Functional Consequences of Truncating Amino Acid Side Chains Located at a Calmodulin-Peptide Interface*

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David Chin‡, David J. Sloan‡, Florante A. Quiocho§, and Anthony R. Means¶

From the ‡Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710 and the §Department of Biochemistry, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030

Calmodulin (CaM)† is an intracellular protein that binds to a wide variety of enzymes in a Ca\(^{2+}\)-dependent manner and serves as a regulatory subunit. Three examples of such enzymes are the protein kinases smooth muscle myosin light chain kinase (smMLCK), CaM-dependent protein kinase II\(a\) (CaMKII\(a\)), and skeletal muscle myosin light chain kinase (skMLCK). Since peptides derived from these enzymes can bind Ca\(^{2+}\)/CaM with high affinity \((K_d = \text{nm})\), they have been treated as representative models of their respective CaM-enzyme interactions. The three-dimensional structures of the Ca\(^{2+}\)/CaM-peptide complexes derived from these three protein kinases have been solved by x-ray crystallography or NMR spectroscopy \((1–3)\). Although the amino acid sequence of each peptide is distinct, the three structures show similar themes.

The peptides adopt a helical structure and orient antiparallel to the NH\(_2\)- and COOH-terminal domains of CaM. To accommodate the different primary structures, the linker connecting the two domains of CaM serves as a variable expansion joint thus facilitating numerous van der Waals contacts between the peptides and hydrophobic pockets located in the two domains of Ca\(^{2+}\)/CaM. On closer inspection, however, the Ca\(^{2+}\)/CaM-peptide complexes exhibit differences in the specific details of their interactions. A comparison of the CaMKII\(a\) peptide (CaMKII\(a\)p)–CaM complex with the smMLCK peptide (smMLCKp)–CaM complex reveals that the NH\(_2\)-terminal domain of CaM interacts differently with the COOH-terminal ends of either the CaMKII\(a\)p or the smMLCKp \((2)\). The specific contacts between the CaM domains and the peptides are important for the formation of these structures since the same contacts are maintained between the CaMKII\(a\)p and CaM mutants containing multiple residue deletions and insertions in the linker between the CaM domains \((4)\).

The relevance of the CaM-peptide structural models to the interaction between CaM and the intact enzymes remains largely untested. Previous mutagenesis studies on CaM and CaM-binding enzymes did not address this point since most failed to target the binding interface between the two proteins. One exception was an alanine scan of the CaM binding domain of smMLCK. These studies demonstrated that the side chains of Trp-800, Arg-812, and Leu-813, which interact with multiple side chains of CaM in the Ca\(^{2+}\)/CaM–smMLCKp structure, are critical for CaM binding and activation of the enzyme \((5)\). Collectively, the available data presented an opportunity to test whether the different CaM-peptide complexes provide information on how CaM interacts with and activates the target enzymes. To address this question two complementary approaches were taken. First, in an attempt to perturb the CaM-smMLCK interaction without interfering with the CaM-CaMKII\(a\) complex we studied the effects of truncating amino acid side chains in the NH\(_2\)-terminal domain of CaM which differentially interact with the smMLCKp and the CaMKII\(a\)p. Consequently Ala residues were sequentially substituted for CaM residues Leu-32, Met-51, Val-55, and Met-71. These side chains interact with residues in the smMLCKp, corresponding to Arg-812 and Leu-813 in the enzyme smMLCK but have no contacts with the CaMKII\(a\)p. Both enzymes were then tested for activation by the resulting double, triple, or multiple substitution mutants of CaM. Second, we tested whether simultaneous Ala substitutions of the Trp-5 and Arg-17 residues in the smMLCKp would eliminate CaM binding as was demonstrated when similar changes were made at the corresponding Trp-800 and Arg-812 in the context of the enzyme \((5)\).

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of CaM—Mutants of CaM were generated by the combination of two protocols. First the double mutant of CaM,
M51A, V55A, was introduced into the pcMaM23N plasmid containing the chicken CaM cDNA (6) by unique restriction site elimination mutagenesis (7). This procedure simultaneously eliminated the unique AccI restriction site. The protein coding region was then subcloned into the pcMaMl vector (8) via the unique 5’ NcoI and 3’ XbaI restriction sites to facilitate bacterial expression. The triple Ala mutant of CaM, L32A, M51A, V55A, was created by the method of site-directed polymerase chain reaction mutagenesis (9) using the pcMaM23N construct containing the M51A, V55A mutant as template. The resulting NcoI-XbaI fragment was then ligated into the pcMaMl expression vector. The L32A, M51A, V55A, F68L, M71A multiple mutant of CaM was also created by polymerase chain reaction mutagenesis, but instead using the plasmid from the L32A, M51A, V55A mutant as DNA template. The identities of the mutant cDNA constructs were verified by double-stranded DNA sequencing of the entire protein coding region of CaM.

Expression and Purification of CaM—CaM mutant proteins were expressed in the heat-inducible bacterial strain N5151 and purified by conventional column chromatography as described previously (10). Briefly, bacteria were grown in 1 liter of LB broth at 30°C to an A600 of 1 and induced to express protein by heat induction at 42°C for 1.5 h. The bacterial pellet was then collected after centrifugation, suspended in a solution of 2.4 M sucrose, and subjected to overnight lysis in a lysis solution containing 1 M CaCl2, 1% phenyl-Sepharose, and gel filtration chromatography. The purified proteins subsequently were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Concentrations of CaM were determined by UV absorbance (11) and by the method of Bradford (12).

CaM-binding Peptides of smMLCK—The peptides containing the sequence of the CaM binding domain of smMLCK residues 796–813 (smMLCKp) ARKKWQRTDOHAIRAGRL and the two alanine substitutions (W57A,R17A) ARRAQKTKHAIRAGAL were made by automated solid phase synthesis and purified by reverse phase high performance liquid chromatography. The compositions of the peptides and their respective concentrations were verified by amino acid analysis.

Assay of CaM-dependent Activation of MLCK Kinase and Autophosphorylation of CaMKII—smMLCK was purified from a baculovirus expression system and assayed for light chain phosphorylation in the presence of increasing amounts of CaM as described previously (10). 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 50 mM Heps (pH 7.5), 5 mM MgCl2, 1 mM CaCl2, 1 mM dithiothreitol, 0.1% Tween 80, 0.5 mg/ml bovine serum albumin, 0.1 mM ATP (0.2 µCi), and 50 µM MLC20. Aliquots of 40 µl were loaded onto a Whatman 3MM filter and washed with four or five changes in a solution of 10% trichloroacetic acid and 2% sodium pyrophosphate. Assays were also conducted under similar conditions except with 200 µM of the MLC peptide MLC20 as substrate. In this case aliquots were loaded onto Whatman P81 filters and washed in 7 M nmol imidazole. Filters were counted on a Beckman LS 6000 scintillation counter. Kinetic constants for CaM and the mutant CaM were derived from the equation

\[ v = \frac{V_{max}[\text{CaM}]] + [\text{Act}]}{K_{CaM} + [\text{Act}]} \]  

(Eq. 1)

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

The inhibition constant for the CaM mutant multiple mutant L32A,M51A,V55A,F68L,M71A with smMLCK was derived from a modified version of the assay with MLC as substrate. A competition assay was performed with either 1, 2, 4 or 8 nM wild type CaM and increasing concentrations of the CaM mutant protein. Inhibition constants were derived from double-reciprocal plots and linear regression analysis. The enzyme inhibition constants for the smMLCK peptides were obtained by adding increasing concentrations (20–1000 nM) of either peptide to the smMLCK assay containing 200 nM CaM and 100 nM enzyme. Inhibition constants were derived by fitting the results from the competition assay with the CURVE-FIT program to an equation describing an activator depletion model.

Ca2+/CaM-dependent autophosphorylation of baculovirus-expressed CaM kinase II was measured at 1 mM CaCl2 as described (10). Kinetic constants (\( K_{CaM} \) and percent maximum activity) were derived in a manner similar to that for smMLCK.

RESULTS AND DISCUSSION

The aim of this study was to investigate the functional implications of the CaM-peptide structures by correlating incremental structural changes at the protein-peptide interface with changes in CaM binding or enzyme activation. Therefore perturbations were introduced at the CaM-smMLCKp binding site by either shortening side chains of CaM which exhibited differences in contacts between the smMLCKp and the CaMKIIP or by shortening side chains on the smMLCKp which were required for binding and activation of the parent smMLCK enzyme.

In the first case mutations were introduced into the CaM NH2-terminal domain by combining the techniques of unique site elimination and polymerase chain reaction mutagenesis. The resulting CaM mutant constructs contained either two amino acid side chain changes, the double mutant M51A,V55A; three side chain substitutions, the triple mutant L32A,M51A,V55A; or five side chain substitutions, the multiple mutant L32A,M51A,V55A,F68L,M71A. The different CaMs were indubitably expressed in bacteria and purified using chromatographic procedures identical to those for the wild type CaM. This resulted in similar yields for all proteins of approximately 10–20 mg of protein/liter of liquid bacterial culture.

The mutant proteins were then compared with authentic CaM in their respective abilities to support autophosphorylation of the enzyme CaMKIIa. The resulting activation profiles at increasing concentrations of CaM are presented in Fig. 1A. Similar to authentic CaM, all mutant CaMs were capable of maximal (100%) activation of CaMKIIa autophosphorylation. In the specific case of the double mutant M51A,V55A, the activation constant (\( K_{CaM} = 50 \text{ nm} \)) was almost identical to that of CaM (45 nm). In contrast, the triple and multiple mutants of CaM exhibit larger increases in \( K_{CaM} \), which are approximately 3–8-fold greater than the normal protein, at 120 and 350 nm, respectively.

The results from testing the ability of the CaM mutants to activate smMLCK phosphorylation of MLCs are plotted in Fig. 1B. In contrast to CaMKIIa, none of the CaM mutant protein was capable of maximally activating smMLCK. The double mutant M51A,V55A achieved 60% of the maximal activity of the wild type, whereas the triple mutant L32A,M51A,V55A resulted in only 25% of maximal activity. The multiple mutant L32A,M51A,V55A,F68L,M71A showed the largest change with less than 1% of maximal activity. Interestingly, the \( K_{CaM} \) values for the double and triple CaM mutants were not significantly different from CaM (1.5 nm) as they increased less than 3-fold to 4.0 nm for the double mutant and 3.0 nm for the triple mutant. Since the multiple mutant L32A,M51A,V55A,F68L,M71A showed scant ability to activate smMLCK, an inhibition constant of 4.5 nm was determined from competition assays with authentic CaM. In addition, the use of the 13-residue MLC peptide MLC11–23 as substrate in the assays gave essentially the same results as with the full-length 20-kDa light chains.

A comparison of the parameters for CaM and mutant CaM proteins is presented in Table I, listing activation constants and maximal activity for autophosphorylation of CaMKIIa and for phosphorylation of MLC or MLC peptide 11–23 by smMLCK. Sequential mutations of the side chains Leu-32, Met-51, Val-55, and Met-71 of CaM to Ala and Phe-68 to Leu in the NH2-terminal domain of CaM had little effect on the activation of CaMKIIa since the most deleterious CaM mutant, L32A,M51A,V55A,F68L,M71A, produced less than an 8-fold increase in \( K_{CaM} \) for CaMKIIa while retaining 100% maximal activity. These results are consistent with the CaM-CaMKIIa structural model since the truncated amino acid side chains of CaM do not interact with the CaMKIIa. They also correlate
closely with results from earlier studies on the NH₂-terminal domain of CaM where chemical modification of Lys-75 with the phenothiazine affinity reagent norchlorpromazine isothiocyanate had little effect on the activation of CaMKII (13). In addition a chimeric protein containing the NH₂-terminal domain E-F hand I of cardiac troponin C joined in-frame with the remaining portion of CaM (TaM-BM1) had at most a 5-fold increase in the activation constant for CaMKII (14).

In contrast with the results from CaMKIIa, the same CaM mutant proteins produced large reductions (40–99%) in the maximal activity of smMLCK with little change in the activation or inhibitory constants (Table I). The mutated residues on CaM interact with a residue on the smMLCKp, corresponding to Leu-813 on the intact enzyme, which has been proposed to play an important role in the recognition of CaM (1). The small effect of these mutations on the affinity of smMLCK for CaM, as reflected in the similar activation constants (Table I), suggests that the van der Waals packing with smMLCK Leu-813 is important for activating the enzyme and less critical in the formation of a high affinity CaM-smMLCK complex. This result also agrees with those from the norchlorpromazine isothiocyanate derivative of the CaM NH₂-terminal domain which was unable to activate smMLCK but still bound to the enzyme with high affinity (15). Finally, the results from a complementary study on the kinase domain of smMLCK by alanine scanning mutagenesis provide support for this point since the L813A mutant lost more than 85% of maximal activity while retaining some ability to bind CaM as assessed by a gel overlay assay (5).

It is remarkable to observe the extent to which the molecular details derived from these and other recent mutagenesis studies on the Ca²⁺/CaM-smMLCK interaction closely follow an earlier two-step model proposing a mechanism of CaM function based on the gross thermodynamics of the Ca²⁺/CaM-skMLCK interaction (17). The first event in this model involves the hydrophobic interaction between undetermined groups of the enzyme and Ca²⁺/CaM which function to immobilize partially both proteins while stabilizing the formation of a high affinity complex. The site of the initial, high affinity interaction between smMLCK and Ca²⁺/CaM has been localized to the COOH-terminal hydrophobic pocket of Ca²⁺/CaM by scanning mutagenesis of the nine widespread Mets of CaM (10). These experiments demonstrated that a solvent accessible hydrophobic surface in the COOH-terminal domain of Ca²⁺/CaM, which includes Met-109 and especially Met-124, is critical for high affinity binding with smMLCK. Importantly, this region of Ca²⁺/CaM has hydrophobic interactions with nonpolar residues in skMLCKp (3) and smMLCKp (1), including the equivalent of Trp-580 in skMLCK and the homologous Trp-800 in smMLCK, which is required for binding Ca²⁺/CaM (5).

The second step in the proposed model involves the operation of short range forces including van der Waals, hydrogen bonding, and ionic interactions that lead to enzyme activation. The results presented herein and elsewhere (10) provide compelling evidence that the disruption of van der Waals interactions between nonpolar residues of smMLCK and those in the NH₂-terminal domain of CaM have dramatic effects on the maximal activity of the enzyme while scarcely affecting the affinity of the Ca²⁺/CaM-smMLCK complex. Other studies also suggest that van der Waals interactions between smMLCK and nonpolar residues in the COOH-terminal domain of CaM such as Met-124 and Leu-112 play a similarly important role in smMLCK activation as well (10, 18, 19). Evidence for the role of hydrogen bonding and ionic interactions comes primarily from CaM-troponin C chimeric proteins. These studies reveal that mutation of polar or charged residues located on an external “latch” surface of CaM, distinct from the CaM-skMLCKp interface, will impair activation of smMLCK but still maintain a high affinity complex (14, 16, 18, 19).

Mutagenesis studies on an enzymatically active fragment of smMLCK had shown that Trp-800 and Arg-812 were individually necessary for Ca²⁺/CaM-dependent binding and enzyme activation as the substitution of either residue markedly decreased both parameters (5). Since both residues interact with CaM in the Ca²⁺/CaM-smMLCKp complex, we prepared a peptide in which Ala substitutions were introduced simultaneously at positions corresponding to Trp-800 and Arg-812. The resulting double Ala peptide was compared with the original peptide for their respective abilities to bind to Ca²⁺/CaM and thus

**Table I**

| CaMKIIa | smMLCK | Enzyme [substrate] |
|---------|--------|---------------------|
|        |        | CaMKIIa | [LC] | [LC peptide] |
| Wild type | 45 | 1.5 | 1.5 |
| M51A,V55A | 50 | 4.0 (60%) | 5.0 (55%) |
| L32A,M51A,V55A | 120 | 3.0 (25%) | 3.0 (25%) |
| L32A,M51A,V55A | 350 | 4.5 (8%) | 4.5 (8%) |

| M51A | F68L,M71A |
|------|-----------|
| 350 | 4.5 (8%) | 4.5 (8%) |

*a* Values in parentheses are % maximum activity.  
*b* Inhibition constant.
inhibit the activation of smMLCK. The two amino acid changes did not abolish the ability of the smMLCKp to bind CaM, but rather resulted in only an 8-fold diminution in the enzyme inhibition constant ($K_i$) from 1.3 nM to 10.4 nM (Table II).

This result is consistent with those from other studies which have determined Ca$^{2+}$/CaM to be capable of high affinity binding to peptides with diverse amino acid sequences (20). A major feature of peptides that bind to Ca$^{2+}$/CaM is the capability to form an amphipathic helix following the binding of Ca$^{2+}$/CaM. Using this premise it was possible to design a high affinity CaM-binding peptide de novo consisting only of Leu and Lys residues (LK peptide) (21). The substitution of Trp for Leu at the position of the LK peptide corresponding to Trp-5 in the smMLCKp resulted in a 10-fold increase in affinity for Ca$^{2+}$/CaM (22). This result is comparable to the effect of the double Ala-substituted smMLCKp (Table II). Other studies on CaM-peptide binding demonstrate even greater plasticity on the part of the peptides. The smMLCKp composed solely of $\alpha$-amino acids binds just as well to Ca$^{2+}$/CaM as the peptide composed of normal L-amino acids (23). Also, a study of the skMLCKp shows that no individual Ala substitutions decreased but in all cases instead increased the affinities of the peptide for Ca$^{2+}$/CaM (24).

The small effects of the double Ala substitutions on the CaM binding properties of the smMLCKp, however, do not agree with the abolition of CaM binding due to individual substitutions at the equivalent positions of Trp-800 and Lys-813 in the smMLCK enzyme. This discrepancy is an indicator of significant differences in the binding of Ca$^{2+}$/CaM by nonnative peptide sequences compared with the same sequence in the context of an intact enzyme. Insight into the differences in the molecular recognition process between a free CaM-binding peptide and the same peptide segment in the environment of a folded enzyme is provided by the recently determined x-ray structure of Ca$^{2+}$/CaM-dependent protein kinase I (CaMKI) (25). In this structure, the CaM binding domain of CaMKI is held in place on the surface of the kinase and makes numerous contacts with neighboring groups in both major domains of the enzyme. In one exceptional case, Trp-303 (the structural homolog to Trp-800 in smMLCK) is exposed to solvent and is thus thought to play an important role in the binding of Ca$^{2+}$/CaM. In contrast to the enzyme, the free CaM-binding peptide, in the absence of constraints imposed by the remainder of the protein, would likely present a markedly different target for recognition and binding by Ca$^{2+}$/CaM. However, since the peptide can form an amphipathic helix it could still bind to Ca$^{2+}$/CaM with similar or even higher affinities than the parent enzyme.

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