Structural requirements for N-trimethylation of lysine-115 of calmodulin.

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Running title: Methyltransferase recognition of mutant calmodulins

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Summary

Calmodulin is trimethylated at lysine 115 by a highly specific methyltransferase that utilizes S-adenosyl methionine as a co-substrate. Lysine 115 is found within a highly conserved six amino acid loop (LGEKL) that forms a 90° turn between EF hand III and EF hand IV in the carboxyl terminal lobe. In the present work a mutagenesis approach was used to investigate the structural features of the carboxyl terminal lobe that lead to the specificity of calmodulin methylation. Three structural regions within the carboxyl terminal lobe appear to be involved in methyltransferase recognition: the highly conserved six amino acid loop-turn region that contains lysine 115, as well as the adjacent α-helices (helix 6 and helix 7) from EF hands III and IV. Site-directed mutagenesis of residues in the loop show that three residues, glycine-113, glutamate-114, and leucine-116 are essential for methylation. In addition, subdomain (individual helix or Ca²⁺-binding loop) exchange mutants show that the substitutions of either helix 6 (EF hand III) with helix 2 (EF hand I), or helix 7 (EF hand IV) with helix 3 (EF hand II) compromises methylation. Charge to alanine mutations in helix 7 show that substitution of conserved charged residues at positions 118, 120, 122, 126, and 127 reduced lysine 115 methylation rates, suggesting possible electrostatic interactions between this helix and the methyltransferase. Single substitutions in helix 6 did not affect calmodulin methylation, suggesting this region may play a more indirect role in stabilizing the conformation of the methyltransferase recognition sequence.
Introduction

Calmodulin is a highly conserved calcium sensor protein that modulates the activities of multiple enzymes. Calmodulin is a monomer consisting of two structurally similar globular calcium-binding lobes (1) connected by 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 amino-terminal lobe and EF hands III and IV constituting the carboxyl-terminal lobe. Many naturally occurring calmodulins are posttranslationally trimethylated on a single lysine residue at position 115 (reviewed in 4). Lysine-115 is a solvent-exposed residue that is found on a highly conserved six amino acid loop-turn region (LGEKLT) located between helix 6 of EF hand III and helix 7 of EF hand IV (Fig. 1). Trimethylation of calmodulin at lysine 115 is catalyzed by an N-methyltransferase that utilizes S-adenosyl methionine (\(^\text{1}\)AdoMet) as a co-substrate (5-9, reviewed in 4). This enzyme appears to have the dedicated function of trimethylating lysine 115 in a wide variety of species. From a functional perspective, calmodulin methylation selectively affects the regulation of certain enzymes, such as NAD kinase (10-12), and might also influence posttranslational ubiquitination of the protein (13).

Previous work has shown that the site on calmodulin recognized by the calmodulin N-methyltransferase resides solely on the COOH-terminal lobe (residues 78-148) (7). Mutations or chemical modifications that affect the hydrophobic core and conformation of calmodulin disrupt methylation (7, 14), and it seems as if the enzyme requires more than a linear sequence of amino acids or the simple surface exposure of lysine-115 for recognition and methylation.
An examination of the calmodulin structure shows that the amino terminal and carboxyl terminal lobes share remarkable structural similarity and symmetry (1). In previous work we performed a series of domain duplication and exchange mutagenesis experiments in which EF hands III and IV were substituted with the symmetry-related EF hands I and II (15). These experiments showed that structural features unique to both EF hands are required for methyltransferase binding and methylation. To define more precisely the regions responsible for calmodulin methyltransferase specificity, we exploited this domain exchange approach further, and performed site directed mutagenesis of various residues in the carboxyl terminal lobe surrounding the methylation site. The results implicate specific regions in the methylation loop/turn region between EF hands III and IV as well as residues within the adjacent α-helices, in the binding and recognition of the enzyme.
Experimental Procedures

Molecular Cloning Techniques  All mutagenesis and expression experiments were done with the calmodulin expression plasmid pVUCH (16) which contains the cloned synthetic VU-1 calmodulin gene (17). Synthetic oligonucleotides were obtained either from Oligos Etc. or Gibco/BRL. All site-directed substitutions of calmodulin were generated by oligonucleotide-directed mutagenesis using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Substitution of entire subdomains of calmodulin was done by cassette mutagenesis as described previously (18). Briefly, targeted regions in the coding region for the carboxyl terminal lobe of calmodulin were removed by digestion with restriction enzyme pairs that flank the regions of interest. Synthetic oligonucleotide cassettes containing the homologous sequences from the amino terminal lobe were engineered with complementary ends of the corresponding restriction enzyme. These were inserted by ligation into digested VU-1 gene as described previously (18). To enable greater latitude in the restriction sites available for cassette mutagenesis, two restriction sites were removed from pVUCH-1 by site-directed mutagenesis: an AatII site within the vector and a BamHI site flanking the ptac promoter. Neither mutation affected vector performance or calmodulin expression. Subdomain mutants and corresponding cassettes and enzymes are:

CaM<sub>CL3</sub> (Stu/EagI) residues 90-101

| Sanger Coding | 5' CC TTC TCT CTG TTT GAC AAA GAC GGT GAC GGT ACC ATC AC 3' |
|---------------|--------------------------------------------------------|
| TIGR Coding   | 3' GG AAG AGA GAC AAA CTG TTT CTG CCA CTG CCA TGG TAG TGC CGG 5' |

CaM<sub>H6</sub> (Eag/HindIII) (residues 105-111)

| Sanger Coding | 5' G GCC GCT GAA CTC GGC ACC GTT ATG CGC AGC CTT GGT GAA A 3' |
|---------------|--------------------------------------------------------|
| TIGR Coding   | 3' CGA CTT GAG CCG TGG CAA TAC GCG TCG GAA CCA CTT TTC GA 5' |
**Protein Purification** Calmodulin was isolated from *E. coli* expression clones by phenyl-Sepharose chromatography as described previously (15,17). Calmodulin N-methyltransferase was purified from rat testes by a modification of a previous procedure (7). The enzyme was extracted and purified through the differential ammonium sulfate step as in Han et al. (1993). At this step, the enzyme was applied to a Sephadex G-10 (6 x 5 cm) column equilibrated in 10 mM Hepes-NaOH, pH 7.4, 4 mM β-mercaptoethanol, 0.01% (w/v) triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride. The absorbance of the effluent was continuously monitored at 280 nm. The fractions containing protein were combined and were applied to a 2.5 x 16 cm column of DEAE-cellulose (Whatman DE-53) equilibrated in the same buffer. The non-binding protein fraction, which contains the calmodulin-N-methyltransferase, was collected. Calcium was added to 2 mM and calmodulin–Sepharose chromatography was done as described previously (7). The fractions with calmodulin methyltransferase activity were pooled and
concentrated by ultrafiltration on a Centricon-30 unit (Amicon). Calmodulin-dependent cyclic nucleotide phosphodiesterase (sheep brain) and NAD kinase (pea seedlings) were prepared as previously described (14).

**Other Analytical Methods** Calmodulin methyltransferase was assayed as described previously (7) in a standard assay buffer of 0.1 M glycylglycine-NaOH (pH 8.0), 0.15 M KCl, 2 mM MgCl$_2$, 5 mM dithiothreitol, 0.01% (w/v) triton X-100 and either 1 mM CaCl$_2$ or 1 mM EGTA. The determination of kinetic parameters for the methyltransferase were derived under pseudo-first order conditions with a constant concentration of 12 µM [methyl-$^3$H]- AdoMet (1.25 µCi/nmol) and various concentrations of calmodulin (14). Apparent K$_m$ and V$_{max}$ parameters were determined by fitting the data to the Michaelis Menten equation, and k$_{cat}$ was determined by using 38,000 as the molecular weight of the methyltransferase as previously described (7).

Pea NADK activity was assayed as described previously (17). Cyclic nucleotide phosphodiesterase assays were done as described previously (19). The NADK and PDE activation curves were generated by best fits to the data using the Hill equation.

\[
\frac{v}{V_{max}} = \frac{[CaM]^n}{K_{0.5} + [CaM]^n}
\]

Where v is the initial enzyme rate, K$_{0.5}$ is the concentration of calmodulin for half-maximal activation. V$_{max}$ is the enzyme rate at maximal activation of the enzyme, [CaM] is the concentration of calmodulin, and n represents the Hill coefficient. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Biochemicals) by using bovine serum albumin as a standard.
Results

Mutations in the methylation loop.

Lysine 115 is located on a highly conserved, six amino acid (LGEKLT, residues 112-117) loop-turn region between EF hands III and IV (Fig. 1). To test whether this conserved six amino acid motif is required for calmodulin methylation, a series of mutations were generated (Fig. 1). Three mutations severely affected lysine 115 methylation: G113S, E114A and L116T. In all three cases, the substitutions resulted in a complete loss of the ability of calmodulin to serve as a methylation substrate (Fig. 2; Table I). Examination of the other two conserved residues (L112, T117) in the methylation loop showed that they are less important in activity (Table I). Of the two, substitution of a threonine at L112 showed the most significant effect, with a 4.5 fold reduction in the catalytic efficiency of methylation when assayed in the absence of calcium (Fig. 2B, Table I). However, unlike calmodulin with substitutions at residues 113, 114, and 116, the methylation properties of L112T were essentially identical to wildtype VU-1 calmodulin in the presence of saturating calcium (Fig. 2A), suggesting that this mutation selectively affects the substrate properties of apo-calmodulin.

In contrast to their influence on methylation, all methylation loop mutants showed essentially indistinguishable NAD kinase and PDE activation profiles (not shown), suggesting that the substitutions of these highly conserved residues in the methylation loop do not significantly alter calmodulin-activator functions.

Production and analysis of sub-domain exchange mutations of calmodulin

While the conserved residues in the methylation loop are essential for trimethylation of lysine 115, it is clear that additional flanking regions within EF hands III and IV are also essential for methyltransferase recognition (15). To identify the critical regions within EF hands
III and IV necessary for methyltransferase recognition, subdomain exchange mutants were generated. In these mutants, the various helices and calcium binding loops of EF hands III and IV were replaced with the homologous regions of EF hands I and II (Fig. 1A).

The conservative nature of these substitutions is underscored by the observation that all subdomain mutant exchange mutants activated PDE and NAD kinase similar to VU-1 calmodulin with the exception of CaM<sup>H6</sup> which showed a modest reduction in maximal activation of NAD kinase (Table II). In contrast to the activator properties, the methylation properties of calmodulin were substantially affected by the exchange of certain subdomain elements (Fig. 3). The two subdomain mutant calmodulins with substitutions adjacent to the methylation loop, CaM<sup>H6</sup> and CaM<sup>H7</sup> showed the most substantial effects on the rate of calmodulin methylation (Fig. 3). CaM<sup>H6</sup> was a poor methylation substrate in both its apo and calcium-saturated state (Fig. 3), showing a catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub>) that was 25-fold (apo) or 13-fold (Ca<sup>2+</sup>-bound) lower than that of VU-1 calmodulin (Table III). In contrast, the ability of CaM<sup>H7</sup> to serve as a methylation substrate was strictly dependent on calcium. In the absence of calcium, CaM<sup>H7</sup> was incapable of being methylated (Fig. 3B). However, in the presence of saturating calcium, the methylation kinetics of CaM<sup>H7</sup> were nearly restored to the level of VU-1 CaM (Fig. 3A).

The other subdomain mutants exhibited a smaller effect on methyltransferase kinetics (Table III). The mutations within the two calcium binding loops (CaM<sup>CL4</sup> and CaM<sup>CL3</sup>) showed catalytic efficiencies with the methyltransferase that were comparable with VU-1 (Table III). The CaM<sup>H8</sup> mutant showed slightly defective methylation in its apo form, with a slightly lower k<sub>cat</sub> (0.0172 vs 0.0124 sec<sup>-1</sup>) and four-fold higher K<sub>m</sub>. However, relatively normal kinetic behavior with CaM<sup>H8</sup> was restored under conditions of saturating calcium (Table III).
Scanning mutagenesis of helix 6

A comparison of the sequence in helix 6 of wild type calmodulin [LRHVMTN/(105-111)] with the substituted sequence in mutant CaM^{H6} [LGTVMRS] reveals no change in the hydrophobic residues that are involved in the packing of helix 6 with the other helices in the carboxyl-terminal lobe (Fig.1B). The possibility that surface residues in helix 6 are important for calmodulin interaction with the methyltransferase was therefore addressed. The CaM^{H6} mutant has shows three principal changes compared to wild type: The removal of two positive charged residues (R106 and H107 replaced with G and T) and the introduction of a bulky, charged arginine for T110. The remaining substitution, a N111 to S change has previously been shown not to affect methylation (5, 20). To test whether these residues are important in methylation, charge to alanine mutants were generated for R106 and H107, and T110 was changed to an arginine. In contrast to CaM^{H6}, these point mutants show little effect on calmodulin methylation (Fig. 4A). Overall, the data suggest that the removal or alteration of individual surface charge residues on helix 6 does not appear to affect the interaction of either apo-CaM or Ca^{2+}-CaM, but rather that the entire substitution of helix 6 with helix 2 results in a structural change that disrupts the site of methylation.

Scanning mutagenesis of helix 7

An examination of helix 7 [DEEVDEMIREA (118-128)] shows that this region possesses an extremely high number (7 out of 11) of conserved charged (mainly acidic) residues. These residues provide a surface-exposed patch of negative charge on the carboxyl-terminal lobe, adjacent to lysine-115 (Fig. 5). This observation and the previous finding that calmodulin methylation is sensitive to ionic strength (7), raises the possibility that charge-charge interactions
could potentially be involved in methyltransferase binding. To test this, a series of mutations of charged residues in helix 7 were generated and assayed for their effects on methylation (Fig. 4B).

The substitution of residues within the charged cluster (residues 118-120) immediately adjacent to the methylation loop showed the greatest effects on methylation. The calmodulin mutant DEE118-20KKK showed the greatest defect, with the methylation rate decreased 10 (in the presence of calcium) to 30 (in absence of calcium) fold relative to VU-1 calmodulin. This calmodulin possesses three lysine substitutions within this conserved cluster, resulting in a neutralization of nearly half of the negative electrostatic potential surface on the carboxyl-terminal lobe (21). Each negative charge was then removed by alanine scanning mutagenesis. E120A and E118A showed the greatest effects with a 6-fold and 3-fold reduction in methylation rate respectively in the absence of calcium (Fig. 4B). E120A was methylated normally in the presence of calcium, whereas E118A showed a two-fold reduced methylation rate. In contrast, E119A was methylated normally (Fig 4B), suggesting this residue is less important in calmodulin methyltransferase recognition.

Other charged residues on helix 7 were also analyzed (Fig. 4B) and showed more modest effects. A decreased activity was observed with E127A to (46% of VU-1) as well as D122A and R126A (60% of VU-1) in the absence of calcium. The effect of these mutations was less apparent in the presence of calcium, suggesting that calcium binding enhances substrate activity and at least partially overcomes the effects of the mutation.
Discussion

Calmodulin is trimethylated at lysine 115 with a high degree of specificity by a dedicated calmodulin lysine N-methyltransferase. In the present study, domain exchange and scanning mutagenesis were done to attempt to identify regions of the protein that contribute to this specificity. The results suggest that three structural regions within the carboxyl terminal lobe appear to be involved: the highly conserved six amino acid loop-turn region that contains lysine 115, as well as the adjacent α-helices (helix 6 and 7) from EF hands III and IV.

The six amino acid methylation loop (LGEKLT) is highly conserved among phylogenetically diverse calmodulins, and it is reasonable to suggest that its structure provides features necessary for calmodulin methyltransferase recognition. Structural studies suggest that the loop shows greater flexibility and dynamics compared to the calcium binding loops and helices of the EF hands (22, 23, 24). The loop provides a 90° hairpin turn between EF hands III and IV, which is facilitated by G-113 (φ/ψ = 93°/10°, 22), and its conformation is stabilized by three hydrogen bonds between the backbone amide nitrogens of G-113 and E-114 and the backbone carbonyl oxygens of M-109 and T-110 in helix 6 of EF hand III (22). In addition, L-116 is imbedded in the core of the carboxyl terminal lobe, forming hydrophobic interactions with residues on the hydrophobic faces of the helices from EF hands III and IV (1). E-114 and K-115 are solvent-exposed charged residues with no apparent contacts with other parts of the calmodulin structure (Fig. 5).

Calmodulin methylation is exquisitely sensitive to the substitutions of G113S, E114A, and L116T, which essentially abolish the methylation of lysine 115. These defects were observed regardless of calcium concentration and thus both the recognition of calcium-bound as well as apo-calmodulin was affected. Based on the structural features of these residues discussed
above, some potential roles for these residues in methyltransferase recognition can be suggested. The substitution of glutamate-114 with an alanine removes a surface negative charge adjacent to the site of methylation and, as discussed further below, could provide an electrostatic contact for the enzyme. The substitution of a serine for the highly conserved glycine-113 likely alters the conformational flexibility of the loop-turn structure, and might prohibit the residues within the loop from adopting an orientation suitable for methyltransferase binding and catalysis. The substitution of a L116T, which is one of 14 residues comprising the hydrophobic core of the carboxyl terminal lobe, could alter the packing of the hydrophobic side chains and the interaction of the methylation loop with the hydrophobic core. Interestingly, none of these mutations significantly affects activation of two calmodulin dependent enzymes, suggesting that their structural effects are subtle, selectively affecting methyltransferase recognition but not other calmodulin functions.

The conserved residues of the methylation loop are not in themselves adequate to confer methylation. For example, previous work (15) showed that the introduction of the methylation loop at a symmetrical position within the amino terminal lobe did not result in lysine methylation. Further, the replacement of either EF hand III or IV with the homologous EF hand I or II also results in the loss of lysine methylation (15). In the present study, we find that the critical regions are the α-helices adjacent to the methylation site, helix 6 of EF hand III and helix 7 of EF hand IV.

The substitution of helix 2 (LGTVMRS) for helix 6 (LRHVMTN) resulted in a substantial reduction in the rate of lysine methylation in both the presence and absence of calcium. A comparison of the structure of these related regions show that they have remarkably similar backbone structures and form nearly identical packing interactions in their respective helical bundles within the amino or carboxyl termini (1, 23, 25). Thus, the loss of methylation was
thought to be the result of alterations of surface residues that presumably interact with the methyltransferase. However, individual charge to neutral substitutions at these positions showed essentially normal methylation. Thus, the conservation of these surface residues is not apparently required for methyltransferase activity, however the packing interactions of helix 6 with others in the carboxyl terminal lobe may be important for stabilizing the conformation of the residues that are recognized and bound by the methyltransferase. The substitution of helix 2 apparently perturbs these interactions, a result that was not anticipated based on the similarity of the two structures. The reason for this defect in CaM$^{H6}$ is not yet clear.

In contrast, helix 7 shows a much different influence on the methylation of lysine 115. This helix introduces a high density of electrostatic charge on the carboxyl terminal lobe (21), adjacent to the site of methylation (Fig. 5). Mutagenesis of these various charged groups show that the removal of charges at position 118 and 120, and to a lesser degree from positions 122, 127 and 128, results in a reduction in the rate of methylation. These findings, along with the E114A results discussed above, suggest that electrostatic interactions may play a role in the binding of the methyltransferase.

Interestingly, many of the defects associated with the substitutions of within helix 7 apparently are more severe in apo-CaM compared to Ca$^{2+}$-CaM. Additionally, other mutations, such as the L112T substitution within the methylation loop, are only defective in apo-CaM. This difference in the recognition of apo-CaM and Ca$^{2+}$-CaM by the calmodulin methyltransferase is supported by several previous findings. For example, the methylation of apo-CaM shows different kinetics and considerably greater sensitivity to conditions of increasing ionic strength than Ca$^{2+}$-CaM (7). Conversely, the methylation of Ca$^{2+}$-CaM, but not apo-CaM, is sensitive to peptides and ligands that bind to the hydrophobic cleft (7-9). The inability of the cam2 mutant of Paramecium
to be methylated normally *in vivo* (26) was found to be due to an inability to selectively recognize the apo-form of calmodulin (14). Thus, while both Ca$^{2+}$- and apo-CaM are trimethylated by the calmodulin methyltransferase, they interact with the enzyme in a distinct fashion.

Based on these previous studies and the present mutagenesis work, we propose a model for the interaction of the methyltransferase with the two forms of calmodulin (Fig. 5). Apo-CaM exists predominantly in a closed conformation consisting of the four $\alpha$-helices of the EF hand pair packed in an antiparallel (128°-137°) orientation relative to one another (23, 25). This results in fewer exposed hydrophobic residues and a high density of surface charge residues (Fig. 5). Based on the substitutions, electrostatic interactions between the methyltransferase and the charged residues of helix 7 and the methylation loop of apo-CaM may help contribute to binding/orientation of the calmodulin substrate. This supports previous findings that the interaction of apo-CaM with the calmodulin methyltransferase (7) is sensitive to ion concentrations. Interestingly, the interaction of apo-CaM with target proteins shows a similar sensitivity (27-30).

The binding of calcium induces a conformational change in the lobe, resulting in a shift of the EF hand interhelical angles to an almost perpendicular state (86°-101°). While, the relative conformation of helix 6-methylation loop-helix 7 (residues 106-126) undergoes a small change between apo-CaM and Ca$^{2+}$-CaM (rms deviation is 2.6 Å), the major change between the two structures is the surface exposure of a pronounced hydrophobic pocket adjacent to the site of methylation (Fig. 5). This surface might provide additional interactions with the methyltransferase. This could explain why reagents, such as drugs and peptides, which interact selectively with the hydrophobic cleft, block the binding of the methyltransferase to Ca$^{2+}$-CaM but not apo-CaM (7). Further, this could also help explain why several of the charge to alanine mutations have less severe effects on calmodulin methylation in the presence of calcium.
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Footnotes

1 AdoMet, S-adenosyl methionine; CaM, calmodulin; 3H-AdoMet, [3H-methyl]-S-adenosyl methionine; IPTG, isopropyl β-D-thiogalactopyranoside; NADK, pea NAD kinase; PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase; VU-1 calmodulin, calmodulin derived from a cloned synthetic gene (17).
FIGURE LEGENDS

FIGURE 1. Subdomain and site-specific calmodulin mutants. A. The sequence of EF hands III and IV of VU-1 CaM (17) are aligned with symmetry-related sequences of EF hands I and II. The boxed areas represent sequence in the carboxyl-terminal lobe that were replaced with the homologous sequence from the amino-terminal lobe for the generation of subdomain mutants: CaM^{CL3}, CaM^{H6}, CaM^{H7}, CaM^{CL4}, and CaM^{H8}. B. Site-directed substitutions within the methylation loop and adjacent α-helices 6 and 7. (#) indicates residues on the hydrophobic faces of the helices 6 and 7. The arrows below the sequence alignment indicate residues that have been mutated and the star indicates the site of methylation, K115.

FIGURE 2 Methylation kinetics of VU-1 and methylation loop mutants. Pseudo-first-order methyltransferase kinetics comparing VU-1 (●), L112T (○), and G113S (▲) at a fixed saturating concentration of S-adenosylmethionine (12 µM). Calmodulin is the varied substrate, and the assay was performed under standard conditions in A. 1 mM CaCl₂ or B. 1 mM EDTA. Means ± S.E. are shown.

FIGURE 3 Methylation of subdomain mutant calmodulins CaM^{H6} and CaM^{H7} in the presence and absence of calcium. Pseudo-first-order methyltransferase kinetics comparing VU-1 calmodulin (●), CaM^{H7} (○), and CaM^{H6} (▲) at a fixed saturating concentration of S-adenosylmethionine (12 µM). Calmodulin is the varied substrate, and the assay was performed under standard conditions in A. 1 mM CaCl₂ or B. 1 mM EDTA. Means ± S.E. are shown.

FIGURE 4. Comparison of the methylation of calmodulins with single substitutions in helix 6 and 7. The rate of methylation with 3 µM of the indicated calmodulin mutants was measured
under standard conditions in the presence of 1 mM calcium (filled bars) or 1 mM EDTA (clear bars). Error bars show the S.E.

FIGURE 5. Comparison of the carboxyl-terminal lobe of apo-CaM and Ca\textsuperscript{2+}-CaM. The carboxyl-terminal (residues 77-148) lobe from A. the NMR structure of apo-CaM (25) and B. the X-ray crystal structure of Ca\textsuperscript{2+}-CaM (1) are shown. Hydrophobic residues are indicated in yellow. The methylation site (K115) and charged residues in the methylation loop and helix 7 are highlighted in red (negatively charged) or blue (positively charged). Hydrophobic residues in the methylation loop (L116 and L112) are indicated in green. Glycine 113, which facilitates a 90° turn in the loop, is shown in pink. The images were generated using a Silicon Graphics Indigo system and Insight II software.
Table I. Kinetic parameters\textsuperscript{a} of the methylation of loop mutant calmodulins in the presence and absence of calcium.

| CaM    | relative rate\textsuperscript{b} | $K_m$ (nM) | $k_{cat}$ (s\textsuperscript{-1}) | $k_{cat}/K_m$ (s\textsuperscript{-1} M\textsuperscript{-1}) |
|--------|-------------------------------|------------|--------------------------------|-------------------------------------------------|
|        | (pmole/min/ml)                 |            |                                 |                                                 |
| absence of calcium                           |            |                                 |                                                 |
| VU-1   | 260                           | 231 (±74.7) | .0189 (.002)                    | 8.18 $\times 10^4$                              |
| L112T  | 269                           | 523 (±81.4) | .0091 (.001)                    | 1.74 $\times 10^4$                              |
| G113S  | 0                             | -----      | -----                          | -----                                           |
| E114A  | 0                             | -----      | -----                          | -----                                           |
| L116T  | 0                             | -----      | -----                          | -----                                           |
| T117A  | 260                           | 314 (±59.1) | .0190 (.0013)                  | 6.12 $\times 10^4$                              |
| presence of calcium                           |            |                                 |                                                 |
| VU-1   | 260                           | 144 (±28.3) | .0205 (.0011)                  | 14.2 $\times 10^4$                              |
| L112T  | 269                           | 232 (±29.8) | .0217 (.0010)                  | 9.35 $\times 10^4$                              |
| G113S  | 12.3                          | -----      | -----                          | -----                                           |
| E114A  | 5.5                           | -----      | -----                          | -----                                           |
| L116T  | 17.1                          | -----      | -----                          | -----                                           |
| T117A  | 260                           | 148 (±35.2) | .0208 (.0014)                  | 14.1 $\times 10^4$                              |

\textsuperscript{a} Assays were performed under standard conditions in the presence of 1 mM calcium or 1 mM EDTA.

\textsuperscript{b} For the sake of comparison to mutants with extremely low activity, the rate at 1 µM CaM is shown.

\textsuperscript{c} Apparent $K_m$ and $k_{cat}$ in the presence of a constant concentration (12 µM) of AdoMet were determined from plots similar to those shown in Fig. 2 as described in the Materials and Methods.
Table II. Activation parameters of calmodulin dependent enzymes by subdomain mutant calmodulins

| CaM   | PDE % maximal rate<sup>a</sup> | K<sub>0.5</sub><sup>(CaM)</sup> | NADK % maximal rate | K<sub>0.5</sub><sup>(CaM)</sup> |
|-------|-------------------------------|-------------------------------|----------------------|-------------------------------|
| VU-1  | 100                           | 58 (± 1.2)                    | 100                  | 2.6 (± .15)                  |
| CaM<sup>CL3</sup> | 106                           | 70 (± 2.8)                    | 103                  | 1.9 (± .31)                  |
| CaM<sup>H6</sup> | 97.3                          | 50 (± 2.2)                    | 66.3                 | 1.2 (± .40)                  |
| CaM<sup>H7</sup> | 89.6                          | 68 (± 1.4)                    | 95.0                 | 1.5 (± .67)                  |
| CaM<sup>CL4</sup> | 81.3                          | 59 (± 1.5)                    | 97.2                 | 2.3 (± .34)                  |
| CaM<sup>H8</sup> | 99.0                          | 59 (± 1.6)                    | 94.5                 | 2.8 (± .23)                  |

<sup>a</sup> % maximal rate: maximal activation of enzymes (PDE or NAD kinase) by calmodulin standardized to VU-1 calmodulin (100%).

<sup>b</sup> K<sub>0.5</sub><sup>(CaM)</sup> Concentration of calmodulin (nM) giving half maximal activation. The S.E. value is shown in parentheses.
Table III. Kinetic parameters\(^a\) of the methylation of subdomain mutant calmodulins in the presence and absence of calcium.

| CaM      | \(K_m\) (nM) | \(V_{max}\) (nmol/min/mg) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) (s\(^{-1}\) M\(^{-1}\)) |
|----------|---------------|-----------------------------|---------------------------|----------------------------------------|
| **absence of calcium** |               |                             |                           |                                        |
| VU-1     | 272 (±13.4)   | 27.3 (±1.4)                 | .0172                     | 6.32 X 10\(^4\)                       |
| CaM\(^{CL3}\) | 652 (±43.1)  | 33.3 (±2.2)                 | .0209                     | 3.25 X 10\(^4\)                       |
| CaM\(^{H6}\) | 505 (±9.34)  | 2.01 (±0.39)                | .0012                     | 0.238 X 10\(^4\)                      |
| CaM\(^{H7}\) | -------      | 0                           | 0                         | 0                                      |
| CaM\(^{CL4}\) | 117 (±4.13)  | 17.0 (±3.1)                 | .0107                     | 9.14 X 10\(^4\)                       |
| CaM\(^{H8}\) | 1350 (±14.1) | 19.7 (±1.3)                 | .0124                     | 9.18 X 10\(^4\)                       |
| **presence of calcium** |               |                             |                           |                                        |
| VU-1     | 151 (±15.1)   | 28.9 (±0.89)                | .0182                     | 12.1 X 10\(^4\)                       |
| CaM\(^{CL3}\) | 154 (±20.4)  | 29.2 (±1.1)                 | .0184                     | 11.9 X 10\(^4\)                       |
| CaM\(^{H6}\) | 475 (±9.73)  | 7.25 (±1.0)                 | .0046                     | .970 X 10\(^4\)                       |
| CaM\(^{H7}\) | 262 (±70.4)  | 31.7 (±2.7)                 | .0200                     | 7.63 X 10\(^4\)                       |
| CaM\(^{CL4}\) | 153 (±13.4)  | 27.7 (±0.77)                | .0175                     | 11.4 X 10\(^4\)                       |
| CaM\(^{H8}\) | 200 (±20.9)  | 28.7 (±1.7)                 | .0181                     | 9.05 X 10\(^4\)                       |

\(^a\) Assays conditions were as described in Table I. Kinetic parameters were determined as described in the Materials and Methods. For subdomain designations, see Fig. 1.
A

\[ \text{H-methyl incorporation (pmole/min/ml)} \]

B

\[ \text{H-methyl incorporation (pmole/min/ml)} \]
A

methyl incorporation (nmol/min/mg)

1 mM CaCl$_2$

1 mM EDTA

VU-1  R106A  H107A  RH106-7AA  T110R

B

methyl incorporation (nmol/min/mg)

VU-1  DEE18-20KKK  D118A  E119A  E119D  E120A  D122A  E123A  R126A  E127A
