

Structural elements within the methylation loop (residues 112–117) and EF hands III and IV of calmodulin are required for Lys¹¹⁵ trimethylation

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Calmodulin is trimethylated by a specific methyltransferase on Lys¹¹⁵, a residue located in a six amino acid loop (LGEKLT) between EF hands III and IV. To investigate the structural requirements for methylation, domain exchange mutants as well as single point mutations of conserved methylation loop residues (E114A, Glu¹¹⁴ → Ala; L116T, Leu¹¹⁶ → Thr) were generated. E114A and L116T activated cyclic nucleotide phosphodiesterase (PDE) and NAD⁺ kinase (NADK) similar to wild-type calmodulin, but lost their ability to be methylated. Domain exchange mutants in which EF hand III or IV was replaced by EF hand I or II respectively (CaM¹²¹⁴ and CaM¹²³² respectively) showed a modest effect on PDE and NADK activation (50 to 100 % of wild-type), but calmodulin methylation was abolished. A third domain exchange mutant, CaM^{EKL}, has the methylation loop sequence placed at a symmetrical position between EF hands I and II in the N-terminal lobe [residues QNP^(41–43) replaced by

EKL]. CaM^{EKL} activated PDE normally, but did not activate NADK. However, CaM^{EKL} retained the ability to bind to NADK and inhibited activation by wild-type calmodulin. Site-directed mutagenesis of single residues showed that Gln⁴¹ and Pro⁴³ substitutions had the strongest effect on NADK activation. Additionally, CaM^{EKL} was not methylated, suggesting that the introduction of the methylation loop between EF hands I and II is not adequate for methyltransferase recognition. Overall the data indicate that residues in the methylation loop are essential but not sufficient for methyltransferase recognition, and that additional residues unique to EF hands III and IV are required. Secondly, the QNP sequence in the loop between EF hands I and II is necessary for NADK activation.

Key words: calcium, methyltransferase, NAD⁺ kinase.

INTRODUCTION

Calmodulin is a highly conserved calcium sensor protein that is ubiquitous among eukaryotes, and modulates the activities of multiple enzymes. Calmodulin is a monomer consisting of two structurally similar globular calcium-binding lobes [1] attached with a flexible linker region [2,3]. Each lobe consists of two helix-loop-helix EF hand calcium binding sites, with EF hand domains I and II constituting the N-terminal lobe and EF hands III and IV constituting the C-terminal lobe of the protein. EF hands I and II share sequence similarity [4] and structural similarity [1,5] with EF hands III and IV respectively. Additionally, this symmetry is also evident in the structures of the mutant calmodulin (CaM)-peptide complexes in which these EF hand pairs are related by an approximate two-fold axis [6,7].

Calmodulin is commonly found to be post-translationally trimethylated on a single lysine residue at position 115 (reviewed in [8]). Lys¹¹⁵ is a solvent exposed residue that is found on a highly conserved six amino acid loop-turn region (LGEKLT) located between the helix 6 of the EF hand III and the helix 7 of the EF hand IV (Figure 1). Although post-translational methylation occurs on many calmodulin proteins, its biological significance has remained a mystery. Previous work showed that trimethylation can affect certain *in vitro* activities of calmodulin, such as the ability to activate plant NAD⁺ kinase (NADK) [9,10], as well as the ability to undergo ubiquitination *in vitro* [11]. Further, mutations at residue 115 lead to abnormal phenotypes and enhanced defence responses in plants [10,12,13], and to a

reduced stability of the protein and growth arrest in *Schizosaccharomyces pombe* [14].

Trimethylation of Lys¹¹⁵ is catalysed by an *N*-methyltransferase that utilizes *S*-adenosyl methionine as a co-substrate, and which shows a high specificity for calmodulin ([15–19]; reviewed in [8]). This suggests that a highly specific calmodulin methyltransferase co-evolved with calmodulin with the dedicated function of trimethylating Lys¹¹⁵. Previous kinetic studies have shown that the site on calmodulin recognized by the calmodulin *N*-methyltransferase resides solely on the C-terminal lobe (residues 78–148) [17]. Further, methyltransferase binding and catalysis is affected by ionic strength, calcium-binding to calmodulin, and calmodulin antagonists [16–19]. Additionally, mutations or oxidation of residues in the hydrophobic core of the C-terminal lobe eliminate methyltransferase recognition [17,20,21]. Thus, it is likely that the site on calmodulin bound by the enzyme is more complex than the simple availability of a solvent exposed lysine residue. However, beyond these preliminary studies, the specific features of the calmodulin C-terminal lobe that lead to methyltransferase recognition and specificity remain unresolved.

In the present study we have investigated the structural requirements for calmodulin methylation by: 1) site-directed mutagenesis of conserved residues in the methylation loop-turn sequence; 2) attempting to engineer a calmodulin methyltransferase recognition site at the symmetrical loop-turn region between EF hands I and II in the N-terminal lobe; and 3) substitution of EF hands III and IV in the C-terminal lobe with EF hands I and II respectively. Additionally, during the course

Abbreviations used: CaM, mutant calmodulin; NADK, NAD⁺ kinase; PDE, cyclic nucleotide phosphodiesterase; VU-1, calmodulin derived from a synthetic gene.

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All expression plasmids were transformed into *Escherichia coli* JM101 as described previously [25]. Domain exchange mutants were identified by restriction mapping: the loss of an *MscI* site for CaM^{EKL}, and the introduction of a new *KpnI* site for CaM^{I214} and a new *PstI* site for CaM^{I232}. All mutants were confirmed by automated DNA sequencing on a Perkin Elmer Applied Biosystems 373 DNA sequencer at the University of Tennessee Molecular Biology Research Facility (Knoxville, TN, U.S.A.). Sequencing reactions were prepared with a Prism Dye Terminator Cycle sequencing kit (Perkin Elmer Applied Biosystems).

Protein purification

Calmodulins were prepared from *E. coli* expression clones as described previously [23,25]. Calmodulin methyltransferase was purified from sheep or pig brain through the calmodulin-Sepharose step as described previously [17]. Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE) was purified through the DEAE-cellulose step as described in [26]. Calmodulin-dependent NADK was purified from pea seedlings as described previously [20].

Other analytical methods

Calmodulin methyltransferase was assayed as described previously [17] in a standard assay buffer containing 0.1 M glycylglycine/NaOH, pH 8.0, 0.15 M KCl, 2 mM MgCl₂, 5 mM dithiothreitol, 0.01 % (w/v) Triton X-100, 12 μM [methyl-³H]S-adenosylmethionine (1.25 μCi/nmol), 1 mM CaCl₂, and various amounts of calmodulin. Pea NADK activity was assayed as described previously [23]. PDE assays were carried out as described previously [26]. NADK and PDE activation curves were generated by best fits to the data using the following equation for calmodulin activation:

$$\frac{v}{V_{\max}} = \frac{[\text{CaM}]^n}{K_{0.5} + [\text{CaM}]^n} \quad (1)$$

where v is the initial enzyme rate, $K_{0.5}$ is the concentration of calmodulin for half-maximal activation, V_{\max} is the maximal activation of the enzyme, $[\text{CaM}]$ is the concentration of calmodulin, and n represents the Hill coefficient.

Assays for the inhibition of NADK activation by CaM^{EKL} were done under standard conditions with various amounts of CaM^{EKL} at several fixed concentrations of VU-1 in 1 mM CaCl₂. The data were plotted as a Dixon plot using the following equation:

$$\frac{1}{v} = \frac{K_{0.5}}{V_{\max} K_1 [\text{CaM}]} \cdot [\text{CaM}^{\text{EKL}}] + \frac{1}{V_{\max}} \left(1 + \frac{K_{0.5}}{[\text{CaM}]} \right) \quad (2)$$

where v is the initial enzyme rate, $K_{0.5}$ is the concentration of VU-1 for half-maximal activation. V_{\max} is the maximal activation of the enzyme, $[\text{CaM}]$ is the concentration of VU-1, $[\text{CaM}^{\text{EKL}}]$ is the concentration of CaM^{EKL}, and K_1 is the inhibition constant. Assays for the calcium dependence of NADK activation were carried out in 50 mM Hepes/NaOH, pH 7.5, 3 mM MgCl₂, 3 mM EGTA, 3 mM ATP, 2 mM NAD⁺, 0.1 mM calmodulin, and various concentrations of CaCl₂ that yielded a free calcium concentration ranging from 10⁻⁸ to 10⁻³ M determined as described previously [20]. Free calcium concentrations in the reactions were verified and quantified with fura-2 (Molecular Probes) as described in [27]. All buffers and reagents were decalcified by Chelex-100 resin treatment as described in [28]. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Biochemicals) using BSA as a standard.

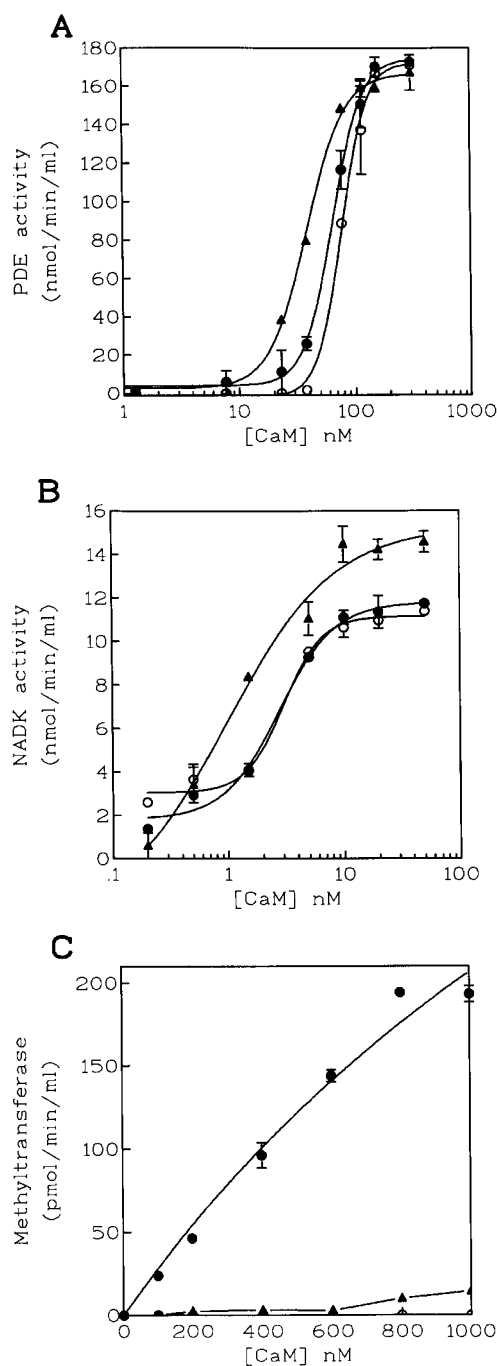


Figure 2 Enzyme activation and methylation of E114A and L116T

Activation of (A) PDE and (B) NADK by VU-1 (●), E114A (○), and L116T (▲) calmodulins, and (C) pseudo-first-order methyltransferase kinetics at a constant saturating concentration of S-adenosylmethionine (12 μM) with calmodulin as the varied substrate. All assays were carried out in 1 mM CaCl₂. Means ± S.E.M. are shown.

RESULTS

Effect of point mutations (E114A and L116T) in the methylation loop

The six amino acid loop-turn sequence that contains the site of methylation (Figure 1A), contains a surface exposed glutamate at position 114 as well as a leucine at position 116 which is

Table 1 Activation parameters of calmodulin dependent enzymes by methylation loop and domain exchange mutant calmodulins

V_{max} , maximal activation of enzymes (PDE or NADK) by calmodulins standardized to VU-1 (100%); $K_{0.5}^{CaM}$, concentration of calmodulin (nM) giving half-maximal activation; $K_{0.5}^{Ca^{2+}}$, concentration of calcium (nM) giving half-maximal activation of enzyme NADK in the presence of a constant amount of calmodulin (0.1 μ M). The S.E.M. value is shown in parentheses; n.d., not determined.

CaM	PDE		NADK		
	V_{max} (%)	$K_{0.5}^{CaM}$ (nM)	V_{max} (%)	$K_{0.5}^{CaM}$ (nM)	$K_{0.5}^{Ca^{2+}}$ (nM)
VU-1	100	54 (4.2)	100	1.8 (0.07)	96
CaM ^{EKL}	100	91 (7.8)	0	0	n.d.
Q41E	97	85 (9.0)	36	1.8 (0.06)	n.d.
N42K	101	67 (7.4)	80	0.8 (0.12)	n.d.
P43L	101	66 (8.8)	57	2.3 (0.06)	n.d.
CaM ^{I214}	98	98 (6.9)	66	1.1 (0.10)	120
CaM ^{I232}	52	72 (10.1)	79	0.7 (0.05)	148
E114A	99	75 (11.1)	97	3.0 (0.07)	n.d.
L116T	96	38 (6.6)	130	1.0 (0.18)	n.d.

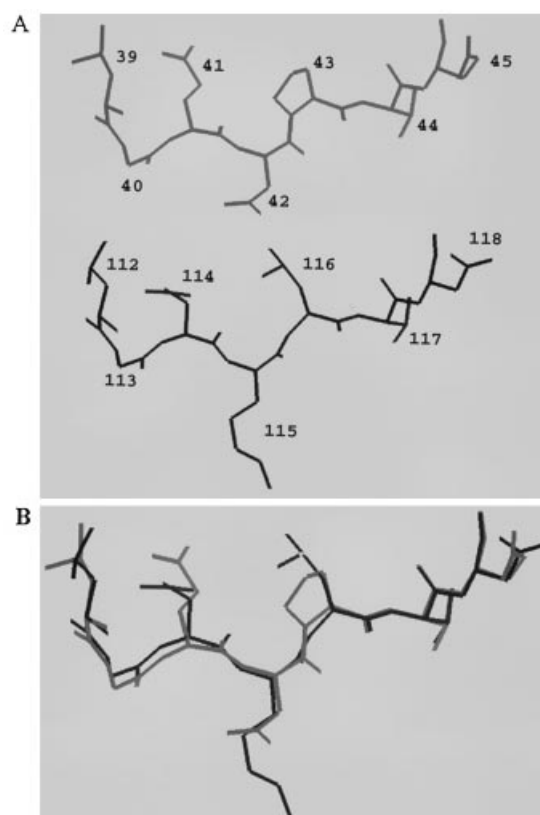
embedded within the hydrophobic core of the C-terminal lobe [1]. To test the role of these invariant flanking residues in calmodulin methyltransferase recognition, Glu¹¹⁴ was substituted with alanine, and Leu¹¹⁶ was replaced by threonine.

The effect of these mutations on the activation of calmodulin-dependent enzymes was assessed to determine whether calmodulin function was disrupted. The activation of NADK and PDE by E114A was essentially indistinguishable from that by VU-1 (Figures 2A and 2B, Table 1). L116T also retained the ability to fully activate NADK and PDE, and actually showed a slightly enhanced activation of NADK (Figures 2A and 2B, Table 1). In contrast, the E114A and L116T mutations showed a drastic effect on Lys¹¹⁵ methylation and neither calmodulin could serve as a substrate for methylation by the calmodulin methyltransferase (Figure 2C). The data suggest that the substitutions of these highly conserved residues on either side of Lys¹¹⁵ do not significantly alter their ability to activate calmodulin-dependent enzymes, but that they are essential for methyltransferase recognition.

Attempts to generate a methylation site within the N-terminal lobe using CaM^{EKL}

While the data in the previous section show that the integrity of the residues in the loop-turn region are essential for methylation, it is not clear whether other structural elements unique to the C-terminal lobe are required. Calmodulin shows symmetry between the N-terminal and C-terminal lobes (Figure 1A), including the presence of a six amino acid loop (LGQNPT) in the N-terminal lobe that is located at a position similar to the methylation loop in the C-terminal lobe (Figure 1A). A comparison of the backbone structure of the two loop regions (residues 39–44 and 112–117) shows that they are superimposable (Figure 3). Taking advantage of the symmetry between the two lobes we investigated whether we could engineer a methylation recognition sequence into the loop region in the N-terminal lobe.

VU-3 [9,10] was used as the starting point for the generation of the mutant CaM^{EKL} (Figure 1). Since it has a substitution of an arginine for a lysine at residue 115, it is not methylated by the calmodulin methyltransferase [9,29]. The sequence of the methylation loop (LGEKLT) of the C-terminal lobe was placed in the loop of the N-terminal lobe by substituting the coding region for QNP^(41–43) with EKL by cassette-based mutagenesis (Figure 1B).

**Figure 3** Comparison of the backbone structures of the loop-turn regions in the N- and C-terminal lobes

The methylation-site loop (LGEKLT, residues 112–117 shown in black) of the C-terminal lobe and its symmetrical loop region (LGQNPT, residues 39–44 shown in grey) of the N-terminal lobe are shown apart (A) and superimposed (B). The two structures superimpose with a root-mean-square difference of 0.33 Å. The images were generated using a Silicon Graphics Indigo system and Insight II software.

This substitution showed a complex effect on calmodulin activator activity. Both VU-1 and CaM^{EKL} activate PDE to the same extent, and compared to VU-1, CaM^{EKL} shows only a slight shift in the concentration required for half-maximal activation ($K_{0.5}^{CaM}$; Figure 4A, Table 1). This suggests that the three substitutions within this loop in the N-terminal lobe do not drastically affect PDE activator properties. In contrast, CaM^{EKL} showed essentially no activation of NADK (Figure 4B). Furthermore, CaM^{EKL} was a poor methyltransferase substrate showing essentially no detectable methylation (Figure 4C). Thus, the substitution of the methylation loop sequence at an analogous position in the N-terminal lobe was not adequate to confer methyltransferase recognition, even though the two loops have a similar conformation.

To investigate whether the loss of activation of NADK by CaM^{EKL} is the result of an inability of NADK to bind to the mutant calmodulin, the ability of CaM^{EKL} to antagonize the activation of NADK by VU-1 was tested (Figure 5A). CaM^{EKL} was able to inhibit the activation of NADK by VU-1 ($K_i = 4 \pm 0.25$ nM). Thus, CaM^{EKL} binds NADK, but this binding event is non-productive and does not lead to the activation of the enzyme. Previous studies have shown that VU-3 is a potent activator of NADK [9,10]. Thus, the aberrant NADK activator activity of CaM^{EKL} appears to be due to the substitution of EKL at residues 41–43 and not due to the presence of Arg¹¹⁵.

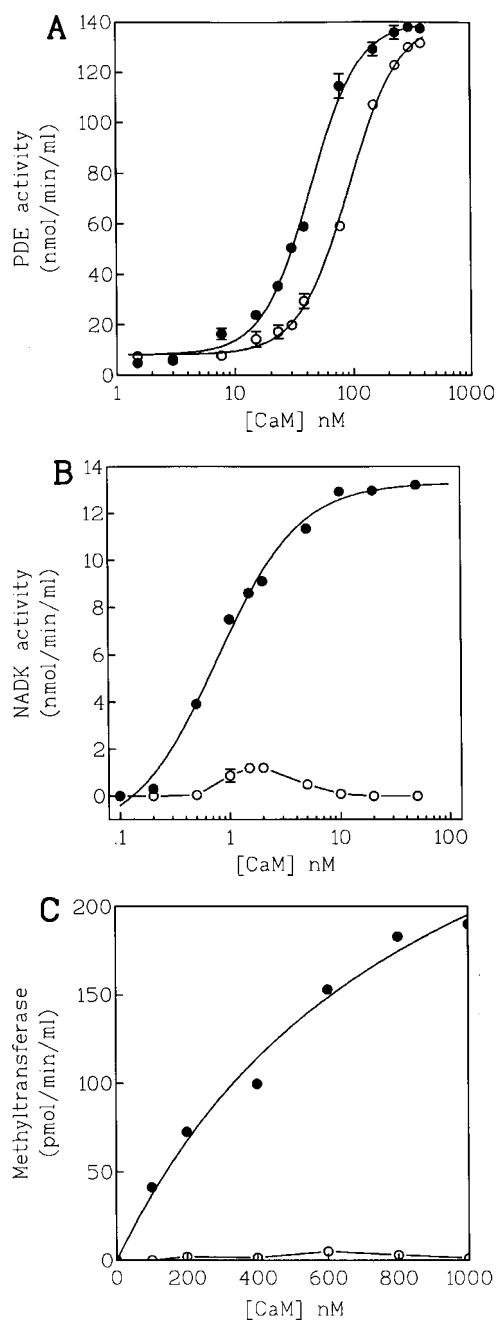


Figure 4 Enzyme activation and methylation of CaM^{EKL}

Activation of (A) PDE and (B) NADK by VU-1 (●) and CaM^{EKL} (○) calmodulins, and (C) pseudo-first-order methyltransferase kinetics at a constant saturating concentration of *S*-adenosylmethionine (12 μM) with calmodulin as the varied substrate. All assays were carried out in 1 mM CaCl₂. Means ± S.E.M. are shown.

To further define which residue(s) are important for the activation of NADK three calmodulin mutants with single amino acid substitutions (Q41E, N42K, P43L) in the loop region were generated. Of the three mutants, Q41E showed the most drastic effect, activating the enzyme to 36% of the level obtained by VU-1 (Figure 5B, Table 1). P43L activates NADK to 57% of the level of VU-1 and also shows a slight shift in the $K_{0.5}^{CaM}$ (Figure 5B, Table 1). Mutation N42K shows the least effect on NADK activation (80% of wild-type). The data show that the inability

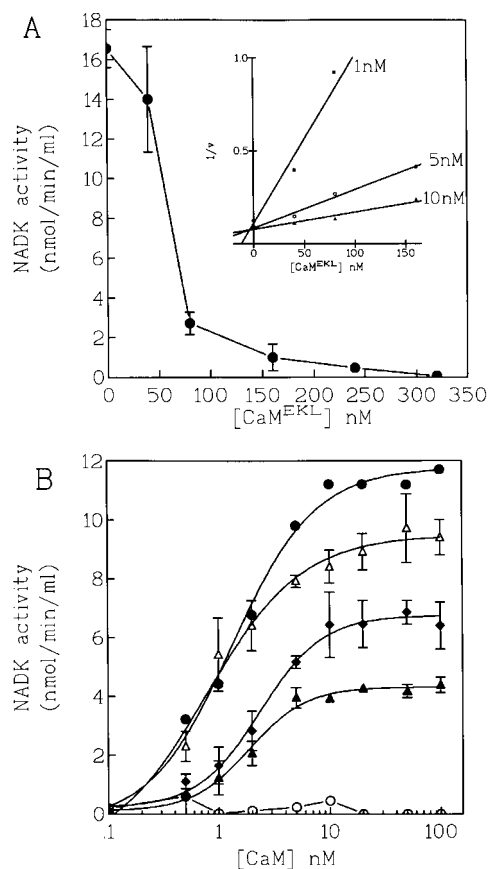


Figure 5 Interaction of CaM^{EKL} and Q41E, N42K, P43L calmodulins with NADK

(A) Activation of NADK by 100 nM VU-1 in the presence of varying amounts of CaM^{EKL}. The inset shows a Dixon plot of the activation of NADK in the presence of fixed concentrations of VU-1 and increasing amounts of CaM^{EKL}. ●, 1 nM VU-1; ○, 5 nM VU-1; ▲, 10 nM VU-1 (B) Activation of NADK by VU-1 (●), CaM^{EKL} (○), Q41E (△), N42K (▲), and P43L (◆). All assays were carried out in 1 mM CaCl₂. Means ± S.E.M. are shown.

of CaM^{EKL} to activate NADK is due to the cumulative effect of the three substitutions, and is not due to any single amino acid change.

Effects of substituting EF hands I and II into the C-terminal lobe using CaM¹²¹⁴ and CaM¹²³²

CaM¹²¹⁴ and CaM¹²³² were constructed by generating a cassette containing EF hand I or EF hand II by PCR, and replacing the coding regions for EF hands III or IV respectively in VU-1 (Figure 1B). Both calmodulins were functional and activated PDE and NAD kinase (Figure 6). CaM¹²¹⁴ activates PDE to the same extent as VU-1 (Figure 6A), however, the $K_{0.5}^{CaM}$ for the activation is two-fold higher (Table 1). CaM¹²³² activates PDE to only 52% of the level of VU-1 and also shows a slight shift in the $K_{0.5}^{CaM}$ (Figure 5A, Table 1). CaM¹²¹⁴ and CaM¹²³² also show a lower NADK activation, activating the enzyme to 66% and 79% respectively of the level obtained with VU-1 (Figure 6B, Table 1). Furthermore, calcium dependence for enzyme activation by VU-1, CaM¹²³² and CaM¹²¹⁴ show similar $K_{0.5}^{Ca^{2+}}$ values between 90 and 150 nM (Figure 6C, Table 1), suggesting that these domain substitutions do not drastically compromise calcium binding activity.

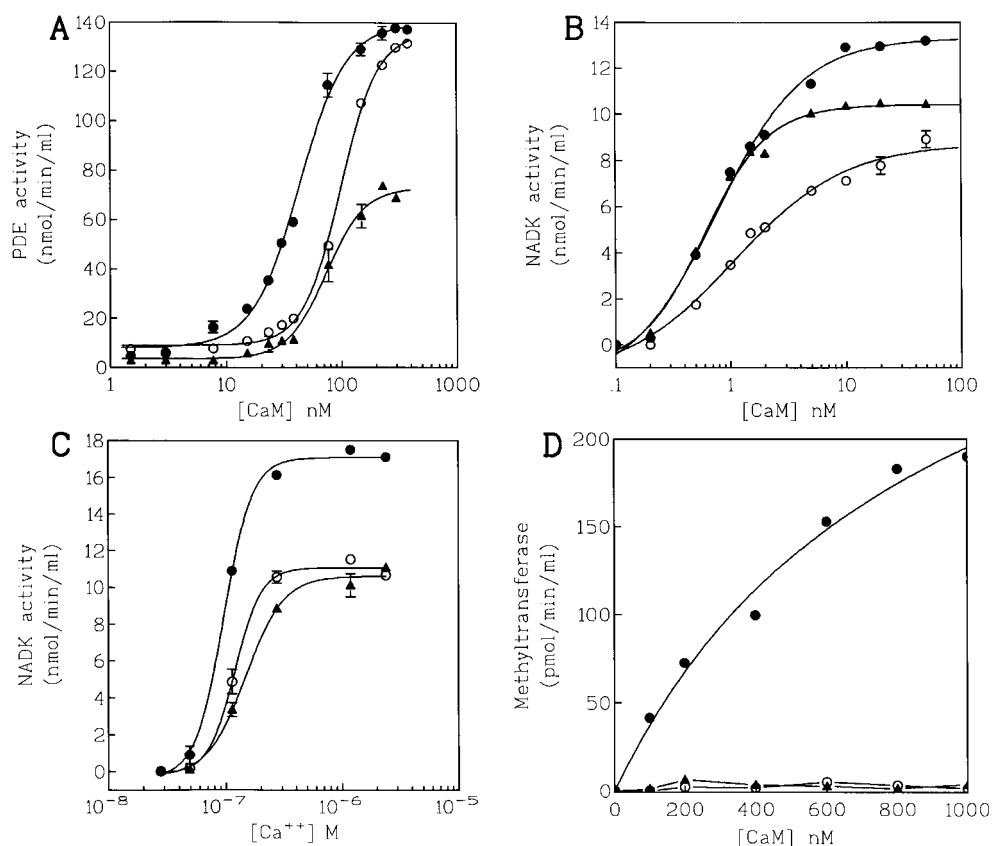


Figure 6 Enzyme activation by and methylation of CaM¹²¹⁴ and CaM¹²³²

Activation of (A) PDE and (B) NADK in 1 mM CaCl₂ with varied concentrations of calmodulin. (C) Activation of NADK by 50 nM of each calmodulin with varied concentrations of free calcium using the EGTA/Ca²⁺ buffering system described in the Materials and methods section. (D) Pseudo-first-order methyltransferase kinetics at a constant saturating concentration of *S*-adenosylmethionine (12 μM) with calmodulin as the varied substrate, and carried out in 1 mM CaCl₂. Means ± S.E.M. are shown. ●, VU-1; ○, CaM¹²¹⁴; ▲, CaM¹²³².

In contrast to the modest effects on enzyme activation, neither CaM¹²³² nor CaM¹²¹⁴ serves as a substrate for methylation by the calmodulin methyltransferase (Figure 6D). Together with the data for CaM^{EK14}, these findings suggest that amino acid residues unique to both EF hands III and IV are required for recognition by the calmodulin methyltransferase.

DISCUSSION

Calmodulin methyltransferase recognition

Previous studies have shown that a tryptic fragment of calmodulin comprising residues 78–148 is sufficient for methyltransferase recognition and Lys¹¹⁵ methylation with kinetics indistinguishable from those with intact calmodulin [17]. Thus, the residues that comprise the recognition site for calmodulin methyltransferase are localized to the C-terminal lobe of the molecule. The loop region (residues 112–117) containing the unique site of methylation is highly conserved among calmodulins, and is a logical place to begin to investigate residues required for methyltransferase binding and recognition.

The methylation loop (LGEKLT) sequence represents a turn between adjacent EF hands III and IV. The turn appears to be facilitated by Gly¹¹³ ($\Phi/\psi = 93^\circ/10^\circ$ [30]), which explains the high conservation of glycine at this position. Additionally, the loop conformation appears to be stabilized by hydrogen bonding between the backbone amide nitrogens of Gly¹¹³ and Glu¹¹⁴ and

the backbone carbonyl oxygens of Met¹⁰⁹ and Thr¹¹⁰ in helix 6 (F helix of EF hand III, Figure 1) [30]. In addition, the side chain of Lys¹¹⁶ in the loop sequence is embedded in the core of the C-terminal lobe where it forms hydrophobic interactions with residues on the hydrophobic faces of the helices of EF hands III and IV [1]. Lys¹¹⁵ is solvent exposed and does not appear to form contacts with any other part of the calmodulin structure [1,31].

The present results show that the two residues that flank the site of methylation appear to be essential for methyltransferase recognition, but not for calmodulin activation of the two enzymes tested. The replacement of Leu¹¹⁶ with the more polar side chain of threonine would most likely disrupt the interaction of the methylation loop with the hydrophobic core, and this apparently results in a loss of recognition and methylation by the enzyme. Substitution of the negatively charged, solvent exposed Glu¹¹⁴ with uncharged alanine also abolishes Lys¹¹⁵ methylation. Previous work [17] has suggested that electrostatic interactions help stabilize the interaction of calmodulin with the methyltransferase, and the loss of the negatively charged, surface exposed glutamate may eliminate a point of contact for the enzyme.

While the conserved sequence of the methylation loop (LGEKLT) appears to be essential for methyltransferase recognition, it is clear that other structural determinants outside of this region are also essential for methyltransferase binding. This is supported by the finding with CaM^{EK14}, which is not recognized and methylated by the calmodulin methyltransferase. Based on a comparison of the backbone structure of residues 39–44 and

112–117 (Figure 3), the loop sequence introduced into the N-terminal lobe of CaM^{EKL} should have a similar conformation to the methylation loop in native calmodulin. Thus, the inability of the enzyme to methylate CaM^{EKL} strongly suggests that additional determinants outside of the loop are essential for methyltransferase binding and catalysis.

This conclusion is further supported by the results with the domain exchange mutants CaM¹²¹⁴ and CaM¹²³², neither of which serve as a substrate for methylation. As pointed out previously, the backbone structures of the EF hand pair I and II are very similar to those of EF hand pair III and IV (root-mean-square difference of 0.751 Å, [1]). In addition, the present study, as well as previous work [32], shows that the exchange of EF hands between the N- and C-terminal lobes produces folded, functional calmodulin proteins. Thus, the lack of the ability of the two EF hand exchange mutants to be methylated is most likely due to the requirement for specific structural determinants unique to both EF hands III and IV, which are missing in both EF hands I and II. The exchange of subdomains (e.g., E or F helices or loop regions) between EF hands, or the substitution of highly conserved residues unique to both EF hands III or IV, will provide future insight regarding these specific structural determinants.

Determinants for the activation of NADK

The present study sheds new light on residues important for the activation of NADK. CaM^{EKL} does not activate NADK but is a potent antagonist of activation by wild-type calmodulin, suggesting binding but not activation of the enzyme. Calmodulin derivatives that have similar antagonist activities with other calmodulin-dependent enzymes have been generated via chemical modification [33], the production of troponin C/calmodulin chimaeras [34–38] or by EF hand duplication within calmodulin [32,39].

From these observations, it has been proposed that calmodulin activation of enzymes follows a two step mechanism. Initially, calcium interaction with calmodulin leads to interaction with the calmodulin binding domain of the enzyme ([6,7]; reviewed in [40]) that is followed by additional specific interactions which lead to activation [35,41]. The structural determinants for this latter process are proposed to form after calmodulin binding, and recent evidence has implicated a region of calmodulin known as the 'latch domain'. During target protein-peptide binding, the N- and C-terminal lobes of calmodulin converge, forming a hydrophobic channel that surrounds the peptide [6,7]. In the peptide bound structure, helix 2 (EF hand I) interacts with helix 6 (EF hand III), forming a 'latch', linking the two lobes of calmodulin [6].

Site-directed mutagenesis studies have shown that residues within helix 2 and helix 6 are essential for activation of calmodulin-dependent enzymes [35–38]. Interestingly, however, specific mutations within the latch domain region show a differential effect on the activation of various calmodulin-dependent enzymes. Thus while it is clear that this domain is important in the activator activity of calmodulin, each target enzyme has a distinct set of structural determinants for activation.

By using a chimaeric approach with soybean calmodulin (SCaM-1) and a calmodulin-like protein (SCaM-4), residues within helix 2 in EF hand I were implicated as being important for activation of NADK [42]. The present study shows that additional residues in the loop-turn region between EF hands I and II adjacent to helix 2 also play a critical role in activation. The substitution of QNP to EKL in CaM^{EKL} is predicted to result in the same backbone conformation (Figure 3), suggesting

that the defect is probably not due to a disruption of calmodulin conformation in this region. Since these residues are localized close to the latch domain, it is possible that they form part of the structure that is required for activation of NADK upon calmodulin binding. Among the residues in the QNP triad, the mutation Q41E appears to exert the greatest effect on activity, suggesting that the introduction of a negative charge for the conserved amide at position 41 affects NADK activation. Additionally, the replacement of the conserved Pro⁴³ residue with leucine, a larger branched hydrophobic residue, also shows a significant decrease in activation. Evidence for the importance of residue 43 in calmodulin function also comes from genetic studies of *Drosophila melanogaster* in which a point mutation of Pro⁴³ → Leu in calmodulin results in enhanced lethality and abnormal behavioural characteristics [43].

Interestingly, the symmetry related methylation loop sequence (EKL) also appears to be important in activation. The present study shows that the E114A and L116T mutations have only a slight effect on NADK activation. In contrast, Lys¹¹⁵ appears to have a greater effect on NADK, since trimethylation drastically lowers the level of activation of NADK [9,10,23,44]. This post-translational modification has little effect on other calmodulin-dependent enzymes, again emphasizing the unique nature of the determinants for activation of the various calmodulin-dependent enzymes.

As a final point, the use of mutant calmodulins that selectively hyperactivate NADK in transgenic plants has been instructive in the analysis of its function *in vivo* in plant defence responses [12,13]. Based on the present work, CaM^{EKL} may be useful as a potential antagonist of NADK for similar *in vivo* studies.

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