Methionine to Glutamine Substitutions in the C-terminal Domain of Calmodulin Impair the Activation of Three Protein Kinases*

(Received for publication, May 17, 1996, and in revised form, August 1, 1996)

David Chin and Anthony R. Means‡

From the Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

The 9 methionine residues of vertebrate calmodulin (CaM) were individually changed to glutamine residues in order to investigate their roles in enzyme binding and activation. The mutant proteins showed three classes of effect on the activation of smooth muscle myosin light chain kinase, CaM-dependent protein kinase IIα, and CaM-dependent protein kinase IV. First, some mutations had no appreciable effect on the ability of CaM to activate the three protein kinases. Included in this category were glutamine substitutions at residues 36 and 51 in the N-terminal domain, at residue 76 in the domain linker sequence, and at residues 144 and 145 in the C-terminal domain. Second, glutamine substitutions in the N-terminal domain of CaM, particularly those at positions 71 and 72, lowered the maximal activity of smooth muscle myosin light chain kinase while having no effect on the other two enzymes. Finally the affinity of CaM for all three enzymes was lowered by glutamine mutations at the neighboring methionines 109 and 124, located on a solvent-accessible surface of the C-terminal domain of Ca2+/CaM. This last result provides the first demonstration of the involvement of the same hydrophobic groups in the high affinity binding of CaM to three different enzymes.

Vertebrate calmodulin (CaM) is a protein consisting of 148 amino acids, which belongs to the E-F hand class of Ca2+-binding proteins. The crystal structure of Ca2+-bound CaM shows a primarily α-helical protein resembling a dumbbell, with two globular domains separated by an extended helix (1). Two Ca2+-binding sites are located in each domain, with the C-terminal pair having a higher affinity for Ca2+ than the N-terminal pair. The recently solved NMR structures of Ca2+-free CaM demonstrate how the binding of Ca2+ induces the exposure of hydrophobic surfaces in the two separate domains (2). This conformational change enables Ca2+/CaM to bind in a 1:1 complex with target proteins. Thus CaM is capable of activating a diversity of functions such as protein phosphorylation/dephosphorylation via kinases and phosphatases, regulation of cAMP levels via adenylyl cyclases and phosphodiesterases, maintenance of calcium homeostasis by modulating membrane Ca2+-ATPase pumps, and cellular integrity by interactions with cytoskeletal components. Since these target proteins have different CaM-binding sequences, the molecular mechanisms of regulation by CaM have been of intense interest.

Many studies have investigated the involvement of hydrophobic groups of CaM in its function. Affinity labeling of CaM with hydrophobic phenothiazine inhibitors targeted to either one or both domains selectively converted CaM to a partial or complete antagonist depending on the enzyme tested (3). The x-ray and NMR structures of CaM in complex with peptides, which correspond to the CaM binding domains of target enzymes, have provided detailed insights into the hydrophobic binding surfaces of both sets of proteins (4–6). These structures clearly reveal direct van der Waals contacts between the peptides and many hydrophobic residues of CaM, including most of its 9 methionines. Furthermore, chemical studies have demonstrated that the oxidation of an unidentified number of Met residues of CaM to methionine sulfoxides decreased the ability of CaM to activate target enzymes (7, 8). Yet attempts at defining the specific function of individual Mets of CaM by site-directed mutagenesis have provided few insights, since single substitutions of Met to Leu in mammalian CaM had little effect on the function of the protein (9). The apparent discrepancy between the results from structural analysis of CaM-peptide complexes and those of mutagenesis experiments might, in part, be explained by the conservative strategy of replacing one nonpolar side chain of CaM with another nonpolar residue. Since the substitution of Leu affected the size rather than the nonpolar nature of Met, then the functional significance of these hydrophobic residues of CaM remains an open question.

In order to address the importance of Met in CaM function, we decided to systematically survey the effects of mutating individual Mets to less conserved, more polar residues. Each of the 9 Met residues of CaM was changed to a polar Gln by site-directed mutagenesis to introduce an oxygen atom at the same position in the side chain as the harmful sulfoxide. We have tested the mutant proteins with three different CaM-dependent protein kinases. An important advantage of this approach is the fact that the CaM binding properties of two of these enzymes, smooth muscle myosin light chain kinase (smMLCK) and Ca2+/CaM-dependent protein kinase IIα (CaMKIIα), have been extensively characterized. This has allowed greater insight at the molecular level concerning the roles of individual Mets of CaM in the separate functions of enzyme recognition and activation.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes were obtained from Boehringer Mannheim and New England Biolabs. PCR reagents including Taq polymerase and buffers were supplied as a kit from Boehringer Mannheim. Double-
Methionine to Glutamine Mutants of Calmodulin

stranded DNA sequencing was performed with reagents from the Sequenase kit purchased from Amersham. Primers were from a FMC Bioproducts, and acrylamide was purchased from Serva. Bacterial reagents were from Difco and media for maintaining Sf9 cells were from Life Technologies, Inc. Peptide substrates for kinase assays were provided courtesy of Dr. Bruce E. Kemp from the St. Vincents Institute, Holt Laboratory, Melbourne, Australia. Radiochemical reagents were purchased from Amersham. Buffers for enzyme assays and column chromatography were made with Milli-Q purified water. All other reagents and chemicals were of the highest available quality.

Methods

Construction of Calmodulin Methionine to Glutamine Mutants—Mutations were introduced into plasmid pCaMpl (10) by the method of polymerase chain reaction (PCR) mutagenesis (11). Pairs of complementary oligonucleotides (20–mers), both sense (Ms) and antisense (Ma), where the appropriate ATG of Met was changed to CAG of Gin in conjunction with another pair of outer oligonucleotide sense and antisense primers (Os and Oa), were used in two rounds of PCR with pCaMpl as a template to generate DNA containing the mutation of interest. In the first round of PCR, extra 0.5 pmol pCaMpl were combined with primers Ms and Oa (30 μg each), 0.2 mM dNTPs, 1 × reaction buffer, 0.5 units of Taq polymerase in a total volume of 50 μl under the following conditions: 92°C for 1 min, 55°C for 2 min, 72°C for 2 min, repeated 20 times. In a separate tube the same reaction was carried out under parallel conditions except using Ma and Os as PCR primers. The resulting products were separated from the parent plasmid by agarose gel (1.5%) electrophoresis, purified by centrifugation through a Millipore MC microconcentrator tube (0.45 μm), combined, and concentrated by isopropanol precipitation in preparation for an intervening 3′-extension step. The PCR products were subjected to 3′-extension in the presence of 0.5 mM dNTP, 1 unit of Taq polymerase, 2 × reaction buffer in a total volume of 30 μl under the following conditions: 90°C for 1 min, 37°C for 1 min, 72°C for 2 min, repeated 10 times. The resulting mixture was diluted 100-fold and 15 μl used as DNA template in the final round of PCR containing 30 μg of each of outer primers Os and Oa, 0.2 mM dNTP, 1 × reaction buffer, 0.5 units of Taq polymerase in a total volume of 50 μl under the same conditions as the first PCR reaction. An aliquot of the resulting product was analyzed by agarose gel electrophoresis, and the remainder concentrated by alcohol precipitation prior to restriction digestion.

The PCR products were treated with a pair of the appropriate restriction enzymes (Ncol/Acc1 or Acc/IvBaI) to yield DNA cassettes, which were subsequently ligated into the parent pCaMpl vector via the same two unique restriction sites, thus replacing the wild type DNA sequence with the mutagenized fragments. The resulting recombinant plasmids were transfected into Sf9 cells by the DEAE-dextran method and used to transfect competent bacteria (strain MM 294 Cl1). The mutation was verified by double-stranded DNA sequencing of plasmids isolated from the resulting bacterial colonies. In each case sequencing confirmed the presence of only one amino acid substitution from Met (ATG) to Gin (CAG).

Expression and Purification of Calmodulin—CaM mutants were expressed in bacteria by heat induction and purified to homogeneity by conventional column chromatography. Individual pCaMpl plasmids (0.1 μg) were used to transform a strain of Erbcherichia coli (N5151) suitable for expressing the mutant proteins. Several colonies were added to 1 liter of Luria-Bertoni (LB) medium liquid and grown at 30 °C with moderate shaking overnight to an A600 nm of ~1.0. The liquid culture was induced by adding 0.1 mM IPTG, and the cells were grown to an OD600 of ~1.9 (12). The mutation was confirmed by double-stranded sequencing to resolve any possible changes in the PCR product. The transformed cells were then harvested by centrifugation at 10,000 × g for 30 min at 4 °C. The cells were then lysed by sonication and the cell extract subjected to precipitation by addition of ammonium sulfate to 40% saturation. The supernatant was concentrated by the addition of ammonium sulfate to 60% saturation, and the resulting precipitate was dissolved and passed over a Superdex G-200 gel filtration column. The peak activity of smMLCK was then pooled and subjected to affinity chromatography on a CaM-Sepharose column with the final EDTA eluate stored in 40% glycerol at −70 °C. The purity of the S9 expressed protein was confirmed by SDS-PAGE analysis. The recombinant smMLCK demonstrated similar kinetic properties to those of the chicken gizzard enzyme.

Expression and Purification of Calmodulin-dependent Protein Kinase IIa—Rat Ca2+ /CaM-dependent protein kinase IIa (CaMKIIa) was obtained by purifying from transfected Chinese hamster ovary cells using a baculovirus expression vector BlueBac II using methods based on a previously established procedure (15). Briefly, 3 days after viral infection (multiplicity of infection > 5:1) of the S9 cells and incubation with stirring at 27 °C, the cells were harvested by centrifugation. The cells were lysed by sonication, the supernatant collected and the 60% saturated ammonium sulfate precipitate fraction subjected to gel filtration on Sephadex G-200 prior to CaM-Sepharose affinity chromatography.

Expression and Purification of Calmodulin-dependent Protein Kinase IV—The rat calmodulin-dependent protein kinase IV (CaMKIV) gene under the control of a baculovirus expression vector pVL1393 was expressed in S9 cells as described previously (15). The protein kinase was purified from cell extract by a two-step procedure, first by ion exchange chromatography on DEAE-cellulose and then by CaM-Sepharose chromatography. This yielded a highly pure fraction of CaMKIV as judged by SDS-PAGE (1%). In purifications of the CaM-dependent kinases, protein concentrations were determined by the method of Bradford (13) using commercially supplied IgG as protein standard.

Assay of Calmodulin-dependent Activation of Myosin Light Chain Kinase (MLCK) was assayed for light chain phosphorylation in the presence of increasing amounts of CaM as was described previously (16). Assays were initiated by the addition of enzyme (2 nm final concentration) in a total volume of 50 μl and performed for 10 min at 30 °C in the following solution: 50 mM Hepes (pH 7.5), 5 mM MgCl2, 1 mM CaCl2, 1 mM DTT, 0.1% Tween 80, 0.5 mg/ml bovine serum albumin, 0.1 mM ATP (5 mM γ-MP), and 50 mM 3-[U-14C] myosin light chains, which were prepared from a bacterial expression vector as described elsewhere (17). Aliquots of 40 μl were loaded on to Whatman No. 3MM filter and washed with four to five changes in a solution of 10% trichloroacetic acid and 2% sodium pyrophosphate. Assays were also conducted under similar conditions except using 200 μM myosin light chain peptide MLC11–23 as substrate. In this case aliquots were loaded on to Whatman P-81 filters.
and washed in 75 mM phosphoric acid. Filters were counted on a Beckman LS 6000 scintillation counter.

Assay of Calmodulin-dependent Activation of Calmodulin Kinase IIa—Ca2+/CaM-dependent autophosphorylation of CaM
IIa was measured at 1 mM CaCl2 by a modified version of a previously described assay (16) with the exception that reactions were spotted on Whatman No. 3MM paper and washed four or five times in solutions of 25% trichloroacetic acid, 2% sodium pyrophosphate before scintillation counting as described previously. This technique gave identical results for CaM activation constants when compared to those derived from the more conventional analysis of CaMII autophosphorylation by SDS-PAGE.

Assay of Calmodulin-dependent Activation of Calmodulin Kinase IV—The conditions for determining the activity of CaMIV for phosphorylation of the peptide GS-10, in the presence of CaM at 1 mM CaCl2, were based on an earlier established procedure (15).

Analysis of Kinetic Constants—Kinetic constants for CaM and the mutant activator proteins were derived from the equation describing activation of these enzymes: 

\[ v = V_m \frac{[\text{Act}]}{K_{act} + [\text{Act}]} \]

where \( v \) is the rate of the enzyme, \([\text{Act}]\) is the concentration of CaM, \( K_{act} \) is the concentration of CaM at half-maximal velocity and \( V_m \) denotes the maximal enzyme velocity under conditions of saturating concentrations for both substrates and activator. All kinase assays included controls of wild type CaM at a saturating concentration of 1 mM, which was defined as 100% maximal activity.

RESULTS

PCR site-directed cassette mutagenesis was used to generate individual point mutants of vertebrate CaM in which each of the 9 Met residues at positions 36, 51, 71, 72, 76, 109, 124, 144, and 145 was changed to a Gln. The single amino acid substitutions were expected to primarily affect local interactions since the MetS are not positioned in any of the four Ca2+-binding loops of CaM, Gln does not unduly affect the stability of the \( \alpha \)-helices into which it is substituted (18, 19), and Gln does not change the backbone structure of the protein but rather the distal portion of the amino acid side chain. Both CaM and mutant proteins were produced to similar levels in a heat-inducible bacterial expression system and were purified to homogeneity by the identical methods of column chromatography. Furthermore, gross structural analysis of the CaM point mutants by gel filtration chromatography, SDS-PAGE (with or without Ca2+), and scanning UV-spectroscopy did not reveal any significant differences, indicating no detectable changes in the mutant proteins when compared to authentic CaM.

In contrast to the similarities in their structures, some CaM mutants were impaired in their ability to activate three different CaM-dependent kinases when compared to wild type CaM. Tests for the ability of the CaM point mutants to activate autophosphorylation of the CaMKIIa revealed differing effects (Fig. 1). With the exception of M124Q in the C-terminal domain of CaM, all mutants were capable of maximally activating the kinase. The four mutants in the N-terminal domain, M36Q, M51Q, M71Q, and M72Q (Fig. 1A), were quite similar to the wild type protein in their respective activation profiles although M36Q and M72Q exhibited approximately 4-fold increases in their activation constants (\( K_{CaM} \)). Similarly, M76Q in the domain linker behaved almost identically to the wild type CaM in activating CaMKIIa (Fig. 1A). The effects due to mutations in the C-terminal domain (Fig. 1B) can be divided into two classes with the first class represented by the adjacent mutants M144Q and M145Q, which have activation constants approximately 4-fold greater than wild type and closely resemble the activation profiles of M36Q and M72Q in the N-terminal domain. The second class of effects is exhibited by mutants M109Q and M124Q, which show the largest increases in activation constants of all the Gln point mutants tested, at 50- and 25-fold, respectively, over the wild type.

In comparison to CaMIIa, the effects of the Gln point mutants on the activation of smMLCK showed a slightly different pattern (Fig. 2). Similar to CaMIIa, the mutants in the N-terminal domain of CaM produced small increases in the activation constants for smMLCK with M36Q exhibiting the largest, approximately 3-fold, increase (Fig. 2A). However unlike CaMKIIa the adjacent mutants M71Q and M72Q produced 40% and 65% losses in maximal activity respectively. Mutation of the nearby residue 76, located in the domain linker (Fig. 2A), behaved the same as the wild type in activating smMLCK. The effects of the mutants in the C-terminal domain of CaM (Fig. 2B) were reminiscent of CaMKIIa since M144Q and M145Q behaved very similar to wild type, while the largest increases in \( K_{CaM} \) were detected for M109Q and M124Q, which exhibited greater than 7- and 35-fold increases, respectively. Similar to CaMIIa, M124Q produced an accompanying 30% decrease in maximal activity, whereas M109Q produced a smaller 15% loss in activity.

The effects of individual Gln mutants on the activation of CaMIV were not as pronounced as in the two previous cases. An examination of the activation profiles (Fig. 3) revealed that all activation constants were within an order of magnitude of the wild type. Mutants in the N-terminal domain (Fig. 3A) all achieved maximal activity and had at the most a 2.5-fold increase in \( K_{CaM} \) relative to wild type. Again M76Q behaved very
similar to the wild type protein (Fig. 3A). By contrast Gln mutants in the C-terminal domain (Fig. 3B) all showed higher activation constants for this enzyme, with M124Q achieving the greatest effect, a 7-fold increase, followed by M109Q with a 4-fold increase in $K_{\text{CaM}}$. The CaM mutants M144Q and M145Q behaved similar to those in the N-terminal domain with at most a 2.5-fold increase in $K_{\text{CaM}}$. Similar to the other two kinases, M124Q resulted in a 20% loss in maximal activity, whereas in contrast to the other two enzymes, M144Q produced a 15% decrease in the maximal activity of only CaMKIV.

The effects of the individual Met to Gln mutants of CaM are summarized in Table I, which shows the activation constants ($K_{\text{CaM}}$) and maximal activity for the three kinases tested. Some general observations can be drawn from a comparison of the effects of these mutants. Focusing on the separate domains of CaM reveals that, in general, the Gln mutants of the N-terminal domain at residues 36, 51, 71, and 72 produced maximal enzyme activity accompanied by small changes in the affinity of CaM for all three enzymes tested, as evidenced by at most a 4-fold increase in their activation constants. The two major exceptions to this rule were M71Q and M72Q, both of which were unable to activate smMLCK to maximal velocity but behaved normally relative to the other two kinases. The sole Gln mutant in the domain linker at residue 76 behaved almost identically to the wild type protein for all three kinases. Mutations in the C-terminal domain of CaM resulted in one of two effects. M144Q and M145Q produced at most a 4-fold increase in $K_{\text{CaM}}$ with all three kinases, which, with the exception of the 85% activation of CaMKIV by M144Q, were maximally activated. The other class of effect is exhibited by M109Q and M124Q, which produced large increases in $K_{\text{CaM}}$ for all three enzymes and was accompanied by a 20–35% loss in maximal activity.

Since M109Q and M124Q impaired the activation of the protein kinases in a similar manner, their calcium-bound structures were compared to that of wild type Ca$^{2+}$/CaM by CD and fluorescence spectroscopy. Whereas CD measures overall secondary structural content, absorption and emission scanning fluorescence spectroscopy provides information on the microenvironments of Tyr-99 and Tyr-138 in Ca$^{2+}$-binding
Methionine to Glutamine Mutants of Calmodulin

The mechanism of activation of these enzymes by CaMKIV. The mechanism of activation of any of the three protein kinases by CaM is different and the sequence of the CaM binding domains is distinct in each case (see Fig. 4a). For CaMKIIa and smMLCK, the structure of each CaM binding domain complexed with CaM is different and the sequence of the CaM binding domains is distinct in each case (20–22). To directly address the specific contributions of Met side chains in CaM, we individually changed each of its 9 Met residues to a Gln by site-directed mutagenesis. Gln substitutions would affect CaM function by replacing the thiocysteine group of the hydrophobic Met with a polar amide. This apparently small change in the side chain may have significant functional consequences, however, since the sulfur of Met is thought to promote sequence-independent nonpolar interactions between proteins (23).

The enzymes chosen to evaluate the function of the CaM mutants were the protein kinases smMLCK, CaMKIIa, and CaMKIV. The mechanism of activation of these enzymes by CaM is different and the sequence of the CaM binding domains is distinct in each case (see Fig. 4a). For CaMKIIa and smMLCK, the structure of each CaM binding domain complexed with CaM has been solved by x-ray crystallography (4, 6). As summarized in Table II, all 9 Met residues of CaM interact with the smMLCK peptide structure and 6 of the 9 make contact in the CaMKIIa peptide complex. Some of the methionines, such as Met-144, make three or four contacts with both peptides. Therefore, it was surprising to find that the substitutions of Met-36, -51, -76, -144, or -145 had little effect on the activation of any of the three protein kinases by CaM.

Results from an earlier systematic mutagenesis study on the Mets of CaM in general support these results since, with the exception of Met-36, Leu substitutions at the other 8 Mets of vertebrate CaM had little effect on the activation of the CaM-dependent phosphodiesterase (9). This is also compatible with results from a study in yeast in which each of the 8 Phe residues of CaM was changed individually to Ala (24).

The differences in the way CaM interacts with smMLCK and CaMKIV are accounted for by the differing capabilities of the M71Q and M72Q to activate these two enzymes. The 40–65% loss in the maximal velocity of smMLCK with no change in the activation constants due to the adjacent N-terminal domain mutants M71Q and M72Q demonstrated that these residues of CaM participated in enzyme activation without affecting affinity. In comparison, the activation of CaMKIIa with the same two mutants closely resembled the wild type protein. These results

---

**Table I**

| Calmodulin point mutant | CaMKIIa | smMLCK | CaMKIV |
|------------------------|---------|--------|--------|
| Wild type              | 40      | 1.5    | 50     |
| M36Q                   | 150     | 5.0    | 75     |
| M51Q                   | 70      | 4.0    | 120    |
| M71Q                   | 150     | 3.0 (60%) | 80 |
| M72Q                   | 150     | 2.5 (35%) | 90 |
| M76Q                   | 50      | 1.7    | 45     |
| M109Q                  | 2100    | 11.0 (85%) | 220 |
| M124Q                  | 1100 (70%) | 55.0 (65%) | 340 (80%) |
| M144Q                  | 150     | 1.5    | 130 (85%) |
| M145Q                  | 160     | 2.0    | 80     |

---

**DISCUSSION**

Previous studies have implicated hydrophobicity as a significant component in the interaction of CaM/with peptides and enzymes (20–22). To directly address the specific contributions of Met side chains in CaM, we individually changed each of its 9 Met residues to a Gln by site-directed mutagenesis. Gln substitutions would affect CaM function by replacing the thiocysteine group of the hydrophobic Met with a polar amide. This apparently small change in the side chain may have significant functional consequences, however, since the sulfur of Met is thought to promote sequence-independent nonpolar interactions between proteins (23).

The enzymes chosen to evaluate the function of the CaM mutants were the protein kinases smMLCK, CaMKIIa, and CaMKIV. The mechanism of activation of these enzymes by CaM is different and the sequence of the CaM binding domains is distinct in each case (see Fig. 4a). For CaMKIIa and smMLCK, the structure of each CaM binding domain complexed with CaM has been solved by x-ray crystallography (4, 6). As summarized in Table II, all 9 Met residues of CaM interact with the smMLCK peptide structure and 6 of the 9 make contact in the CaMKIIa peptide complex. Some of the methionines, such as Met-144, make three or four contacts with both peptides. Therefore, it was surprising to find that the substitutions of Met-36, -51, -76, -144, or -145 had little effect on the activation of any of the three protein kinases by CaM.

Results from an earlier systematic mutagenesis study on the Mets of CaM in general support these results since, with the exception of Met-36, Leu substitutions at the other 8 Mets of vertebrate CaM had little effect on the activation of the CaM-dependent phosphodiesterase (9). This is also compatible with results from a study in yeast in which each of the 8 Phe residues of CaM was changed individually to Ala (24).

The differences in the way CaM interacts with smMLCK and CaMKIV are accounted for by the differing capabilities of the M71Q and M72Q to activate these two enzymes. The 40–65% loss in the maximal velocity of smMLCK with no change in the activation constants due to the adjacent N-terminal domain mutants M71Q and M72Q demonstrated that these residues of CaM participated in enzyme activation without affecting affinity. In comparison, the activation of CaMKIIa with the same two mutants closely resembled the wild type protein. These results

2 D. Chin and A. R. Means, unpublished observations.
are in agreement with x-ray crystallographic studies, which demonstrated that Met-71 makes two contacts with the CaM-binding peptide of smMLCK, whereas Met-71 does not interact with the CaM-binding peptide of CaMKII (Table II). The crystal structures also revealed that both peptides interacted with Met-72 of CaM. However the larger number of contacts of Met-72 with the smMLCK peptide compared with the CaMKII peptide (four versus two) could account for the larger effect of the M72Q mutant on smMLCK. Other studies demonstrate the necessity of the N-terminal domain of CaM in the maximal activation of smMLCK while still maintaining high affinity binding to the enzyme. For example the introduction of multiple substitutions in the first or second E-F hands, located in the CaM N-terminal domain, either entirely abolished its activator function or produced a 48% loss in maximal activity of smMLCK while maintaining a high affinity for the enzyme (16, 17, 25). In addition affinity labeling of CaM at the N-terminal domain, either entirely abolished its activator function or produced a 48% loss in maximal activity of smMLCK while maintaining a high affinity for the enzyme (16, 17, 25). In addition affinity labeling of CaM at the N-terminal domain Lys-75 with the hydrophobic phenothiazine analog norchlorpromazine isoiothiocyanate changed the protein to a high domain Lys-75 with the hydrophobic phenothiazine analog or 17, 25). In addition affinity labeling of CaM at the N-terminal domain, either entirely abolished its activator function or produced a 48% loss in maximal activity of smMLCK while still maintaining high affinity binding to the enzyme. For example the introduction of multiple substitutions in the first or second E-F hands, located in the CaM N-terminal domain, either entirely abolished its activator function or produced a 48% loss in maximal activity of smMLCK while maintaining a high affinity for the enzyme (16, 17, 25). In addition affinity labeling of CaM at the N-terminal domain Lys-75 with the hydrophobic phenothiazine analog norchlorpromazine isoiothiocyanate changed the protein to a high affinity antagonist of smMLCK without affecting activation of CaMKII (3).

The lowered maximal velocities of the protein kinases due to Gln mutants located in the C-terminal domain is attributed to the autoinhibitory function of the CaM binding domain of these enzymes. The effect of the CaM mutant M124Q was to lower the maximal velocity of all three enzymes between 20 and 35%, whereas M109Q and M144Q had lesser effects on the maximal activity of smMLCK and CaMKIV, respectively (Table I). A review of the crystal structures of the peptides of smMLCK or CaMKII bound to CaM (Table II) reveals that Met-109 and Met-124 make multiple contacts with regions of the two protein kinases that are involved in enzyme autoinhibition (residues 796–803 in smMLCK and residues 1–77 or 78–148 in CaMKIIa). The autoinhibitory sequences of the protein kinases are responsible for repressing the activity of these enzymes by blocking access to substrates (26). The role of CaM is to displace the autoinhibitor, allowing substrate binding and catalysis. If M109Q, M124Q, and M144Q (in the case of CaMKIV) partially interfered with the normal interaction between CaM and the autoinhibitory region of these kinases, this might explain their decreased efficiency in promoting catalysis. For example, Met-124 contacts Ala-796 of smMLCK and Phe-293 of CaMKIIa, which have both been implicated in separate deletion studies to play important roles in repressing the activities of their respective enzymes (27, 28). In addition several other studies have demonstrated the deleterious effects of mutations in the C-terminal domain of CaM on the maximal activity of target enzymes (29, 30).

The large increases in the activation constants ($K_\text{CaM}^*$) of the CaM mutants M109Q and M124Q on the three protein kinases suggest that individually Met-109 and Met-124 are involved in generating a high affinity interaction between the CaM C-terminal domain and different target enzymes. Both Met-109 and Met-124 are spatially adjacent residues located on a solvent-accessible surface of the hydrophobic pocket in the C-terminal domain of Ca$^{2+}$/CaM (2). The magnitude of the changes in $K_\text{CaM}$ for M109Q and M124Q is consistent with a local effect, as opposed to extensive changes in the secondary and tertiary structures of the proteins, since the largest differences in free energy calculated between the wild type and mutant proteins ($\Delta\Delta G = 2.00–2.38$ kcal/mol) is in the range of the calculated difference in the free energy of transfer/solvation between Met to Gln ($\Delta\Delta G = 1.98–2.62$ kcal/mol) (31, 32). This agrees with the lack of effect on the remainder of the protein structure by these mutants when compared to wild type CaM by CD and fluorescence spectroscopy. Several other lines of evidence support a primary role for the C-terminal domain of CaM in the high affinity binding of target enzymes. Studies on proteolytic fragments comprising either the N- or C-terminal domains of CaM (residues 1–77 or 78–148) showed that the C-terminal domain has a higher affinity for target enzymes (33) and is the only domain capable of activating some enzymes (34, 35). Also, chimeras generated by domain swaps between CaM and other E-F hand proteins implicated the C-terminal domain of CaM in promoting a high affinity interaction with target enzymes (25, 36).

The interaction of CaM Met-124 with a conserved, large hydrophobic residue in the CaM binding domains of different enzymes suggests these two complementary hydrophobic residues help to define the specificity of CaM for CaM-binding proteins. Since changing the nonpolar nature of CaM Met-124 led to a loss in affinity for the three enzymes, then the CaM binding domains of these enzymes might contain a complementary hydrophobic residue that interacts with Met-124. Although CaM-binding sequences are notoriously divergent, a conserved bulky hydrophobic side chain, which is absolutely required for CaM binding and activation (37, 38), is present at the same position in the N-terminal half of the CaM binding domains of five CaM-dependent kinases (Fig. 4a) and all known Ca$^{2+}$/CaM-binding proteins (39). It is noteworthy that the results from x-ray crystallography and NMR spectroscopy show that Met-124 interacts with this conserved, large hydrophobic side chain in three CaM-dependent kinases: Leu-299 in CaMKIIa, Trp-800 in smMLCK (Table II), and Trp-580 in skeletal muscle myosin light chain kinase (skMLCK) (5).

An important insight into how CaM is able to recognize this one residue has been provided by the first x-ray crystal structure of a CaM-dependent protein kinase, Ca$^{2+}$/calmodulin-dependent protein kinase I (CaMKI) (40). In this structure the conserved hydrophobic target residue of CaMKI, Trp-303, (Fig. 4a) juts away from the enzyme and into the solvent. This presents an energetically inviting target to the solvent-exposed hydrophobic surface of the CaM C-terminal domain, including Met-124, and thus might allow an initial grip on the enzyme. Since the major effect of M124Q was on the affinity of CaM for all three kinases, we propose that the interaction between this

Interactions of the methionines of calmodulin with the smMLCK and CaM KII peptides

Table II

| Calmodulin methionine | CaM KII residues | smMLCK residues |
|-----------------------|------------------|----------------|
| 36                    | Alanine 309      | Isoleucine 810, serine 814 |
| 51                    | None             | Leucine 813, serine 814 |
| 71                    | None             | Arginine 812, leucine 813 |
| 72                    | Leucine 308, leucine 304 | Histidine 805, arginine 808, alanine 809, arginine 812 |
| 76                    | None             | Arginine 812 |
| 109                   | Alanine 302      | Threonine 803 |
| 124                   | Phenylalanine 293, alanine 295, leucine 299 | Arginine 796, lysine 799, tryptophan 800 |
| 144                   | Arginine 296, leucine 299, lysine 300, isoleucine 303 | Arginine 796, tryptophan 800, glutamine 801 |
| 145                   | Lysine 300, isoleucine 303, leucine 304 | Glycine 804 |

Data are taken from Ref. 6.
conserved, hydrophobic “target” residue on the CaM-binding enzymes and the hydrophobic patch on CaM, which includes Met-124, is a significant factor in defining the specificity between CaM and CaM-binding proteins. Support for this proposal comes from the activation of a glutathione S-transferase fusion protein of CaM Ki with the M124Q mutant of CaM, which has a greater than 40-fold higher activation constant than wild type CaM.²

The x-ray crystallographic and NMR structures for CaM bound to the peptides of either smMLCK, skMLCK, or CaMKIIα also reveal that the hydrophobic target residue (Trp or Leu) of these three enzymes each interact with Leu-105, Met-124, and Met-144 of CaM, respectively. This implies that CaM has maintained a complementary hydrophobic surface in order to recognize its enzyme partners. It is interesting to observe that whereas Met-144 can be conservatively substituted by either Leu or Val in CaM from yeast to mammals, the other two residues of CaM which interact with the proposed target residue, Leu-105 and Met-124, are evolutionarily invariant. Indeed both Met-124 and Met-144 of CaM cross-link with target residues, Leu-105 and Met-124, are evolutionarily conserved, hydrophobic “target” residue on the CaM binding domain of the target enzyme (Fig. 4b). This enables the remainder of the C-terminal hydrophobic domain of CaM to bind to the N-terminal domain of the CaM binding domain of the target enzyme (Fig. 4b). This enables the remainder of the C-terminal hydrophobic domain of CaM to bind to the N-terminal domain of the CaM binding domain (Fig. 4c). In some special cases, the interaction with the C-terminal domain of CaM may be sufficient to displace the enzyme autoinhibitory regions and allow enzyme activation to proceed. However, CaM-dependent enzymes require both domains of CaM for full activity and would require an additional step involving an increase in free Ca²⁺ concentration (Fig. 4d). The excess Ca²⁺ would occupy both the C- and N-terminal Ca²⁺-binding sites of CaM. Finally with the aid of the flexible domain linker of CaM (47, 48), the N-terminal domain of CaM participates in additional interactions with the enzyme, thus completely exposing the active site, which leads to full enzyme activity.

Acknowledgments—We thank Charles R. Mena for help in isolating the mutant proteins, Jonathan L. Schreiber for the baculovirus construct used in expressing the smMLCK enzyme, and Bruce E. Kemp for providing the synthetic peptides used as substrates in the protein kinase assays. We also thank Christine M. Padgett and Shirish Sheno-likar for helpful suggestions in editing the manuscript.

Reference——While this paper was under review, the Met-124 to Leu mutant of CaM was shown to activate smMLCK and neuronal nitric oxide synthase to 80 and 65% of maximal activity, respectively (49).

REFERENCES

The x-ray crystallographic and NMR structures for CaM 30471

While this paper was under review, the Met-124 to Leu mutant of CaM was shown to activate smMLCK and neuronal nitric oxide synthase to 80 and 65% of maximal activity, respectively (49).