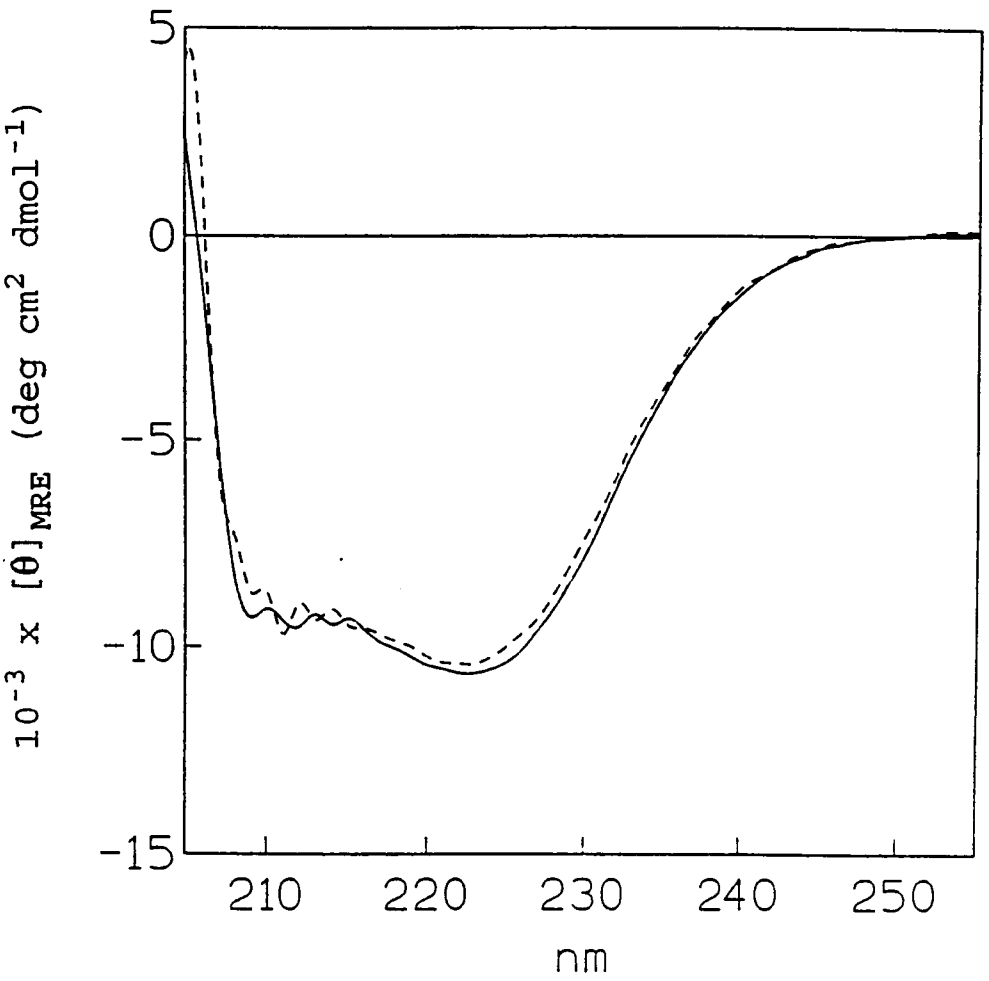


Figure 33. Far-UV CD spectra of VU-1 and V136T calmodulins in the absence of calcium. Spectra are shown for VU-1 **(solid trace)** and V136T **(dashed trace)** calmodulins (0.166 mg/ml) in 20 mM HEPES, pH 7.5 and 1 mM EGTA. The spectra are the average of five scans. The mean residue ellipticity ($[\theta]_{MRE}$) is plotted against wavelength.

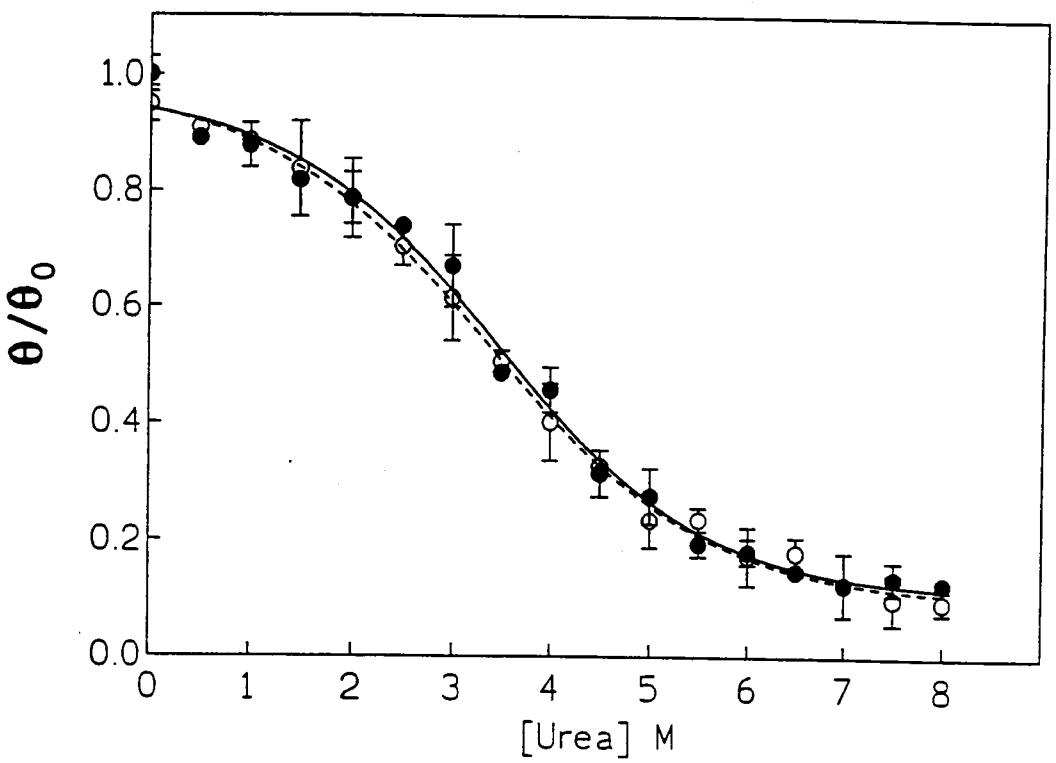


Figure 34. Urea denaturation curves of VU-1 and V136T calmodulins in the presence of 1 mM EDTA. The circular dichroism spectra at 222 nm were recorded over the range of urea concentrations with 5 μM of VU-1 (●) and V136T (○) in 10 mM Tris-HCl, pH 7.0, and 1 mM EDTA buffer. θ/θ_o denotes the ellipicity (θ) at 222 nm of each sample at the urea concentration indicated divided by the ellipticity of native VU-1 calmodulin (θ_o) at 222 nm. Error bars show the standard error of the mean.

for VU-1 calmodulin and 1.76 kcal/mol (S.E. = 0.13) for V136T calmodulin. Midpoints were estimated to occur at 3.5 M urea, which is similar to the value observed by Martin and Bayley (1986) for vertebrate calmodulin. Despite the higher midpoint values, relatively small M_G values were observed for both calmodulins (-559 cal/mol/M, S.E. = 65 for VU-1 calmodulin and -527 cal/mol/M,S.E. = 34 for V136T calmodulin). Based on the relationship:

$$\text{Midpoint of unfolding curve} = \Delta G_{H2O}/M_G$$

these lower M_G values result in a ΔG_{H2O} that is relatively lower than other proteins (Pace et al., 1990). Despite these considerations, the data clearly show that the thr-136 mutation does not appear to significantly affect the average α-helical content nor stability of the protein.

Design of a methylation site on the NH_2-terminal lobe of calmodulin

Sequence analysis of calmodulin (Fig. 1) and comparison of the structures of the NH_2- and COOH-terminal lobes of calmodulin (Figs. 3 and 35) show partial sequence homology and structural similarity. As discussed above, the site of methylation lies on a six amino acid loop (LGEKLT, residues 112-117) that lies between EF hands 3 and 4 (Fig. 1). A similar loop region (LGQNPT, residues 39-44) resides at a homologous region between EF hands 1 and 2 (Fig. 1). As shown in Fig. 35, superimposition of these two regions show that their peptide backbones

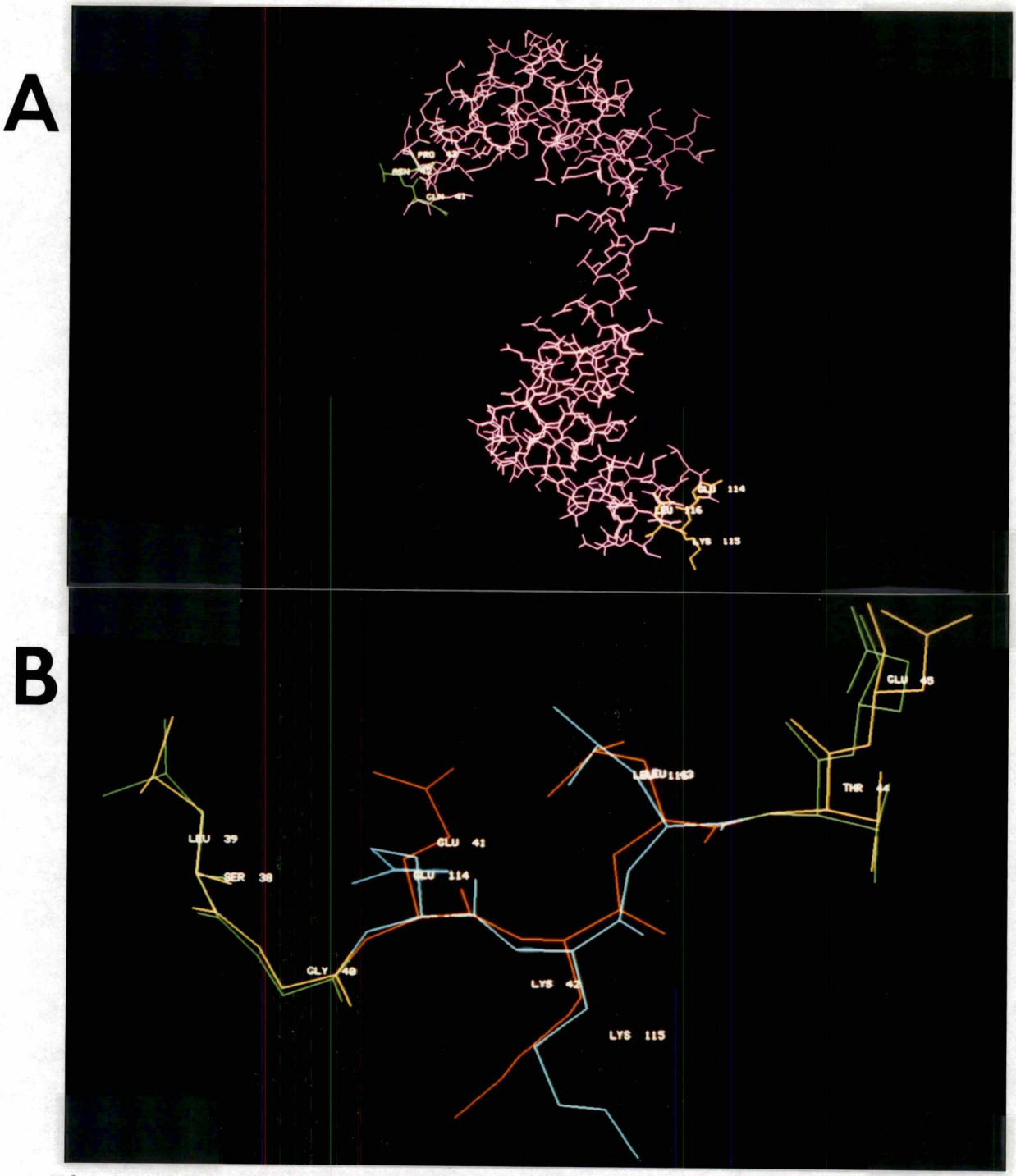


Figure 35. Overlaid backbone structures of residues 40-44 and 113-117 of calmodulin. **A.** X-ray crystal structure of bovine calmodulin (Babu et al., 1988). The methylation site region (GEKLT, residues 113-117) of the COOH-terminal lobe and its symmetric region (GQNPT, residues 40-44) of the NH_2-terminal lobe are shown. **B.** Overlaid backbone structure between residues 40-44 and 113-117 of bovine calmodulin. For the sake of illustration, the EKL sequence was inserted at residues 41-43 of the structure. The structures were superimposed by using a Silicon Graphics Indigo system and the Insight II software.

adopt a similar conformation. Thus, it is conceivable to use the amino terminal lobe as a scaffold to build a methylation site.

To this end, VU-1 calmodulin was engineered to: 1. remove the lys-115 methylation site in the COOH-terminal lobe; and 2. to introduce a methylation site at a homologous position in the loop between EF hand domains 1 and 2. This was accomplished in a double mutant calmodulin by introducing an EKL sequence at positions 41-43 of VU-3 calmodulin (Fig. 23) which has an arginine residue at position 115.

The resulting mutant calmodulin [QNP(41-43)EKL/K115R] was generated and was investigated for its ability to serve as a methylation substrate. No detectable methylation of the mutant calmodulin was observed under any conditions even at high concentrations (e.g., 5 μM, 50-fold of the K_m value of VU-1 calmodulin).

Previous studies show that VU-3 calmodulin is an effective competitive inhibitor of the enzyme (Oh and Roberts, 1990). To test the possibility of competitive inhibition by the COOH-terminal lobe of QNP(41-43)EKL/K115R calmodulin, the NH_2-terminal lobe (residues 1-77) of the mutant calmodulin was produced via tryptic digestion and HPLC fractionation as described above. Although this treatment removes the COOH-terminal region, methylation of the isolated NH_2-terminal tryptic fragment was still not observed. This finding suggests that introduction of a lysine residue at position 42

of the NH_2-terminal lobe is not in itself sufficient for binding and recognition by the methyltransferase.

To ensure that the QNP(41-43)EKL/K115R calmodulin was functional as an enzyme activator, its ability to activate NAD kinase was tested. Interestingly, even at high concentrations of calmodulin (100 nM) the mutant calmodulin [QNP(41-43)EKL/K115R] was only able to activate NAD kinase to a level that was 28 % of the value of VU-3 or VU-1 calmodulin (Fig. 36). Thus, despite the similarity of the predicted backbone conformation of 41-43 and 114-116 of calmodulin, NAD kinase activation is sensitive to the change of loop sequences between EF hand domains I and II.

VU-1 calmodulin-DHFR sandwich protein

As discussed above, calmodulin function is linked to the ability of the two lobes to "clasp" a hydrophobic, basic target structure (O'Neil and Degrado, 1990; Clore et al., 1993). A novel approach to studying calmodulin/protein structure and function was developed by making a "sandwich protein" which was constructed by insertion of VU-1 calmodulin into the β-bulge region of bacterial chromosomal dihydrofolate reductase (DHFR) (Fig. 37).

DHFR catalyzes the NADPH dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8,-tetrahydrofolate (THF). DHFR has a β-bulge region between two consecutive β-type hydrogen bonds which includes positions 136 and 137 on one strand and a position 155 on the other strand (Bolin et al., 1982;

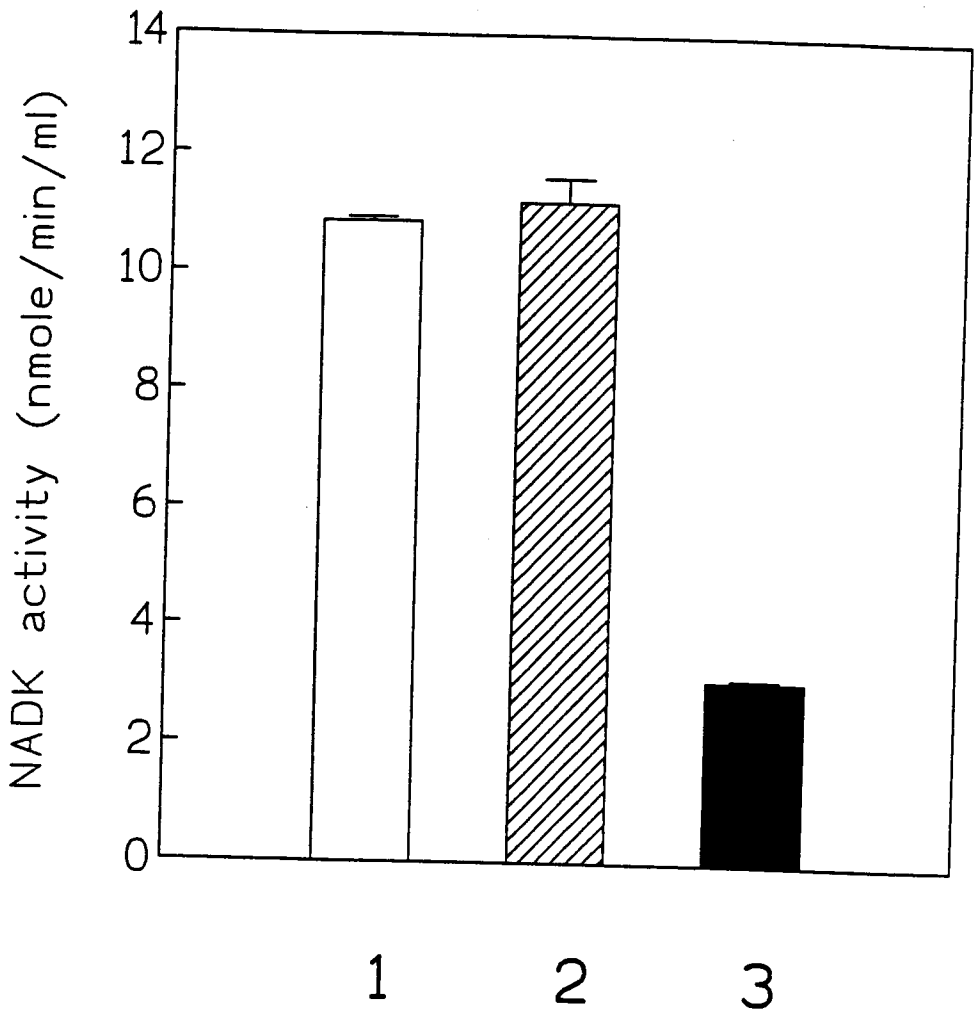


Figure 36. NAD kinase activation by VU-1, VU-3, and [QNP(41-43)EKL/K115R] mutant calmodulin. The histograms show NAD kinase activation by a saturating dose (100 nM) of each calmodulin in the presence of calcium. NAD kinase activation by VU-1 (**1**), VU-3 (**2**), and [QNP(41-43)EKL/K115R] calmodulin (**3**) in the presence of 1 mM $CaCl_2$.

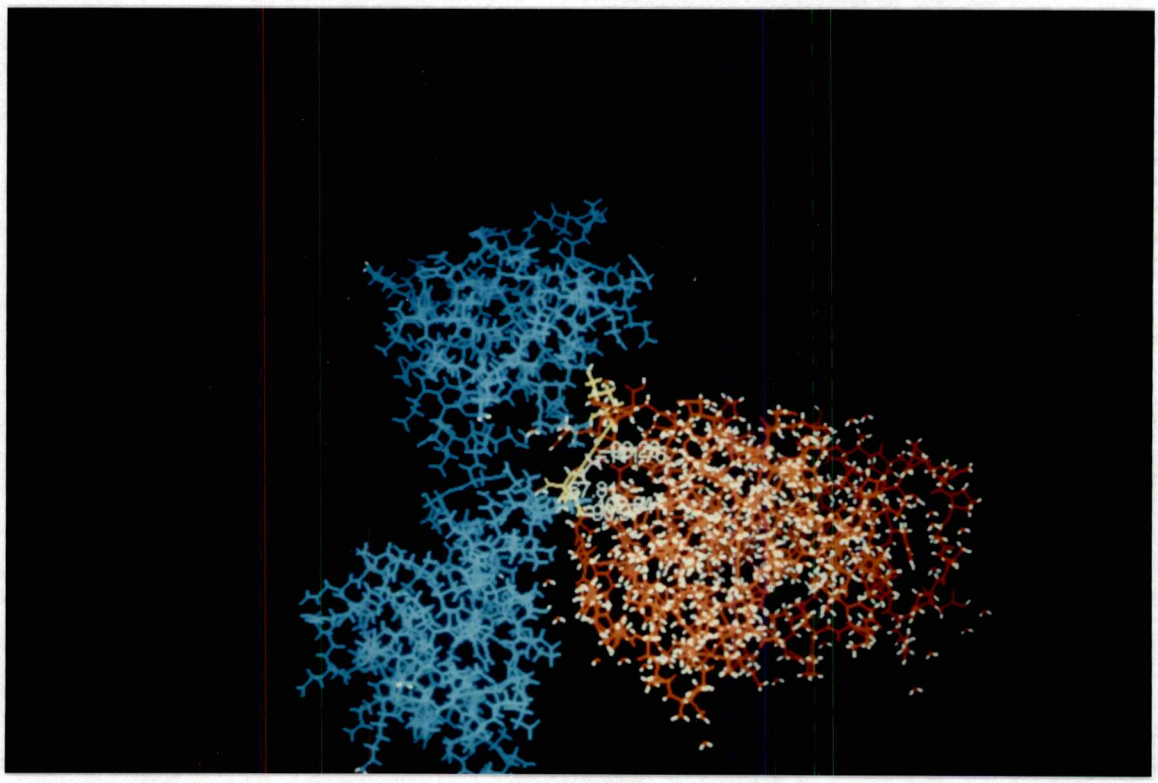


Figure 37. Constructed model structure of VU-1 calmodulin-DHFR sandwich protein. The primary sequence of the sandwich protein is shown in Fig. 4. The model was constructed by insertion of apo-form of calmodulin (blue color, Kuboniwa et al., 1995) into the β-bulge region (position 134 and 135) of bacterial chromosomal dihydrofolate reductase (DHFR) (red color, Filman et al., 1982). The linker region shown in Fig. 4 is not included in the model.

Filman et al., 1982). The role of β-bulges is not clear, however they might facilitate insertions or deletions in β-strands or position crucial residues by accentuating the local twist of the strands (Richardson et al., 1978; Sondek & Shortle, 1990, 1992). In the case of DHFR, insertions are tolerated in the β-bulge region, for example, residues 160 - 167 of human DHFR form a seven-residue disruption of the β-sheet hydrogen bonding, and make β-bulge, so called "β-blow-out" (Volz et al., 1982). The fact suggests that the possibility of the tolerance by insertion of sequences into the β-bulge region of bacterial DHFR. In this study, we took advantage of this property of DHFR to generate a sandwich protein in which VU-1 calmodulin was inserted into the β-bulge region of *E. coli* DHFR and transformants were selected by growth on trimethoprim. The sandwich protein was purified and its abilities to bind calcium, activate calmodulin-dependent enzymes and serve as a substrate for the methyltransferase were evaluated.

The calcium binding properties of the sandwich protein were analyzed by flow dialysis (Fig. 38). Similar to VU-1 calmodulin, the sandwich protein bound four equivalents of calcium, with half saturation ($K_{0.5}$) observed at a $[Ca^{2+}]$ of 8 μM. This value is slightly higher than that of VU-1 calmodulin (10.7 μM). Macroscopic binding constants obtained by fitting the data to the Adair equation were calculated and yielded values similar to VU-1 including a high affinity pair

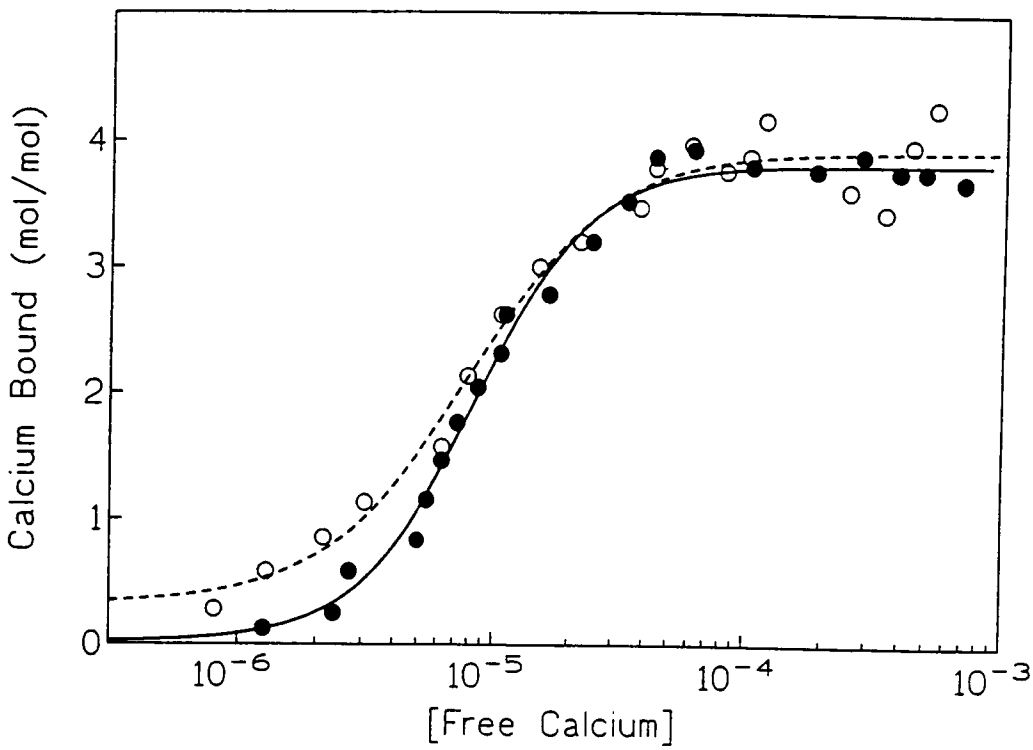


Figure 38. Calcium binding isotherms of VU-1 calmodulin-DHFR sandwich protein. Calcium binding curves of VU-1 (●) and sandwich protein (o) were generated by flow dialysis as described in the Materials and Methods. Protein concentrations were 30 μM in 50 mM Hepes-NaOH buffer, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$.

(K_1 and K_2) and lower affinity pair (K_3 and K_4) of binding sites (Table V). This result suggests that the insertion of VU-1 into the β-bulge of calmodulin does not adversely affect its ability to associate with calcium.

The ability of the sandwich protein to activate two calmodulin-dependent enzymes, NAD kinase (Fig. 39) and cyclic nucleotide phosphodiesterase (Fig. 40) was evaluated. The sandwich protein activated NAD kinase, but to only half of the level obtained with VU-1 calmodulin. The $K_{0.5}$ for both VU-1 and the sandwich protein were approximately the same (1-2 nM). PDE activation was also affected but in a different manner. The sandwich protein fully activated the enzyme, but the $K_{0.5}$ was shifted to a 3-fold higher value (6.8 pmol for VU-1 and 21 pmol for the sandwich protein). The addition of trimethoprim (1 μM) to the assay did not drastically affect the activation profile suggesting that DHFR portion of the sandwich protein is folded in the assay.

Methylation analyses of the sandwich protein show that insertion of VU-1 calmodulin into DHFR results in a large decrease in methylation (Figs. 41-43). The trend of the residual methylation is same, with calcium stimulating the rate of methylation of the sandwich protein (Fig. 41), with barely detectable methylation observed in the absence of calcium (Fig. 41, 42). Similar $K_{0.5}$ values were observed for two proteins (0.796 μM for VU-1 and 0.970 μM for sandwich protein), which is consistent with the similar calcium binding

Table V. Calcium binding parameters for Sandwich Protein

CaM	K_1[a]	K_2	K_3	K_4 (μM^{-1})
VU-1	0.152 (0.049)	0.187 (0.084)	0.022 (0.013)	0.065 (0.031)
SP[c]	0.158 (0.092)	0.252 (0.186)	0.036 (0.024)	0.040 (0.020)

[a] K_1, K_2, K_3 and K_4 are the apparent macroscopic binding constants calculated by fitting the data in Fig. 38 to the Adair-Klotz equation as described in Materials and Methods. The R^2 for the fit was 0.9340 for the sandwich protein and 0.9979 for VU-1 calmodulin. The numbers in parentheses indicate standard deviations of values obtained from fitted binding data.

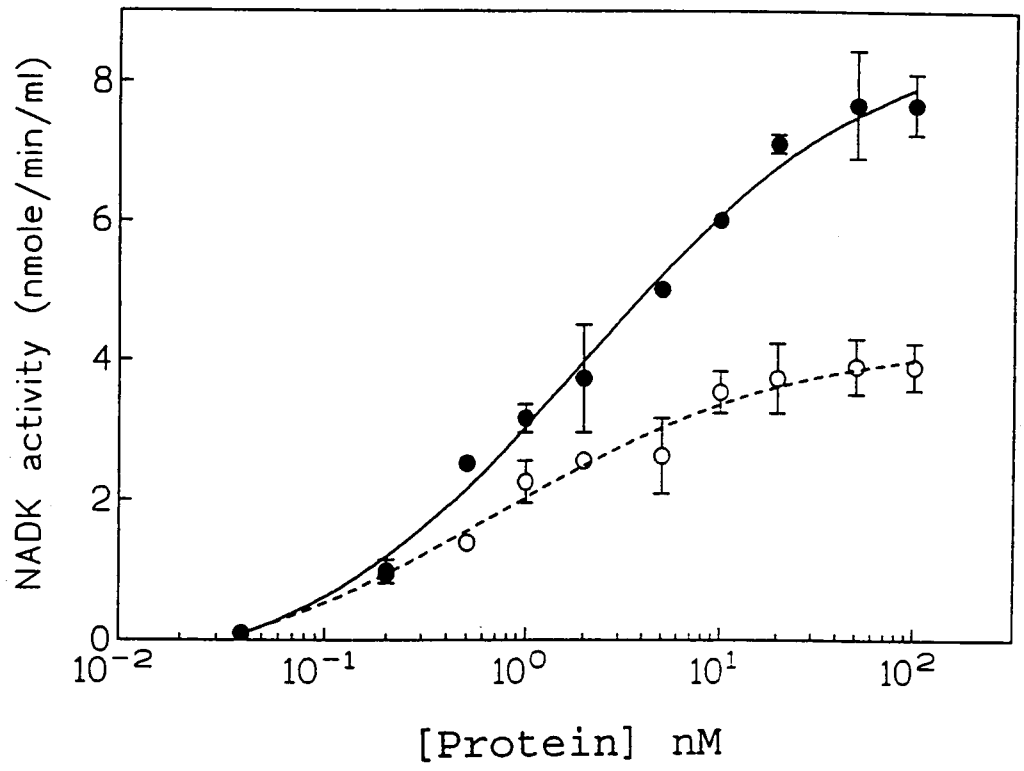


Figure 39. NAD kinase activation by VU-1 calmodulin-DHFR sandwich protein. NAD kinase activation by VU-1 (●) or VU-1 calmodulin-DHFR sandwich protein (o) were measured at various concentrations in the presence of 0.1 mM $CaCl_2$ as described in the Materials and Methods. Error bars show the standard error of the mean.

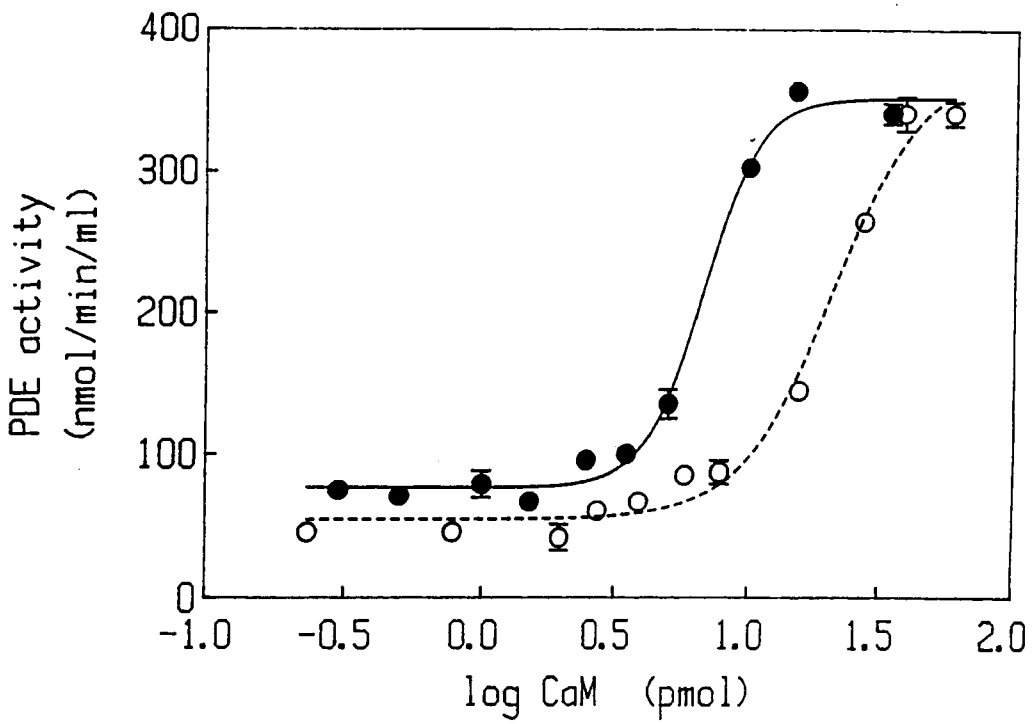


Figure 40. PDE activation by VU-1 calmodulin-DHFR sandwich protein. PDE activation of VU-1 (●) or VU-1 calmodulin-DHFR sandwich protein (○) were measured at various concentrations in the presence of 0.1 mM $CaCl_2$ as described in the Materials and Methods. Error bars show the standard error of the mean.

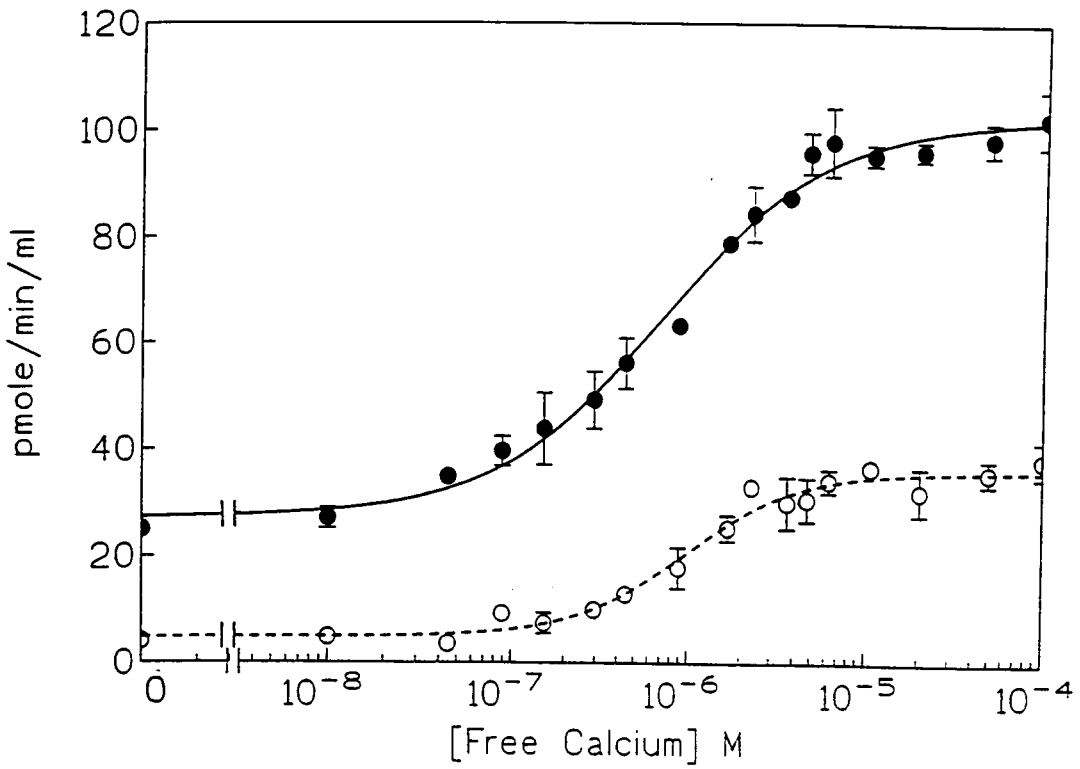


Figure 41. Calcium dependence of the methylation of VU-1 calmodulin-DHFR sandwich protein. Methyltransferase activity was measured over the range of calcium concentrations with 1 μM VU-1 (●) or 1 μM VU-1 calmodulin-DHFR sandwich protein (o). Free calcium concentrations were controlled by using EGTA/Ca^{+2} buffering system as described in the Materials and Methods. The other assay components were: 12 μM S-adenosylmethionine, 2 mM $MgCl_2$, 0.15 M KCl, 0.1 M glycylglycine, pH 8.0. Error bars show the standard error of the mean.

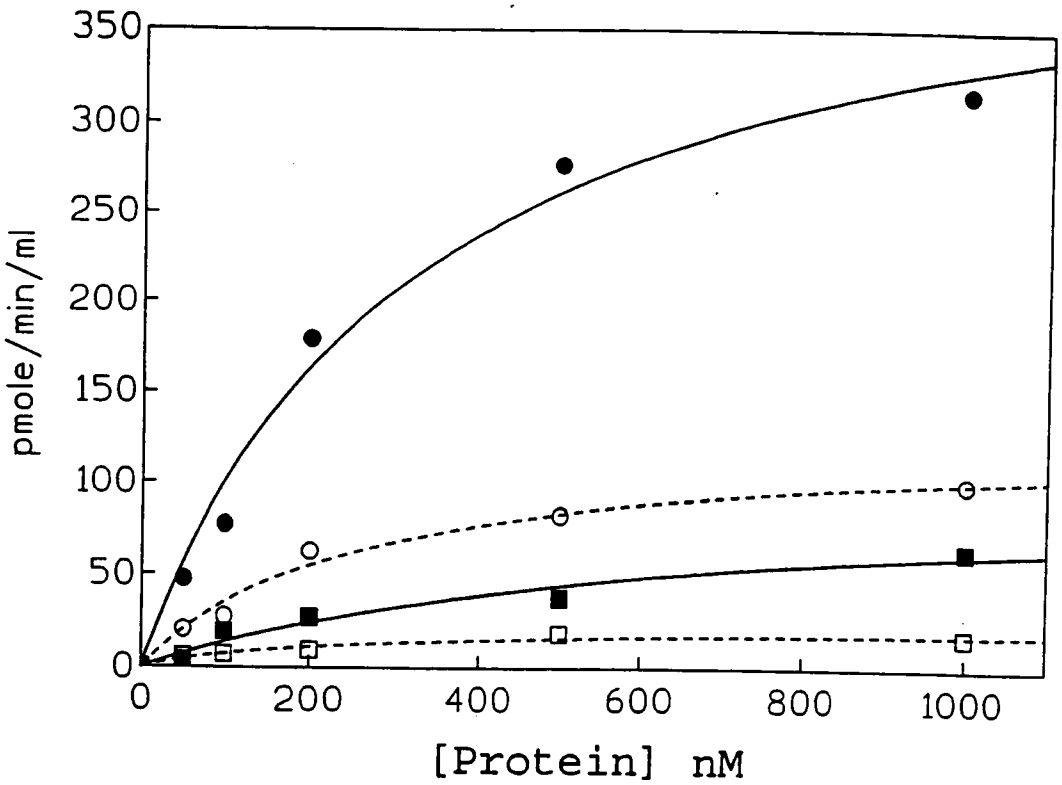


Figure 42. Kinetics of the methylation of VU-1 calmodulin-DHFR sandwich protein. Methyltransferase activities were measured with a saturated concentration (12.1 μM) of AdoMet. VU-1 calmodulin in the presence of 0.1 mM $CaCl_2$ (●); VU-1 calmodulin in the presence of 1 mM EGTA (○); VU-1 calmodulin-DHFR sandwich protein in the presence of 0.1 mM $CaCl_2$ (■); VU-1 calmodulin-DHFR sandwich protein in the presence of 1 mM EGTA (□).

properties of the two proteins based on calcium binding measurements (Fig. 38).

Methyltransferase activities were measured at a high, fixed concentration (12.1 μM) of AdoMet with sandwich protein as variable substrate (Fig. 42). The V_{max} observed with the sandwich protein (95 nmole/min/ml) was only 22 % of the value observed with VU-1 (436 nmol/min/ml) in the presence of calcium. However, the apparent Km for sandwich protein (579 nM) was only 1.7-fold higher than apparent Km for VU-1 (334 nM) in the presence of calcium.

Addition of TMP to the reaction inhibited the observed methylation rate substantially (Fig. 43), raising the possibility that the low level of methylation observed may have been due to a small percentage of sandwich protein that has an unfolded DHFR domain but still has an intact calmodulin domain that allows methyltransferase recognition. However, a modest decrease in the rate of VU-1 calmodulin methylation is also observed with TMP, and a direct effect of this reagent on the methyltransferase or on calmodulin cannot yet be ruled out. Regardless, the results strongly suggest that the insertion of calmodulin into the DHFR protein significantly perturbed the methyltransferase recognition site.

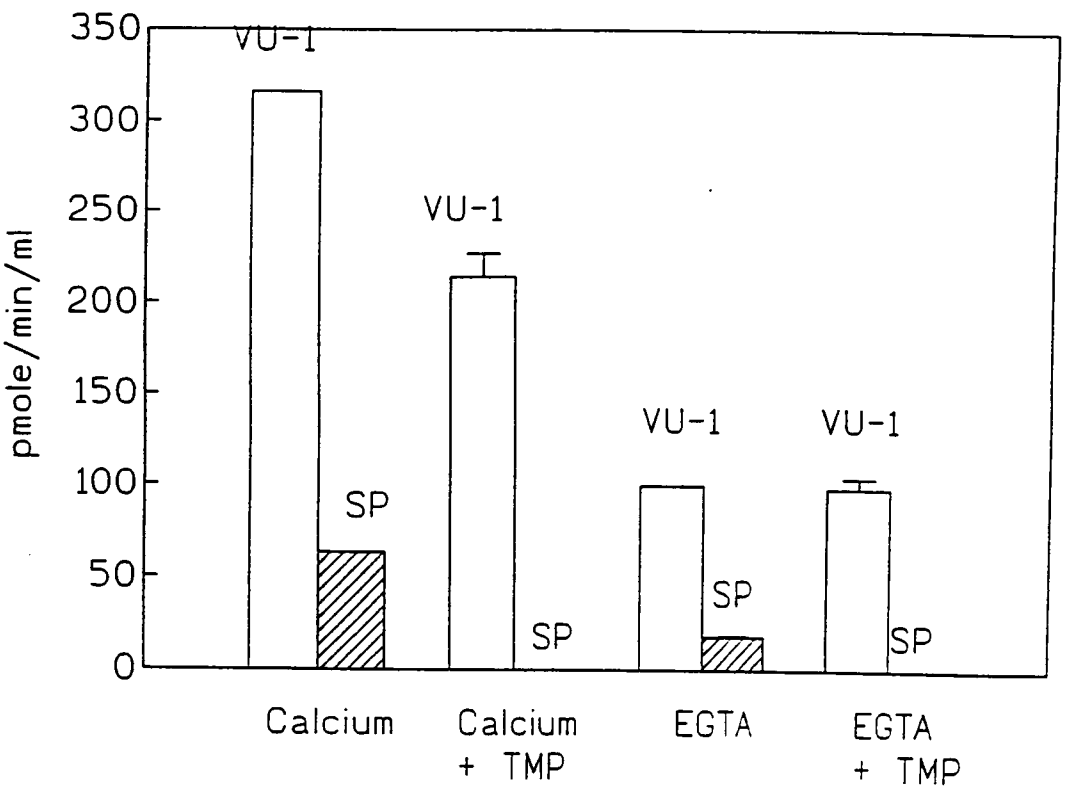


Figure 43. Calmodulin N-methyltransferase activities in the presence or absence of TMP. Methyltransferase activities were measured with a saturated concentration (12.1 μM) of AdoMet with VU-1 (1 μM, open bars) or the VU-1 calmodulin-DHFR sandwich protein (1 μM, cross hatched bars) in the presence or absence of 3.4 μM of trimethoprim (TMP), and in the presence of either 0.1 mM $CaCl_2$ or 1 mM EGTA.

CHAPTER IV

DISCUSSION

The calmodulin N-methyltransferase

In an effort to understand how the calmodulin N-methyltransferase binds and recognizes calmodulin with high specificity, the enzyme was purified from sheep brain and its enzyme activity was investigated under conditions that affect calmodulin structure. Calmodulin N-methyltransferase, the enzyme responsible for the trimethylation of lysine-115 of calmodulin, is a monomer of 38,000 MW based on gel filtration and SDS-PAGE analyses. Under physiological conditions of ionic strength and pH, the calcium-bound form of calmodulin is the preferred substrate for the enzyme. Similar to most calcium/calmodulin-dependent enzyme activities, the ability of the calmodulin methyltransferase to recognize and methylate calmodulin is sensitive to methionine oxidizing agents as well as to peptides and drugs that interact with calmodulin in a calcium-dependent manner. However, unlike most calmodulin-dependent enzymes, removal of the NH_2-terminal lobe (residues 1-77) of calmodulin does not affect the ability of the calmodulin methyltransferase to recognize and methylate lysine-115. Overall, the data suggest that determinants for calmodulin methyltransferase binding reside in the COOH-terminal lobe of calmodulin. Further, structural features within this region that are manifested upon calcium-binding may contribute to the interaction of calmodulin with the

enzyme.

As shown in Table VI, calmodulin N-methyltransferases have been purified from three mammalian tissues (Sitaramayya et al., 1980; Rowe et al., 1986; Morino et al., 1987; Han et al., 1993) and from *Paramecium* (Pech & Nelson, 1994). Only two homogeneous preparations of the enzyme have been obtained, from sheep brain (Han et al., 1993) and *Paramecium* (Pech & Nelson, 1994). Comparison of the enzyme kinetics, specificity, and regulation of the various calmodulin methyltransferase enzymes show considerable similarities, but also clear differences.

For example, while the enzyme purified from *Paramecium* showed a similar molecular weight to that from sheep brain, the enzyme from rat brain (Morino et al., 1987) and rat testis (Rowe et al., 1986) showed different apparent molecular weights (55,000-57,000 MW). The reason for this discrepancy is not clear but may simply be due to species differences. With respect to the kinetics of methylation, similar values of K_m for AdoMet (0.8 -2.0 μM) and calmodulin (20 - 100 μM) have been reported (Table VI). With respect to the highly purified, preparations of the enzyme (Morino et al., 1987; Han et al., 1993; Pech & Nelson, 1994), similarities in fold-purification and V_{max} of the final product (7-14 nmole/min/mg protein) have been reported. From this V_{max} value, we calculated a k_{cat} of 0.0287 sec^{-1} for the sheep brain methyltransferase. This seems to be an usually low turnover rate. Considering the

Table VI. Comparison of calmodulin N-methyltransferase isolated from various sources.

Source	MW	Km for CaM	Km for AdoMet	Activated by	Inhibited by	References
Rat brain	-	-	-	Ca^{2+}, Mn^{2+}	EGTA	(1)
Rat testis	55,000	-	-	-	-	(2)
Rat brain	57,000	22 nM	0.8 μM	Ca^{2+}, Mn^{2+}	EGTA	(3)
Sheep brain	-	50 nM	-	Ca^{2+}	VU-3, VU-4	(4)
Sheep brain	38,000	100 nM	2.22 μM	Ca^{2+}	EGTA, W-7 CaMK II peptide	(5)
Paramecium	37,000	-	1.0 μM	Ca^{2+}, Mg^{2+}, Mn^{2+}	calmidazolium mellitin, SAH Zn^{2+}	(6)

(1) Sitaramayya et al. (1980), (2) Rowe et al. (1986), (3) Morino et al. (1987), (4) Oh and Roberts (1990), (5) Han et al. (1993), (6) Pech & Nelson (1994).

reported lability of the enzyme (Rowe et al., 1986; Morino et al., 1987), one possible explanation for the low k_{cat} is that the final product only retains partial activity (e.g. only a fraction of the enzyme preparation is active). However, we consistently observe the same specific activity in our preparations and the enzyme remains stable under the buffer and storage conditions used. Another possibility may be related to the kinetic mechanism of methyltransferases. For example, studies with the *EcoRI* DNA methyltransferase show that it also has a relatively low k_{cat} (0.124 sec^{-1}) (Reich & Mashhoon, 1991). Pre-steady state kinetics of this enzyme show that the rate constant for methyltransfer is 300-fold higher than k_{cat}, suggesting that steps following methyltransfer (e.g., product release) limit the catalytic rate (Reich & Mashhoon, 1993). Since methylated calmodulin has a similar affinity for the enzyme as unmethylated calmodulin (Oh & Roberts, 1990; Roberts et al., 1990), the rate of product release might also be limiting for the calmodulin methyltransferase.

All calmodulin methyltransferases tested (Table VI) show stimulation by divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+} or Ni^{2+}) and inhibition by EGTA. However, there is not a consensus concerning the types of ions that stimulate the activity. Ca^{2+} and Mn^{2+} stimulate all activities tested (Table VI), whereas Mg^{2+} is more variable and either stimulates strongly (Pech & Nelson, 1994), weakly (Morino et al., 1987) or not at

all (Sitaramayya et al., 1980). Further, Pech and Nelson (1994) have showed that Ni^{2+}, which has a similar ionic radius as Mg^{2+}, also selectively stimulates the *Paramecium* methyltransferase activity, whereas Zn^{2+} is a potent inhibitor of this enzyme. These complex effects of the various metal ions might result from their interaction with calmodulin, the methyltransferase, or both proteins. Ca^{2+}, Mn^{2+}, and Zn^{2+} have similar ionic radii and bind strongly to calmodulin causing the typical calcium-dependent conformational change in the protein (Cheung, 1984). Ni^{2+} and Mg^{2+} have smaller ionic radii and do not interact strongly with calmodulin and do not activate it, nor cause conformational changes (Cheung, 1984). One possible interpretation of these data is that Ca^{2+} and Mn^{2+} stimulate the methylation rate through their interaction with calmodulin. Zn^{2+}, on the other hand, inhibits enzyme activity, possibly by interacting directly with the methyltransferase. Mg^{2+} and Ni^{2+}, which seem to selectively stimulate the *Paramecium* methyltransferase, may activate methylation through direct interaction with the enzyme.

Among these various metal ions, Mg^{2+} and Ca^{2+} are more prevalent in cells and are the most physiologically relevant. To avoid potential confusion about the significance of metal ion stimulation, we adopted assay conditions that mimic the physiological Ca^{2+}, Mg^{2+}, and K^{+} conditions of the cytosol. Under these conditions, the effect of calcium on the stimulation of methyltransferase activity seems to be due to

an interaction with calmodulin. This is supported by several observations: 1. the $K_{0.5}$ for calcium stimulation is similar to the K_d of calcium for calmodulin, 2. Ca^{2+}-overlay binding experiments show that there is no direct interaction of calcium with the calmodulin methyltransferase, and 3. inhibition studies with calmodulin antagonists that can interact selectively with calcium-bound calmodulin only inhibit the methylation of calmodulin in the presence of calcium (Han et al., 1993). The differences in the recognition of calcium-bound and calcium-depleted calmodulin structures is discussed in greater detail below.

While the present study has revealed new information regarding enzyme specificity and determinants of calmodulin structure that are required for methyltransferase recognition, much remains to be understood regarding the structure and function of the calmodulin methyltransferase. Much of our understanding of the structure and function of methyltransferases has come from the analysis of DNA, small molecule, or protein carboxyl methyltransferases (Kagan & Clark, 1994). Using multiple alignments of the sequence of 84 distinct enzymes, Kagan and Clark (1994) observed three conserved regions of consensus sequences (motif I, II, and III). Among the sequences examined, all three motifs are found in 45 methyltransferases, and motifs I and III are found in an additional 15 enzymes. Motif I has a nine residue conserved region (V/I/L)(L/V)(D/E)G (G/C)G(T/P)G which is

present in most methyltransferases sequenced to date. This motif has a glycine rich region (GXGDXG) which is similar to the nucleotide binding fold in protein kinases. Motif II has an eight-residue block with the consensus sequence (P/G)(Q/T)(F/Y/A)DA(I/V/Y)(F/I)(C/V/L) which is abundant in aromatic residues. It is usually found approximately 60 residues after motif I. The consensus sequence of motif III is LL(R/K)PGG(R/I/L)(L/I)(L/F/I/V)(I/L). Motif III is found approximately 20 residues after motif II. These motifs also are found in certain nonmethyltransferases which utilize either AdoMet or SAH, including AdoMet decarboxylase, AdoMet synthetase, and SAH hydrolase. It was suggested that these conserved motifs contribute to the binding of the substrate AdoMet and/or the product SAH (Kagan & Clark, 1994). This is supported by the X-ray crystal structure of the DNA methyltransferase M.*Taq*I in complex with AdoMet (Labahn et al., 1994). The structure shows α/β folding of the polypeptide into two domains of about equal size (Labahn et al., 1994). A wide cleft in the C-terminal domain is thought to accommodate the DNA substrate. The N-terminal domain is the site of catalysis and contains the two conserved motifs I and II which form a pocket for binding of AdoMet (Labahn et al., 1994).

Essentially no sequence information is currently available for lysine methyltransferases. The isolation of a cDNA clone for the calmodulin methyltransferase will be necessary to determine whether the enzyme is structurally

homologous to other AdoMet-utilizing methyltransferases, and to facilitate structure and function experiments to determine the sites that lead to the high specificity of this enzyme for calmodulin.

Recognition of calcium-bound and calcium-depleted calmodulin

Lysine 115 can be methylated regardless of whether calcium is bound or not. This is not surprising given the solvent accessibility of lysine 115 in both calcium-bound and calcium-depleted calmodulin structures (Babu et al., 1988; Chattopadhyaya et al., 1992; Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995). However, as summarized in Table VII, calcium-bound and calcium-depleted calmodulin clearly differ in their interaction with the calmodulin methyltransferase. It is important to interpret these differences from the perspective of the conformational change that takes place upon calcium binding to calmodulin, and how these changes affect interaction with target structures.

Upon calcium binding, the helices of the EF hands shift, and the interhelical angles become more perpendicular resulting in the exposure of a large hydrophobic surface (Fig. 3B) (Babu et al., 1988; Chattopadhyaya et al., 1992) which is involved in the interaction with calcium-dependent regulatory targets (Fig. 3C) (Ikura et al., 1992; Meador et al., 1992; reviewed in Clore et al., 1993). Each hydrophobic cleft of calmodulin is surrounded by a polar rim which is rich in negatively charged residues. In addition to extensive

Table VII. Comparison of properties of calcium-bound and calcium-free calmodulin as methyltransferase substrates.

Parameters	calcium-bound CaM	calcium-free CaM
k_{cat} (sec^{-1})	0.0278	0.0106
K_m for CaM (nM)	100	220
drug/peptide inhibition	yes	no
Met oxidation sensitivity	yes	yes
V136T mutant sensitivity	no	yes
ionic strength sensitivity	no	yes

hydrophobic interactions, electrostatic interactions between the negatively charged residues in the polar rim of calmodulin and positively charged residues of the calmodulin-binding domain of target proteins contribute to peptide binding (Ikura et al., 1992; Meador et al., 1992, 1993).

In contrast, the α-helices of the EF-hands in calcium-depleted calmodulin structure are packed so that the interhelical angles are smaller and the hydrophobic residues are sequestered in the core of each lobe (Fig. 3A) (Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995). This results in a less exposed hydrophobic surface and a higher density of charged surface residues (Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995). The highly charged surface of calcium-depleted calmodulin has been proposed to be involved in ionic interactions of calmodulin with proteins, such as neuromodulin (Chapman et al., 1991) neurogranin (Baudier et al., 1991), and myosin I (Swanljung-Collins & Collins, 1994) that interact specifically with calcium-depleted calmodulin but not with calcium-saturated calmodulin. The calmodulin binding sites in these proteins share an IQ motif with a consensus sequence of IQxxxRGxxxR (Mercer et al., 1991) which forms a highly basic amphipathic α-helix upon binding calmodulin (Gerendasy et al., 1995). Based on binding measurements (Chapman et al., 1991) and NMR studies (Urbauer et al., 1995), the interaction of the neuromodulin-derived target peptide with apo-calmodulin was extremely sensitive to

ionic strength. NMR measurements also show that the overall conformation of calcium-free calmodulin does not change upon binding to the neuromodulin-derived peptide (Urbauer et al., 1995). Thus, calcium-depleted calmodulin is proposed to interact with these targets mainly through ionic interactions. Since calcium binding results in a rearrangement of surface charge residues on calmodulin (discussed in Ikura, 1996), this may account for the selective interaction of these proteins with calcium-depleted calmodulin.

These differences between calcium-bound and calcium-free calmodulin are consistent with the observed differences in requirements for methylation in the presence and absence of calcium (Table VII). For example, the methylation of calcium-depleted calmodulin is ionic strength sensitive, whereas the methylation of calcium-bound calmodulin is not. Thus, similar to neuromodulin, ionic interactions between the basic methyltransferase and negatively charged calmodulin appear to be necessary for binding and catalysis.

However, unlike neuromodulin, calcium does not inhibit methyltransferase binding to calmodulin. In contrast, binding appears to be somewhat strengthened (Table VII). In addition, calcium-binding to calmodulin results in an interaction with the enzyme which is no longer ionic strength sensitive. As described above, the conformational change (i.e., exposure of the hydrophobic cleft) accompanying calcium binding to calmodulin is well defined, and it is logical to propose that

hydrophobic interactions may help stabilize the binding of calmodulin to the methyltransferase. This is supported by experiments which show that both a calmodulin-binding synthetic peptide and the naphthalene sulfonamide derivatives W-7 inhibit the calmodulin N-methyltransferase activity in the presence of calcium. These compounds bind to the exposed hydrophobic clefts of calcium-bound calmodulin, but show little or no interaction with calcium-depleted calmodulin. Consistent with this, the compounds show no effect on the methylation of calmodulin in the absence of calcium. This is strong evidence that the inhibition of calmodulin methylation is due to the interaction of these compounds with calmodulin and not with the methyltransferase. Similar findings were also made by Pech and Nelson (1994), who observed an inhibition of the enzyme activity by the calmodulin-antagonist mellitin and calmidazolium, but curiously not by W-7.

A possible interpretation of the drug/peptide data is that binding results in a conformational change that indirectly affects methyltransferase recognition. However, the overall conformation of calmodulin does not change upon peptide or drug binding with the exception of a disordering of the central helix (compare Fig. 3B to 3C) (Ikura et al., 1992; Meador et al., 1992, 1993; Cook et al., 1994). This change is probably not a case since the methylation experiments with the COOH-terminal lobe of calmodulin (discussed below) argue against the importance of the central helix in calmodulin

binding with the methyltransferase. The only other major structural difference between the two calmodulin structures is that the peptide (or drug) occupies the hydrophobic cleft (Fig. 3C). Lysine 115 is still solvent exposed on this structure and presumably is available for methylation. Overall, the data suggest that the hydrophobic cleft may contribute to binding of the methyltransferase in the presence of calcium.

A model summarizing the different interactions between the various forms of calmodulin, and the methyltransferase is shown in Fig. 44. In this model, the interaction of the calcium-depleted form of calmodulin with the methyltransferase is stabilized mainly through ionic interactions with the enzyme that can be disrupted by high ionic strength. However, in the calcium-bound form of calmodulin, the hydrophobic cleft can serve as an additional site for the binding by the methyltransferase. The peptide or drug-bound form of calmodulin cannot be properly recognized by the enzyme because the antagonist occupies the hydrophobic cleft, blocking access of the methyltransferase to its recognition site.

Although it is clear that calcium enhances the rate of methylation of calmodulin, the physiological significance of this observation is not yet known. Lysine methylation is commonly thought to be an irreversible enzymatic reaction (Paik and Kim, 1990). Indeed, turnover studies with histone methylation show that methylation has the same turnover rate

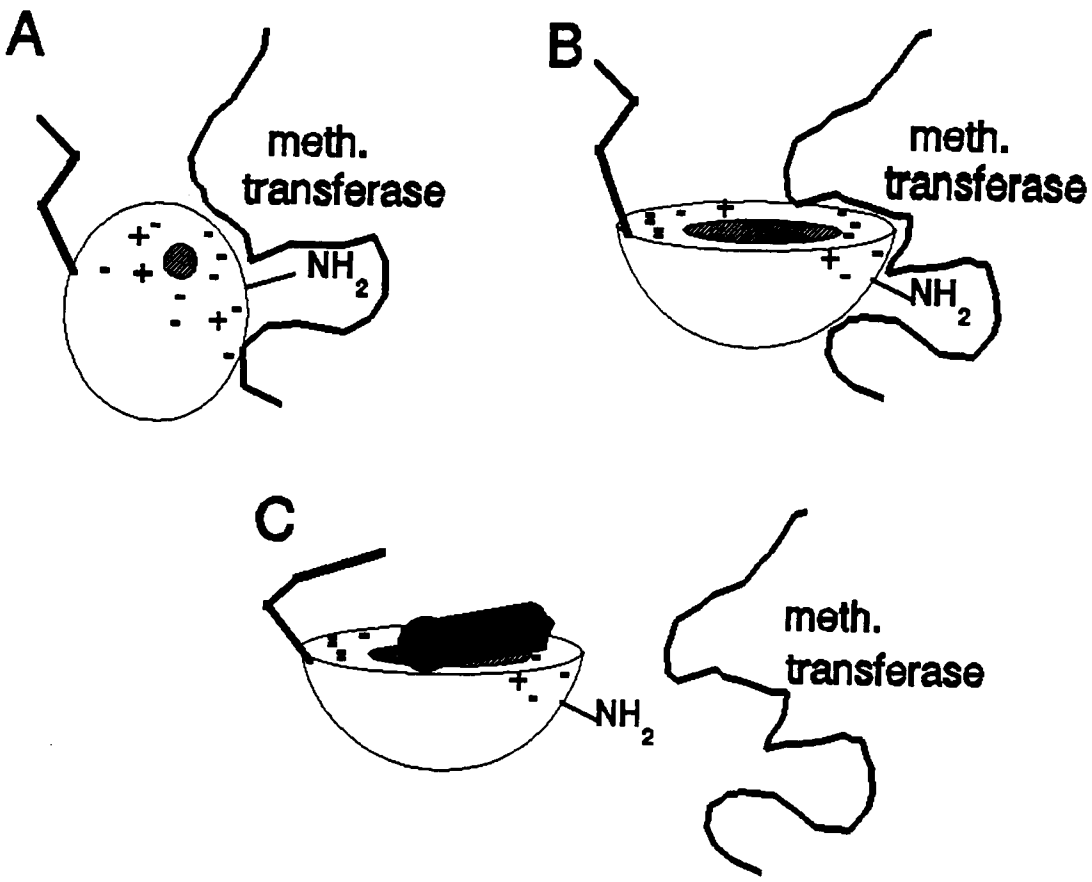


Figure 44. Binding models of the various forms of calmodulin with the methyltransferase. Schematic diagrams show potential binding modes of the methyltransferase with **A**) calcium-depleted, **B**) calcium-bound, and **C**) peptide (or drug) bound forms of calmodulin. For the sake of simplicity, only the COOH-terminal lobe is represented. The number and location of the exposed charge residues are based on Ikura (1996). The cross hatched area indicates exposed hydrophobic residues.

as the protein, suggesting that demethylation does not take place independent of protein turnover (Waterborg, 1993). However, it is apparent that calmodulin methylation *in vivo* can be a dynamic process that varies depending upon the developmental state of the tissue. For example, by using plant cell models, Oh and Roberts (1990) and Oh et al. (1992) have observed large changes in the level of calmodulin methylation that depend upon the stage of growth, and that the turnover of methylated calmodulin can occur rapidly. Rowe et al. (1986) also have reported differences in calmodulin methylation between various animal tissues that correlate with endogenous levels of the methyltransferase.

Since the effect of calcium is exhibited at micromolar concentrations, it is conceivable that calcium fluxes might enhance the methylation rate of calmodulin *in vivo*. Certainly, the work with *cam2* (Lukas et al., 1989) and V136T calmodulin (this study) underscore the importance of calcium-binding in endogenous calmodulin methylation. It is possible that the methylation of calmodulin could be influenced by several cellular parameters including calcium, the endogenous level of the methyltransferase, and the rate of synthesis and turnover of calmodulin.

Methionine residues in calmodulin function and methylation

Performic acid oxidation of the methionines of calmodulin essentially abolishes recognition by the methyltransferase in both the presence or absence of calcium. Thus, calmodulin

methylation is similar to calmodulin-dependent activation of enzymes which is also extremely sensitive to methionine oxidation (Walsh & Stevens, 1978; Guerini et al., 1984; Yao et al., 1996). The methionine residues in calmodulin are principally found within the hydrophobic core of the NH_2- and COOH-terminal lobes (Babu et al., 1988; Chattopadhyaya et al., 1992; Zhang et al., 1995; Kuboniwa et al., 1995; Finn et al., 1995) and contribute substantially to the hydrophobic interactions between target peptides and calmodulin (O'Neil et al., 1989; Ikura et al., 1992; Meador et al., 1992, 1993; reviewed in Vogel, 1994). Methionine constitutes a flexible hydrophobic side chain that has been proposed to mediate flexible, sequence independent interactions between proteins (Gellman, 1990). As such, it has been proposed that methionines in the hydrophobic clefts of calmodulin are necessary to facilitate its interaction with multiple protein targets with divergent sequences (O'Neil & Degrado, 1990; Vogel, 1994). NMR spectroscopy of calmodulin support this notion of flexibility and show that the methyl groups of the methionine residues have the highest mobility in calcium-bound calmodulin (Siivari et al., 1995). In addition, the importance of methionines in calmodulin structure and function is apparent from site-directed mutagenesis experiments. Zhang et al. (1994) substituted all nine individual calmodulin methionine residues with leucine (which has a less flexible hydrophobic side chain) and observed their abilities to

activate PDE. Certain mutations (e.g., M36L, M144R/M145L, or M->L at all four positions in the C-terminal lobe) showed a reduced activation of PDE even though NMR spectroscopy showed that these mutants retain their native structures (Zhang et al., 1994). Thus, the substitution of methionine residues with less flexible or more polar residues can yield a substantial decrease in calmodulin-dependent enzyme activation.

Oxidation of methionines to methionine sulfoxide or methionine sulfone could conceivably influence calmodulin structure by increasing the polarity and decreasing the flexibility of these side-chains. Two structural studies have been done on the conformational effects of methionine oxidation on calmodulin. Oxidation of calmodulin with N-chlorosuccinimide results in the oxidation of only four methionine residues at positions 71, 72, 76, and 109. This results in a reduced ability to activate PDE. However, these modifications do not seem to cause a major disruption of calcium-bound calmodulin structure since CD spectra of oxidized calmodulin was almost identical to that of native calmodulin in the presence of calcium (Walsh & Stevens, 1978). In another study, it was found that selective oxidation of a vicinal methionine pair (M146 and M147) in the COOH-terminal lobe of wheat germ calmodulin by hydrogen peroxide abolishes activation of the plasma membrane Ca-ATPase (Yao et al., 1996). However, Yao et al. (1996) observed that calmodulin

binding peptide still can interact with calmodulin albeit with lower affinity. Further, oxidized calmodulin acts as an antagonist for wild-type calmodulin activation, which suggests that oxidized calmodulin still binds its target proteins in a calcium-dependent manner, but can no longer activate them (Yao et al., 1996). Yao et al. (1996) probed the structural change associated with methionine oxidation by fluorescence spectroscopy of the intrinsic tyrosine 139 and a pyrenylmaleimide analogue (modified at cys 27). Oxidized calmodulin showed a decreased fluorescence intensity and a lower average fluorescence lifetime of both fluorophores either in the presence or absence of calcium. Yao et al. (1996) proposed that these results suggest an increased solvent accessibility of these fluorophores and thus a more polar environment. Collectively, these studies suggest that methionine oxidation inhibits calmodulin-dependent enzyme activation, but the oxidized calmodulin still retains its secondary structure, and its ability to bind calcium and target peptides.

In the present study, oxidation of calmodulin was done with performic acid which is a stronger oxidizing agent, converting all methionines irreversibly to methionine sulfone rather than methionine sulfoxide (Van Eldik & Watterson, 1981). However, performic acid-oxidized VU-1 calmodulin still interacts with phenyl-Sepharose in a calcium-dependent manner. This argues that oxidized VU-1 calmodulin, similar to the

oxidized calmodulin derivatives discussed above, can still bind calcium, undergo a calcium-dependent conformational change, and recognize hydrophobic ligands. The loss of binding or recognition of oxidized calmodulin by the methyltransferase might result from the loss of hydrophobicity or flexibility of the methionine residues leading to subtle conformational changes that disrupts the methyltransferase recognition site.

The methylation site is located on the COOH-terminal lobe

Based on the present results with isolated tryptic fragments, it appears as if the calmodulin methyltransferase recognition site is located within residues 78-148, which contains the two EF-hand structures that comprise the COOH-terminal calcium-binding lobe. In this regard, the calmodulin methyltransferase differs from most calmodulin-dependent enzymes. In the case of most calmodulin-dependent enzymes, the NH_2- as well as COOH-terminal lobes of calmodulin are required since both hydrophobic clefts engage in calmodulin-target protein binding (Ikura et al., 1992; Meador et al., 1992; reviewed in Clore et al., 1993). As a result, fragments that contain either the COOH-terminal or NH_2-terminal lobe alone, while retaining most of the structural and calcium binding properties of the intact molecule (Dalgarno et al., 1984; Ikura et al., 1984; Minowa & Yagi, 1984; Linse et al., 1991), are generally poor activators of calmodulin-dependent enzymes (Drabikowski et al., 1982; Guerini et al., 1984;

Kuznicki et al., 1981; Newton et al., 1984; Wall et al., 1981; Walsh et al., 1977). Thus, it has been proposed that the long central helix between the two lobes is a flexible tether that maintains the lobes in close proximity for coordinate binding to calmodulin-target proteins (Persechini & Kretsinger, 1988).

Certainly, the results here show that the NH_2-terminal lobe is not essential for the recognition by the methyltransferase. However, since the NH_2-terminal lobe shares high homology with the COOH-terminal lobe in its sequence and structure (Fig. 2, 35), we attempted to engineer a methylation recognition site using the NH_2-terminal lobe as a scaffold. We generated a double mutant calmodulin by introducing the EKL sequence at positions 41-43 of the NH_2-terminal lobe (corresponding to positions 114-116 of the COOH-terminal lobe) of VU-3 calmodulin which has an arginine residue at position 115. Although the NH_2-terminal lobe of this mutant calmodulin [QNP(41-43)EKL/K115R] possesses the sequence of the loop (residues 112-117, LGEKLT) that is methylated in the COOH-terminal lobe, it was not methylated by the methyltransferase under any conditions tested. This was somewhat surprising since the backbone structure of the two loops appears identical based on modeling predictions (Fig. 35B). Thus, although calmodulin is a symmetrical molecule with EF hands I and II being homologous to EF hands III and IV respectively, there is likely to be unique features (sequence or conformation) in domains III and IV that lead to selective

recognition and methylation by the calmodulin methyltransferase.

The lack of functional symmetry between the NH_2- and COOH-terminal lobes is supported by studies of engineered calmodulins that have two NH_2-terminal domains (CaMNN), two COOH-terminal domains (CaMCC), and exchanged domains (CaMCN) (Persechini et al., 1996). Both CaMNN and CaMCC mutant calmodulins poorly activated smooth muscle MLCK whereas CaMCN fully activates the enzyme. However, the CaMCN only partially activated skeletal muscle MLCK, whereas CaMCC fully activated and CaMNN poorly activated the enzyme. Nitric oxide synthase gave a different pattern from both MLCK enzymes showing full activation by CaMNN and CaMCN but poor activation by CaMCC (Persechini et al., 1996). The findings show that different calmodulin-dependent enzymes show a distinct interaction with the various mutants. In addition, the results clearly show that there are a structural and functional differences between the two lobes despite their high homology.

Alignment of the backbone structures of the complete NH_2-terminal and COOH-terminal lobe shows remarkable overlap (Kuboniwa et al., 1995; Strynadka & James, 1989). Structural differences between the NH_2- and COOH-terminal lobe are more prominent in the calcium-depleted forms than that observed in their calcium-bound form (Zhang et al., 1995; Kuboniwa et al., 1995). These include: 1. Helix II (29-38) which has a pronounced kink at position 31 whereas homologous helix VI

(101-112) does not; 2. the interloop β-sheet which is more twisted in the COOH-terminal lobe which partly makes up for the absence of the kink; 3. There is the interaction between Phe 141 and backbone and side-chain atoms in helix VII, whereas no homologous interaction was observed in the N-terminal domain; and 4. the higher flexibility of the COOH-terminal lobe compare with the NH_2-terminal lobe. Interestingly, the calcium-bound NH_2-terminal lobe is structurally more similar to the calcium-bound COOH-terminal lobe than is the calcium-depleted COOH-terminal lobe (Kuboniwa et al., 1995). Overall, the structural comparisons suggest that the inability of the methyltransferase to recognize the NH_2-terminal lobe might result from the differences in sequence rather than in tertiary structure. Further studies, perhaps by exchanging EF hands between the two lobes, might be helpful for narrowing down specific sites for the enzyme recognition. Besides an inability to be methylated, the NAD kinase activation by the mutant calmodulin [QNP(41-43)EKL/K115R] was also decreased to only 28 % of the value of VU-1 or VU-3 calmodulin (Fig. 34). Therefore, NAD kinase activation is quite sensitive to not only methylation state of the loop between EF-hand domains III and IV (Roberts et al., 1986), but also to the change in loop sequence between EF-hand domains I and II. The sites of the QNP --> EKL mutation are proximal to helix II (see Fig. 2). In the crystal structure of calcium-saturated calmodulin, helix II and helix VI are

about 50 Å apart (Babu et al., 1988). However, peptide binding brings helices II and VI into very close approximation (less than 5 Å distance) which forms a "latch" between the NH_2- and COOH-terminal lobes of calmodulin (Meador et al., 1992). The clustering of 5 important residues in this "latch" region is composed of Thr 34, Ser 38, Thr 110, Leu 112, and Lys 115 (Meador et al., 1992; Su et al., 1994). Mutations that disrupt latch formation show altered calcium-dependent activation (Su et al., 1994). In addition, analysis of site-directed mutagenesis of thr 110, leu 112, and lys 115 suggest that they are critical for full activation of calmodulin-target proteins (Su et al., 1994). Reduced NAD kinase activation by the [QNP(41-43)EKL/K115R] mutant calmodulin, which has mutation sites proximal to "latch-forming" region, could be due to disruption of the formation of this upon binding of NAD kinase.

***cam2* calmodulin**

A val to thr-136 mutation in calmodulin results in two principal effects that lead to a lower susceptibility to lysine 115 methylation and defective enzyme activation. First, the mutant calmodulin requires higher calcium concentrations for activation of calmodulin-dependent enzymes and methylation. Based on calcium binding and fluorescence spectroscopic analyses, defective calcium binding to sites in the COOH-terminal lobe was detected. However, upon saturation of the mutant calmodulin in high (and likely unphysiological)

concentrations of calcium, normal activation and methylation were observed. Secondly, in addition to defects in calcium binding, fluorescence spectroscopy and electrophoretic analyses suggest that the structure of calcium-depleted V136T calmodulin differs from that of VU-1 calmodulin. These differences in structure apparently result in an inability of the methyltransferase to methylate V136T in the absence of calcium. However, calcium-depleted V136T calmodulin does not differ significantly from VU-1 calmodulin with respect to its CD spectrum or urea-induced denaturation curves. Overall the data argue that threonine 136 alters the conformation of calmodulin, particularly in its calcium-depleted state, but does not drastically affect stability or folding, and the average secondary structural content of the two calmodulins is the same.

As discussed above, the valine to threonine-136 mutation is similar to the *cam2* behavioral mutant of *Paramecium* which has an isoleucine to threonine mutation at this position (Lukas et al., 1989). In wild type *Paramecia,* mechanical or chemical stimuli result in a transient membrane depolarization and the activation of voltage-dependent Ca^{2+} channels (reviewed in Hirichsen et al., 1995). This leads to the reversal of ciliary motion, causing a backward swimming response (Brehm & Eckert, 1978). A Ca^{2+}-dependent K^{+} inward current prolongs the action potential and lengthens the backward swimming (Saimi, 1986). A Ca^{2+}-dependent K^{+} current

plays a major role in membrane repolarization and shortens the backward swimming time (Satow & Kung, 1980; Richard et al., 1986; Preston et al., 1990). Various calmodulin mutants have been characterized that show defects in both of these currents resulting in exaggerated or reduced behavioral response (Hinrichsen et al., 1995).

cam2 is an example of the pantophobiac class of mutants. *cam2* has a substitution of a threonine residue for isoleucine 136 within the fourth calcium-binding loop (Lukas et al., 1989). The altered behavior results from a reduced Ca^{2+}-dependent K^+ efflux of *pantophobiac cam2* (Kink et al., 1990; Preston et al., 1990; Ling et al., 1994). The microinjection of DNA fragment carrying wild-type calmodulin gene into macronuclei caused a reversion of the phenotype (Kanabrocki et al., 1991), which indicates that the defect might result from the poor activation of a Ca^{2+}-dependent K^+ channel activity by I136T calmodulin (Kink et al., 1990). Other pantophobiac calmodulin mutants have been defined, all having various mutations in the carboxyl terminal lobe of calmodulin (Kink et al., 1990; Ling et al., 1994). Interestingly, another class of *Paramecium*, the *fast-2* behavioral mutants, have abbreviated responses and a decreased calcium-dependent sodium currents that result from mutations of calmodulin in the amino terminal end of the molecule (Kink et al., 1990; Ling et al., 1994).

Among the calmodulins leading to the pantophobiac phenotype, the *cam1* mutant (ser to phe 101 in the ninth

position of the third calcium binding loop) and *cam2* are the most well studied biochemically (Lukas et al., 1989; Burgess-Cassler et al., 1987; Kink et al., 1990; Haiech et al., 1991; Schaefer et al., 1987; Hinrichsen et al., 1990). Both have normal enzyme activation properties *in vitro* in the presence of mM calcium concentrations (Lukas et al., 1989; Hinrichsen et al., 1990). *cam1* also shows stoichiometric calcium-binding, but similar to *cam2* the K_d is slightly higher (Haiech et al., 1991). Presumably a higher threshold of calcium is necessary for the activation of K^+ currents in mutant *Paramecia* containing these calmodulins. Interestingly, *cam1* is methylated normally (Schaefer et al., 1987) suggesting that a decrease in calcium affinity in the carboxyl terminal lobe is not in itself sufficient to decrease the calmodulin methyltransferase recognition *in vivo*. Rather it is a combination of a shift in calcium dependence and an inability of the calcium-depleted mutant calmodulin to be methylated that could explain why *cam2* calmodulin is poorly methylated *in vivo*.

As discussed above position 136 in calmodulin is invariably an aliphatic hydrophobic residue, usually valine or isoleucine, that is inserted into the hydrophobic core. The residues at position 7-9 in the loop also form a short antiparallel β-strand structure between pairs of EF hand structures, and thus contributes to the interactions between adjacent calcium binding loops. Therefore, it is proposed

that the highly conserved residue at this position plays a role in anchoring calcium binding loop into the hydrophobic core (Strynadka & James, 1989; Kawasaki & Kretsinger, 1994). This may be particularly important in apo-calmodulin. Unlike calcium-bound calmodulin, in which the loop residues are stabilized by contacts with calcium ions, the first six residues of each calcium binding loop of apo-calmodulin are not well defined due to high flexibility (Zhang et al., 1995; Kuboniwa et al., 1995). However, residues 7-12 in the other part of the calcium-binding loop are relatively stable, and the val to thr-136 substitution might disrupt this stability. This is supported by fluorescence spectroscopy and electrophoretic analyses that suggest that the structure of calcium-depleted V136T calmodulin differs from that of VU-1 calmodulin.

Although the exact conformational change resulting from the substitution of a threonine at position 136 remains to be defined, it is conceivable that it results in an altered packing of the calcium-binding loop IV to the hydrophobic core. This could affect the orientation of the two EF hand structures in the COOH-terminal lobe, and the positioning of key residues which interact with the methyltransferase as well as affecting calcium binding. However, upon saturation of the mutant structure with calcium, the structure is driven into a more active conformation as manifested by normal enzyme activator profiles, normal calmodulin methylation kinetics,

and electrophoretic and fluorescence spectral properties that are indistinguishable from those of wild-type calmodulin.

Calmodulin-DHFR sandwich fusion protein

A calmodulin-DHFR sandwich fusion protein was generated by insertion of VU-1 calmodulin into the β-bulge region of DHFR to observe the effects of structural constraints placed on the calmodulin molecule enforced by DHFR folding. The use of the DHFR protein for this study is ideal because it is structurally well defined, folding can be selected by TMP resistance, and because several inserts have been successfully integrated into the β-bulge (Dion-Schultz and Howell, 1996).

The calcium binding properties of the sandwich protein were virtually indistinguishable from wild type VU-1. This is not surprising since the two lobes of calmodulin do not interact and have similar structural and calcium binding properties regardless of whether they are part of the same molecule or are separate fragments (Finn et al., 1993, 1995; Minowa and Yagi, 1984; Linse et al., 1991). Therefore, if there is no structural perturbation within the lobes of VU-1 calmodulin, the calcium binding characteristics of the calmodulin domain of the sandwich protein should be normal.

Despite normal calcium binding characteristics, the sandwich protein showed modest defects in its ability to activate NAD kinase or PDE, and substantial defects in it ability to recognized by the methyltransferase. Upon binding target peptide, the long central helix (residues 65-93) of

calmodulin is disrupted into two helices connected by a long flexible loop (residues 74-82), thereby enabling the two domains to clamp the bound peptide (Ikura et al., 1992; Meador et al., 1992). Further, the helix can unfold to a various extent to accommodate different target structures (Meador et al., 1993). The lower activator properties of the sandwich protein might result from a constraint of two domains of calmodulin by the DHFR structure. However, this constraint does not drastically affect activation of the two enzymes tested in this study. Previous work with various calmodulin-dependent enzymes show that they are extremely variant in their sensitivities to mutations in calmodulin structure. For example, while some enzymes (e.g. PDE) are very tolerant of alterations in calmodulin structure, others are extremely sensitive to the same mutations (c.f., Craig et al., 1987). In this regard it will interesting to investigate the interaction of the sandwich protein with a variety of calmodulin-binding peptides and calmodulin-dependent enzymes.

The sandwich protein was poorly methylated by the methyltransferase, and this effect seemed to be enhanced in the presence of TMP. Since the recognition site of the methyltransferase resides solely in the COOH-terminal lobe of calmodulin, this result is probably not due to the alterations in the central helix or constraints of the two lobes relative to one another. Perhaps steric hindrance of the COOH-terminal domain within the DHFR-sandwich protein structure prevents the

methyltransferase from binding. Also we cannot exclude the possibility of a distortion of the methyltransferase recognition sequence or even local interactions between calmodulin and the DHFR structure.

Summary

The results of this study have provided important leads in the types of interaction and general structural elements that are necessary for methyltransferase binding. Further, it is clear that the recognition site is not a simple linear peptide sequence, and that it requires structures that are unique to the COOH-terminal lobe. With these findings as a starting point, finer detailed mutagenesis studies can now be designed to start to elucidate further the specific residues within the COOH-terminal lobe that are essential for methyltransferase specificity. In addition, the availability of a purified methyltransferase enzyme should facilitate molecular cloning experiments so that the structural and functional properties of the methyltransferase can be further pursued.

LIST OF REFERENCES

LIST OF REFERENCES

Allen, B.G. & Walsh, M.P. (1994) *Trends Biochem. Sci.* 19, 362-366.

Apel, E.D., & Storm, D.R. (1992) *Perspect. Dev. Neurobiol.* **1**, 3-11.

Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R., & Cook, W.J. (1985) *Nature* **315**, 37-40.

Babu, Y.S., Bugg, C.E., & Cook, W.J. (1988) *J. Mol. Biol.* **204**, 191-204.

Barbato, G., Ikura, M., Kay, L.E., Pastor, R.W., & Bax, A. (1992) *Biochemistry* **31**, 5269-5278.

Baudier, J., Deloume, J.C., Dorsselaer, A.V., Black, D., & Matthes, H.W.D. (1991) *J. Biol. Chem.* **266**, 229-237.

Bazari, W.L., & Clarke, M. (1981) *J. Biol. Chem.* **256**, 3598-3603.

Bean, B.P. (1989) *Annu. Rev. Physiol.* **51**, 367-384.

Beavo, J.A. (1995) *Physiological Reviews* **75**, 725-748.

Berridge, M.J. & Irvine, R.F. (1989) *Nature* **341**, 197-205.

Berridge, M.J. (1993) *Nature* **361**, 315-325.

Bidlingmeyer, B.A., Cohen, S.A., & Tarvin, T.L. (1984) *J. Chromatogr.* **336**, 93-104.

Blumenthal, D.K., Takio, K., Edelman, A.M., Charbonneau, H., Titani, L., Walsh, K.A. & Krebs, E.G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3187-3191.

Bolin, J.T., Filman, D.J., Mattews, D.A., Hamlin, R.C., & Kraut, J. (1982) *J. Biol. Chem.* **257**, 13650-13662.

Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248-254.

Bredt, D.S., & Snyder, S.H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 682-685.

Brehm, P., & Eckert, R. (1978) *Science* **202**, 1203-1206.

Brzeska, H., Szynkiewicz, J., & Drabikowski, W. (1983) *Biochem. Biophys. Res. Commun.* **115**, 87-93.

Burgess-Cassler, A., Hinrichsen, R.D., Maley, M.E., & Kung, C. (1987) *Biochim. Biophys. Acta* **913**, 321-328.

Carafoli, E. (1987) *Annu. Rev. Biochem.* **56**, 395-433.

Chapman, E.R., Au, D., Alexander, K.A., Nicolson, T.A., & Storm, D.R. (1991) *J. Biol. Chem.* **266**, 207-213.

Chattopadhyaya, R., Meador, W.E., Means, A.R., & Quiocho, F.A. (1992) *J. Mol. Biol.* **228**, 1177-1192.

Cheney, R.J., & Mooseker, M.S. (1992) *Curr. Opin. Cell. Biol.* **4**, 27-35.

Cheung, W.Y. (1970) *Biochem. Biophys. Res. Commun.* **38**, 533-538.

Cheung, W.Y. (1971) *J. Biol. Chem.* **246**, 2859-2869.

Cheung, W.Y. (1980) *Science* **207**, 19-27.

Cheung, W.Y. (1984) *FASEB* **43**, 2995-2999.

Clapham, D.E., & Sneyd, J. (1995) in *Advances in Second Messenger and Phosphoprotein Research* **30**, 1-24.

Clore, G.M., Bax, A., Ikura, M., & Gronenborn, A.M. (1993) *Curr. Opinion Structural Biol.* **3**, 838-845.

Cohen, P. (1988) in *Molecular Aspects of Cellular Regulation. Vol. 5: Calmodulin* (Cohen, P., & Klee, C.B. eds.) pp 123-144, Elsevier, Amsterdam.

Colbran, R.J., & Soderling, T.R (1990) *Curr. Top. Cell Regul.* 31, 181-221.

Colowick, S.P., & Womack, F.C. (1969) *J. Biol. Chem.* **224**, 774-777.

Cook, W.J., Walter, L.J., & Walter, M.R. (1994) *Biochemistry* **33**, 15259-15265.

Cox, J.A., Ferraz, C., Demaille, J.G., Perez, R.O., Tuinen, D., & Marme, D. (1982) *J. Biol. Chem.* **257**, 10694-10700.

Craig, T.A., Watterson, D.M., Prendergast, F.G., Haiech, J., & Roberts, D.M. (1987) *J. Biol. Chem.* **262**, 3278-3284.

Crouch, T.H., & Klee, C.B. (1980) *Biochemistry* **19**, 3692-3698.

Davis, T.N., Urdea, M.S., Masiarz, F.R. & Thorner, J. (1986) *Cell* **47**, 423-431.

Davis, T.N. (1992) *J. Cell Biol.* **118**, 607-617.

Dalgarno, D.C., Klevit, R.E., Levine, B.A., Williams, R.J.P., Dobrowolski, Z., & Drabikowski. (1984) *J. Biochem.* **138**, 281-289.

Dahlquist, F.W.(1979) *Methods Enzymol.* **48**, 270-299.

da Silva, A.C., & Reinach, F.C. (1991) *Trends Biochem. Sci.* **16**, 53-57.

da Silva, A.C., Araujo, A.H., Herzberg, O., Moult, J., Sorenson, M., & Reinach, F.C. (1993) *Eur. J. Biochem.* **213**, 599-604.

Dion-Schultz, A., & Howell, E.E. (1996) *Biochemistry*, in press.

Dobrowolski, Z., & Drabikowski, W. (1984) *Eur. J. Biochem.* **138**, 281-289.

Drabikowski, W., Brzeska, H., & Venyaminov, S.Y. (1982) *J. Biol. Chem.* **257**, 11584-11590.

Dretzen, G., Bellard, M., Sassone-Corsi, P., & Chambon, P. (1981) *Anal. Biochem.* **112**, 295.

Eisenberg, D. (1982) *Faraday Symp. Chem. Soc.* **17**, 109-120.

Enslen, H., Sun, P., Bricky, D. Soderling, S.H., Klamo, E., & Soderling, T.R. (1994) *J. Biol. Chem.* 269, 15520-15527.

Filman, D.J., Bolin, J.T., Mattews, D.A., & Kraut, J. (1982) *J. Biol. Chem.* **257**, 13650-13662.

Findlay, W., & Sykes, B.D. (1993) *Biochemistry* **32**, 3461-3467.

Findlay, W.A., Martin, S.R., Beckingham, K., & Bayley, P.M. (1995) *Biochemistry* **34**, 2087-2094.

Finn, B.E., Drakenberg, T., Férsen, S. (1993) *FEBS Lett.* **336**, 368-374.

Finn, B.E., Evenäs, J., Drakenberg, T., Waltho, J.P., Thulin, E., & Forsén, S. (1995) *Nature Struct. Biol.* **2**, 777-783.

Flaherty, K.M., Zozulys, S., Stryer, L., & McKay, D.B. (1993) *Cell* **75**, 709-716.

Fletcher, J.E., Spector, A.A., & Ashbrook, J.D. (1970) *Biochemistry* **9**, 4580-4587.

Gagne, S.M., Tsuda, S., Li, M., Smillie, L.B., & Sykes, B.D. (1995) *Nature Struct. Biol.* **2**, 784.

Gellman, S.H. (1991) *Biochemistry* **30**, 6633-6636.

Gerard, D., Lemieux, G., & Laustriat, G. (1975) *Photochem. Photobiol.* **22**, 89-95.

Gerendasy, N.A., Herron, S.R., Jennings, P.A., & Sutcliffe, J.G. (1995) *J. Biol. Chem.* **270**, 6741-6750.

Gopalakrishna, R., Anderson, W.B. (1982) *Biochem. Biophys. Res. Commun.* **104**, 830-836.

Gregori, L., Marriot, D., West, C.M., & Chau, V. (1985) *J. Biol. Chem.* **260**, 5232-5235.

Gregori, L., Marriot, D., Putkey, J.A., Means, A.R., & Chau, V. (1987) *J. Biol. Chem.* **262**, 2562-2567.

Grover, A.K., & Khan, I. (1992) *Cell Calcium* **13**, 9-17.

Guerini, D. Krebs, J., & Carafoli, E. (1984) *J. Biol. Chem.* **259**, 15172-15177.

Haiech, J., Derancourt, J., Pechere, J.-F., & Demaille, J.G. (1979) *Biochemistry* **18**, 2752-2758.

Haiech, J., Vallet, B., Aquaron, R., & Demaille, J.G. (1980) *Anal. Biochem.* **105**, 18-23.

Haiech, J., Klee, C.B., & Demaille, J.G. (1981) *Biochemistry* **20**, 3890-3897.

Haiech, J., Kilhoffer, M.C., Lukas, T., Craig, T.A., Roberts, D.M., & Watterson, D.M. (1991) *J. Biol. Chem.* **266**, 3427-3441.

Han, C.H., Richardson, J., Oh, S.H., & Roberts, D.M. (1993) *Biochemistry* **32**, 13974-13980.

Head, J.F., Masure, H.R., & Kaminer, B.(1982) *FEBS Lett.* **137**, 71-74.

Hergenhahn, H., Kegel, G., & Sedlmeier, D. (1984) *Biochim. Biophys. Acta.* **787**, 196-203.

Hidaka, H., Yamaki, T., Naka, M., Tanaka, T., Hayashi,H., & Kobayashi, R. (1980) *Mol. Pharmacol.* **17**, 66-72.

Hinrichsen, R., Wilson, E., Lukas, T., Craig, T., Schultz, J., & Watterson, D.M. (1990) *J. Cell Biol.* **111**, 2537-2542.

Hinrichsen, R.D. (1993) *Biochim. Biophys. Acta* **1155**, 277-293.

Hinrichsen, R.D., Fraga, D., & Russel, C. (1995) in *Adv. Second Messenger and Phosphoprotein Res.* **30**, (Means, A.R., ed.), Raven Press, New York, 311-338.

Hirs, C.H.W. (1967) *Methods Enzymol.* **11**, 197-199.

Ikura, M., Hiraoki, T., Hikichi, K., Minowa, O., Yamaguchi, H., Yazawa, M., & Yagi, K. (1984) *Biochemistry* **23**, 3124-3128.

Ikura, M., Minowa, O., & Hikichi, K. (1985) *Biochemistry* **24**, 4264-4269.

Ikura, M., Hasegawa, N.,Aimoto, S.,Yazawa, M., & Yagi, K., & Hikichi, K. (1984) *Biochem. Biophys. Res. Commun.* **23**, 3124-3128.

Ikura, M. Spera, S., Barbato, G., Kay, L.E., Krinks, M., & Bax, A. (1991) *Biochemistry* **30**, 9216-9228.

Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B., & Bax, A. (1992) *Science* **256**, 632-638.

Ikura, M. (1996) *Trends Biochem. Sci.* **21**, 14-17.

Kagan, R.M., & Clarke, S. (1994) *Arch. Biochem. Biophys.* **310**, 417-427.

Kakiuchi, S., & Yamazaki, R. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1104-1110.

Kakiuchi, S., Yamazaki, R., & Nakajima, H. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **46**, 587-592.

Kakiuchi, S., Yamazaki, R., & Teshima, Y. (1972) *Adv. Cyclic. Nucleotide Res.* **1**, 455-477.

Kawasaki, H., & Kretsinger, R. (1994) in *Protein Profile*, Volume **1**, Academic Press, Liverpool.

Kemp, B.E., & Stull, J.T. (1990) in *Peptides and Protein Phosphorylation*. Kemp, B.E., ed. CRC Press, Boca Raton, pp. 115-133.

Kemp, B.E., & Pearson, R.B. (1991) *Biochim. Biophys. Acta* **1094**, 67-76.

Kilhoffer, M.C., Demaille, J.G., & Gerard, D. (1981) *Biochemistry* **20**, 4407-4414.

Kilhoffer, M.C., Roberts, D.M., Adibi, A.O., Watterson, D.M.,

& Haiech, J. (1989) *Biochemistry* **28**, 6086-6092.

Kink, J.A., Maley, M.E., Preston, R.R., Ling, K.Y., Wallen-Friedman, M.A., Saimi, Y., & Kung, C. (1990) *Cell* **62**, 165-174.

Klee, C.B., & Krinks, M.H. (1978) *Biochemistry* **17**, 120-126.

Klee, C.B., & Vanaman, T.C. (1982) *Adv. Protein Chem.* **35**, 213-321.

Klee, C.B., & Cohen, P. (1988) **in** *Molecular Aspects of Cellular Regulation, Volume 5*, (Klee, C.B. & Cohen, P., eds.), Elsevier, Amsterdam, pp. 123-144.

Klee, C.B. (1988) **in** *Molecular Aspects of Cellular Regulation, Volume 5*, (Klee, C.B. & Cohen, P., eds.), Elsevier, Amsterdam, pp. 35-56.

Klevit, R.E., Blumenthal, D.K., Wemmer, D.E., & Krebs, E.G. (1985) *Biochemistry* **24**, 8152-8157.

Kretsinger, R.H. (1975) **in** *Calcium transport in contraction and secretion* (Carafoli, E., ed), North-Holland Publishing, Amsterdam, 469-478.

Kretsinger, R.H. (1980) *CRC Crit. Rev. Biochem.* **8**, 119-174.

Kretsinger, R.H., Rudnick, S.E., & Weissman, L.J. (1986) *J. Inorg. Biochem.* **28**, 289-302.

Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C.B., & Bax, A. (1995) *Nature Struct. Biol.* **2**, 768-776.

Kumar, V.S., & Cook, W.J. (1992) *J. Mol. Biol.* **224**, 413-426.

Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W., & Cohen, P. (1981) *FEBS Lett.* 130, 141-145.

Labahn, J., Granzin, J., Schluckebier, G., Robinson, D.P., Jack, W.E., Schildkraut, I., & Saenger, W. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 10957-10961.

Laemmli, U.K. (1970) *Nature* 227, 680-685.

Levin, R.M., & Weiss, B. (1977) *Mol. Pharmacol.* **13**, 690-697.

Levin, R.M., & Weiss, B. (1979) *J. Pharmacol. Exp. Ther.* **208**, 454-459.

Ling, K.-Y., Maley, M.E., Preston, R.R., Saimi, Y. & Kung, C. (1994) *Eur. J. Biochem.* **222**, 433-439.

Linse, S., Helmersson, A., & Forsén, S. (1991) *J. Biol. Chem.* **266**, 8050-8054.

Linse, S., & Forsén, S. (1995) in *Advances in Second Messenger and Phosphoprotein Research* **30**, 89-151, Raven Press, NEW York.

Lukas, T.J., Friedman, M.W., Kung, C., & Watterson, D.M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7331-7335.

Martin, S.R., & Bayley, P.M. (1986) *Biochem. J.* **238**, 485-490.

Masters, B.S., McMillan, K., Sheta, E.A., Nishmura, J.S., Roman, L.J., & Martasek, P. (1996) *FASEB J.* **10**, 522-528

Maune, J.F., Klee, C.B., & Beckingham, K. (1992) *J. Biol. Chem.* **267**, 5286-5295.

Meador, W.E., Means, A.R., & Quiocho, F.A. (1992) *Science* **257**, 1251-1255.

Meador, W.E., Means, A.R., & Quiocho, F.A. (1993) *Science* **263**, 1718-1721.

Mercer, J.A., Seperack, P.K., Strobel, M.C., Copeland, N.G. & Jenkins, N.A. (1991) *Nature* **349**, 709-713.

Meyer, T., Hanson, P.I., Stryer, L., and Schulman, H. (1992) *Science* **256**, 1199-1202.

Minowa, O., Yagi, K. (1984) *J. Biochem.* **96**, 1175-1182.

Moews, P.C., & Kretsinger, R.H. (1975) *J. Mol. Biol.* **91**, 201.

Moncrief, N.D., Kretsinger, R.H., and Goodman, M. (1990) *J. Mol. Evol.* **30**, 522-562.

Morino, H., Kawamoto, T., Miyake, M., & Kakimoto Y. (1987) *J. Neurochem.* **48**, 1201-1208.

Nairn, A.C. & Greengard, P. (1987) *J. Biol. Chem.* **262**, 7273-7281.

Nairn, A.C., Hemmings, H.C.Jr., & Greengard, P. (1985) *Annu. Rev. Biochem.* **54**, 931-976.

Nakayama, S., & Kretsinger, R.H. (1994) *Annu. Rev. Biophys. Biomol. Struct.* **23**, 473-507.

Newton, D.L., Oldewurtel, M.D., Krinks, M.H., Shiloach, J., & Klee, C.B. (1984) *J. Biol. Chem.* **259**, 4419-4426.

Oh, S.-H., & Roberts, D.M. (1990) *Plant Physiol.* **93**, 880-887.

Oh, S.-H., Steiner, H.Y., Dougall, D.K., & Roberts, D.M. (1992) *Arch. Biochem. Biophys.* **297**, 28-34.

Oh, S.-H. (1992) Ph.D. Desertation, The University of Tennessee, Knoxville.

Olwin, B.B., & Storm, D.R. (1985) *Biochemistry* **24**, 8081-8086.

Olivera, B.M., Ramachandran, J., Miljanich, G.P., & Adams M.E. (1994) *Annu. Rev. Biochem.* **63**, 823-867.

O'Neil, K.T., Erickson-Viitanen, S., & DeGrado, W.F. (1989) *J. Biol. Chem.* **264**, 14571-14578.

O'Neil, K.T., & DeGrado, W.F. (1990) *Trends Biochem. Sci.* **15**, 59-64.

Pace, C.N., Shirley, B.A., & Thomson, J.A. (1990) in *Protein Structure* (Creighton, T.E. ed.) IRL press, Oxford, pp 311-330.

Paik, W.K., & Kim, S. (1990) in *Protein Methylation* CRC press, Inc. Boca Raton, Florida.

Pearlstone, J.R., Borgford, T., Chandra, M., Oikawa, K., Kay, C.M., Herzberg, O., Moult, J., Herklotz, A., Reinach, F.C., & Smillie, L.B. (1992) *Biochemistry* **31**, 6545-6553.

Pech, L.L. and Nelson, D.L. (1994) *Biochim. Biophys. Acta* **1199**, 183-194.

Persechini, A. & Kretsinger, R.H. (1988) *J. Biol. Chem.* **263**, 12175-12178.

Persechini, A., Gansz, K.J., & Paresi, R.J. (1996) *Biochemistry* **35**, 224-228.

Perrin, D.D., & Sayce, I.G. (1967) *Talanta* **14**, 833-842.

Potter, J.D., Johnson, J.D., Dedman, J.R., Schreiber, W.E., Mandel, F., Jackson, R.L., & Means, A.R. (1977) in *Calcium-Binding Proteins and Calcium Function*, Wasserman, R.H., Corradino, R., Carafoli, E., Kretsinger, R.H., McLennan, D., & Siegel, F. Eds. North-Holland, New York, 239.

Porumb, T. (1994) *Anal. Biochem.* **220**, 227-237.

Preston, R.R., Wallen-Friedman, M.A., Saimi, Y., & Kung, C. (1990) *J. Membrane Biol.* **115**, 51-60.

Reich, N.O., & Mashhoon, N. (1991) *Biochemistry* **30**, 2933-2939.

Reich, N.O., & Mashhoon, N. (1993) *J. Biol. Chem.* **268**, 9191-

9193.

Richard, E., Saimi, Y., & Kung, C. (1986) *J. Membr. Biol.* **91**, 173-181.

Richardson, J.S., Getzoff, E.D., & Richardson, D.C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2574-2578.

Roberts, D.M., Crea, R., Malecha, M., Alvarado-Urbina, G., Chiarello, R.H., & Watterson, D.M. (1985) *Biochemistry* **24**, 5090-5098.

Roberts, D.M., Lukas, T.J., & Watterson, D.M. (1986a) *CRC Crit. Rev. Plant Sci.* **4**, 311-339.

Roberts, D.M., Rowe, P.M., Siegel, F.L., Lukas, T.J., & Watterson, D.M. (1986b) *J. Biol. Chem.* **261**, 1491-1494.

Roberts, D.M., Zimmer, W. E., & Watterson, D.M. (1987) *Methods Enzymol.* **139**, 290-303.

Roberts, D.M., Oh, S.H., Besl, L., Weaver, C.D., & Stacey, G. (1990) *Curr. Top. Plant Biochem. Physiol.* **9**, 67-84.

Roberts, D.M., & Harmon, A.C. (1992) *Annu. Rev. Plant Physiol. Mol. Biol.* **43**, 375-414.

Roberts, D.M., Besl, L., Oh, S.-H., Masterson, R.V., Schell, J., & Stacey, G. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8394-8398.

Rowe, P.M., Murtaugh, T.J., Bazari, W.L., Clarke, M., & Siegel, F.L. (1983) *Anal. Biochem.* **133**, 394-400.

Rowe, P.M., Wright, L.S., & Siegel, F.L. (1986) *J. Biol. Chem.* **261**, 7060-7069.

Saimi, Y. (1986) *J. Membr. Biol.* **92**, 227-236.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Santoro, M.M., & Bolen, D.W. (1988) *Biochemistry* **27**, 8063-8068.

Satow, Y., & Kung, C. (1980) *J. Exp. Biol.* **88**, 293-303.

Satyshur, K.A., Rao, S.T., Pyzalska, D., Drendel, W., Greaser, M., & Sundarlingam, M. (1988) *J. Mol. Biol.* **263**, 1628-1647.

Schaefer, W.H., Hinrichsen, R.D., Burgess-Cassler, A., Kung,

C., Blair, I.A., & Watterson, D.M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3931-3935.

Schleicher, M., Lukas, T.J., & Watterson, D.M. (1984) *Arch. Biochem. Biophys.* **229**, 33-42.

Schreier, P.H., & Cortese, R. (1979) *J. Mol. Biol.* **129**, 169-172.

Sharma, R.K., & Wang, J.H. (1979) in *Adv. Cyclic Nucleotide Res.* (Brooker, G., Greengard, P., & Robert, G.A. eds.) **10**, pp 187-198, Raven Press, New York.

Sharma, R.K., Wang, T.H., Wirch, E., & Wang, J.H. (1980) *J. Biol. Chem.* **255**, 5916-5923.

Shattuck, R.L., & Storm, D.R. (1988) **in** *Molecular Aspects of Cellular Regulation, Volume 5*, (Klee, C.B. & Cohen, P., eds.), Elsevier, Amsterdam, pp. 249-264.

Siivari, K., Zhang, M., Palmer III, A.G., & Vogel, H.J. (1995) *FEBS Letters* **366**, 104-108.

Siegel, F.L., Vincent, P.L., Neal, T.L., Wright, L.S., Heth, A.A., & Rowe, P.M. (1990) in *Protein Methylation* (Paik, W.K., & Kim, S., Eds.) pp. 33-58, CRC Press, Boca Raton, Florida.

Sitaramayya, A., Wright, L.S., & Siegel, F.L. (1980) *J. Biol. Chem.* **255**, 8894-8900.

Skelton, N.J. (1994) *Nature Struct. Biol.* **1**, 239-245.

Slupsky, C.M., & Sykes, B.D. (1995) *Biochemistry* **34**, 15953-15964.

Soderling, T.R. (1990) *J. Biol. Chem.* **265**, 1823-1826.

Sondek, J., & Shortle, D. (1990) *Proteins* **7**, 299-305.

Sondek, J., & Shortle, D. (1992) *Proteins* **13**, 132-140.

Strehler, E.E. (1991) *J. Membr. Biol.* **120**, 1-15.

Strynadka, N.C., & James, M.N.G. (1989) *Annu. Rev. Biochem.* **58**, 951-998.

Su, Z., Fan, D., & George, E. (1994) *J. Biol. Chem.* **269**, 16761-16765.

Sundaralingam, M.R., Bergstrom, R., Strasbug, G., Rao, S.T., Roychowdhury, P., Greaser, M., & Wang, B.C. (1985) *Science* **227**, 945-948.

Swanljung-Collins, H., & Collins, J.H. (1994) *Adv. Exp. Med. Biol.* **358**, 205-213.

Svensson, B., Jonsson, B., Thulin, E., & Woodward, C.E. (1993) *Biochemistry* **32**, 2828-2934.

Takeda, T., & Yamamoto, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3580-3584.

Tarof, K.D., & Hobbs, C.A. (1987) *Bethesda Res. Lab. Focus* **9**, 12.

Toda, H., Yazawa, M., Sakiyama, F., & Yagi, K. (1985) *J. Biochem.* **98**, 833-842.

Tokumitsu, H., Enslen, H., & Soderling, T.R. (1995) *J. Biol. Chem.* 270, 19320-19324.

Tsien, R.Y., Rink, T.J. & Poenie, M. (1985) *Cell Calcium* **6**, 145.

Urbauer, J.L., Short, J.H., Dow, L.K., & Wand, A.J. (1995) *Biochemistry* **34**, 8099-8109.

Van Eldik, L.J., Piperno, G., & Watterson, D.M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4779-4783.

Van Eldik, L.J. & Watterson, D.M. (1981) *J. Biol. Chem.* **256**, 4205-4210.

Van Eldik, L.J., Zendegui, J.G., Marshak, D.R., & Watterson, D.M. (1982) *International Review of Cytology* **77**, 1-49.

Vogel, H.J., Lindahl, L., & Thulin, E. (1983) *FEBS Lett.* **157**, 241-246.

Vogel, H.J. (1994) *Biochem. Cell Biol.* **72**, 357-376.

Volz, K.W., Matthews, D.A., Alden, R.A., Freer, S.T., Hansch, C., Kaufman, B.T., & Kraut, J. (1982) *J. Biol. Chem.* **257**, 2528-2536.

Wall, C.M., Grand, R.J.A., & Perry, S.V. (1981) *Biochem. J.* **195**, 307-316.

Walsh, M., Stevens, F.C., Kuznicki, J., & Drabikowski, W. (1977) *J. Biol. Chem.* **252**, 7440-7443.

Walsh, M. & Stevens, F.C. (1978) *Biochemistry* **17**, 3924-3930.

Waterborg, J.H. (1993) *J. Biol. Chem.* **268**, 4918-4921.

Weiss, B., Prozialeck, W.C., & Wallace, T.L. (1982) *Biochem. Pharmacol.* **31**, 2217-2226.

Williams, D.A., & Fay, F.S. (1990) *Cell Calcium* **11**, 75-83.

Yao, Y., Yin, D., Jas, G.S., Kuczera, K., Williams, T.D., Schoneich, C., & Squier, T.C. (1996) *Biochemistry* **35**, 2767-2787.

Yazawa, M., Ikura, M., Hikichi, K., Ying, L., & Yagi, K. (1987) *J. Biol. Chem.* **262**, 10951-10954.

Zhang, M., Li, Ming, Wang, J.H., & Vogel, H.J. (1994) *J. Biol. Chem.* **269**, 15546-15552.

Zhang, M., Huque, E., & Vogel, H.J. (1994) *J. Biol. Chem.* **269**, 5099-5105.

Zhang, M., Tanaka, T., & Ikura, M. (1995) *Nature Struct. Biol.* **2**, 758-767.

Ziegenhagen, R., Goldberg, M., Rakutt, W.D., & Jennissen, H.P. (1990) *FEBS Lett.* **271**, 71-75.

VITA

Chang-Hoon Han was born in Seoul, Korea on May 9, 1957. He graduated Kyungdong High School in 1976. He entered Seoul National University and obtained Doctor of Veterinary Medicine degree in 1981. He continued his graduate study in same University and received the Master degree in Veterinary Medicine in 1983. He served in the army as a Reserve Officer from 1983 to 1984. He was employed by YuHan Pharmaceutical Company as a researcher from 1984 to 1986. He also worked in Hyundai Heavy Industry as a researcher from 1986 to 1990.

In August 1991 he entered the Graduate Program in Biochemistry at the University of Tennessee, Knoxville and received a Ph.D. degree in Biochemistry in December, 1996. He was employed by the University of Tennessee as a GA (graduate assistant) or GTA (graduate teaching assistant) from 1991 fall to 1996 spring. He received a Science Alliance Upgrade award from the University in 1994. He presented his research data through poster presentation at 1994 American Society for Biochemistry and Molecular Biology meeting. He also presented his data at several seminars and publications (see below).

He was married to Hee-Kyung Choi in 1989 and they have two sons Dong-Jun and Dong-Hyun.

Publications

1. **C.H. Han**, J. Richardson, S.H. Oh, and D. M. Roberts (1993) Isolation and kinetic characterization of the calmodulin methyltransferase from sheep brain. *Biochemistry* 32, 13974-13980.

2. **C.H. Han**, J. Richardson, S.H. Oh, and D.M. Roberts (1994) Isolation and characterization of the calmodulin methyltransferase from sheep brain. The FASEB Journal 8, A1437.

3. **C.H. Han**, D.M. Roberts (1996)
Activator analysis and methylation kinetics of a calmodulin mutant with a valine to threonine-136 mutation. submitted to *Biochemistry*

Work in preparation

Calcium binding, enzyme activation function and methylation of a calmodulin-DHFR sandwich-fusion protein. in preparation.

Presentation at scientific meeting

Chang-Hoon Han and Daniel M. Roberts
American Society for Biochemistry and Molecular Biology (ASBMB) Annual Meeting, May 21-25 (1994), Washington, D.C.

Seminar

Invited speaker, Korean Scientists and Engineers Association (KSEA) in America, North Carolina Chapter Symposium, February 18 (1995), Raleigh, NC.

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