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Role of Domain 3 of Calmodulin in Activation of Calmodulin-stimulated Phosphodiesterase and Smooth Muscle Myosin Light Chain Kinase*

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CaM[3 TnC] is a calmodulin-cardiac troponin C chimeric protein containing the first, second, and fourth calcium-binding domains of calmodulin (CaM) and the third calcium-binding domain of cardiac troponin C (cTnC) (George, S. E., Su, Z., Fan, D., and Means, A. R. (1993) *J. Biol. Chem.* 268, 25213–25220). CaM[3 TnC] shows altered activation of phosphodiesterase (PDE) and is a potent competitive inhibitor of smooth muscle myosin light chain kinase (smMLCK) activation by CaM. To determine why CaM[3 TnC] exhibits altered target enzyme interactions, we constructed a series of domain 3 CaM mutants. We began with subdomain substitutions, replacing most of CaM's helix 5, Ca^{2+} binding loop 3, and helix 6 with the corresponding subdomains of cTnC. Only CaM[helix 6-TnC] exhibited significant impairment of smMLCK and PDE activation. We then individually substituted the residues in the region of CaM's helix 6 with the corresponding cTnC residue. This revealed that CaM residues Thr-110, Leu-112, and Lys-115 were critical for full smMLCK activation and could not be substituted by the corresponding cTnC residue (Gln, Thr, and Thr, respectively). In contrast, only the L112T substitution significantly affected PDE activation. The CaM-smMLCK peptide structure (Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) *Science* 257, 1251–1255) suggests a relationship between the proposed helix 6 smMLCK-activating residues and those previously described in helix 2 (VanBerkum, M. F. A., and Means, A. R. (1991) *J. Biol. Chem.* 266, 21488–21495).

Calmodulin (CaM)[1] interacts with and regulates a number of intracellular target proteins (1–3). Understanding the structural basis of CaM-target protein interaction is important for understanding how CaM accomplishes its diverse regulatory functions.

In crystal stucture, CaM is a dumbbell-shaped protein with two globular domains separated by a flexible central helix (4–7). Upon complex formation with model binding peptides, the central helix of CaM bends 100° and twists 120°, enveloping the CaM-binding peptide in a hydrophobic tunnel and bringing the two globular domains into close approximation (8, 9). This striking rearrangement may produce new functional domains on the surface of CaM that interact with the target enzyme in regions remote from its calmodulin-binding domain. These putative additional interactions may be essential for enzyme activation (10, 11).

Chimeras between CaM and cardiac troponin C (cTnC) have helped to define CaM-target enzyme interactions (12, 13). The two proteins show remarkable structural similarity (14), but cTnC cannot activate most CaM target enzymes (12, 15). By exchanging functional domains between the two, it is possible to define domains of CaM that are essential for enzyme activation. We replaced each of the four helix-loop-helix Ca^{2+}-binding domains and the central helix of CaM with the corresponding domain of cTnC, naming the resulting chimeras according to the domain replaced, *e.g.* CaM[1 TnC], CaM[2 TnC], CaM[CH-TnC], CaM[3 TnC], and CaM[4 TnC]) (12, 13). We found that CaM[3 TnC] had several distinctive properties (13). First, relative to CaM it had enhanced Ca^{2+} affinity. Second, CaM[3 TnC] completely failed to activate smMLCK and in fact proved to be a potent competitive inhibitor of smMLCK activation by CaM (K_i = 42 nM). Third, CaM[3 TnC] was a full agonist with respect to calmodulin-stimulated phosphodiesterase (PDE), but, relative to CaM, an over 100-fold increase in K_{act} was observed.

In the present study, we sought to determine why the substitution of cTnC's third domain for that of CaM produced such profound detrimental effects on enzyme activation. Our approach was to exchange the subdomain components of domain 3 of CaM and cTnC (*i.e.* helix 5, Ca^{2+} binding loop 3, and helix 6) and to generate a series of CaM domain 3 point mutations in which a cTnC residue was substituted for the corresponding CaM residue. The subdomain chimeras and point mutations were then assayed for ability to activate smMLCK and PDE and to bind to a 20-residue peptide derived from the CaM-binding domain of smMLCK. Our results indicate that some residues in the helix 6 region may play a key role in enzyme activation.

EXPERIMENTAL PROCEDURES

Construction of Mutants—We used overlap extension PCR to construct the bacterial expression plasmids encoding the domain 3 CaM mutants (13, 16). The general strategy for this technique is outlined in Fig. 1. Two overlapping oligonucleotides, one antisense (*oligo 2*) and one sense (*oligo 3*), are designed to incorporate the desired mutations. Each is used, in two separate first-stage PCRs, to amplify a segment of CaM plasmid DNA. Each first-stage reaction includes a second oligonucleotide that is either sense and lies 5′ to the CaM coding region (oligo 1, ACCACACCTATGGTGTATGC, used with oligo 2) or antisense and lies 3′ to the CaM coding region (oligo 4, CCCAAGTCTTCTAGAGAAAGC, used with oligo 3). The sequences of oligos 2 and 3 vary with the particular mutant being constructed (see Fig. 1). The template DNA for all first-stage PCRs was pCaM[*Sal*I] (13), except as follows. For CaM-

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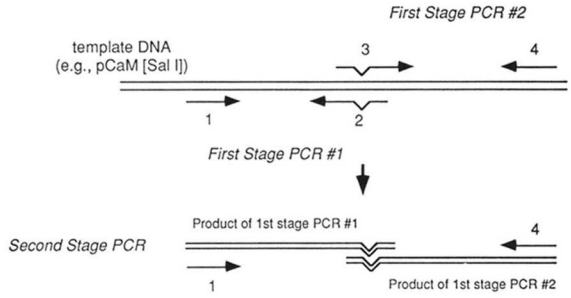
[1] The abbreviations used are: CaM, calmodulin; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; cTnC, cardiac troponin C; PDE, calmodulin-stimulated cyclic nucleotide phosphodiesterase; MLCK, myosin light chain kinase; smMLCK, smooth muscle MLCK.



Mutant	oligo 2	oligo 3
CaM [helix 5-TnC]	CATACGGAACGCTTGTGAGAGCTC	AGCGTTCCGTATGTTTGACAACG
CaM [loop 3-TnC]	GATGTAATCAGCATTCTTGTCAAACAC	GCTGATTACATCGATCTTGAAGAACTTCG
CaM [helix 6-TnC]	TGCCTGTAGCATGATACGAAGTTCTGC	ATGCTACAGGCAACTGGGGAGAAGCTA
H107I	CATCACGATACGAAGTTCTGCAGCACT	CTTCGTATCGTGATGACAAATCTTGGG
V108M	CATCATATGACGAAGTTCTGCAGC	CGTCATATGATGACAAATCTTGGG
M109L	TGTTAGCACATGACGAAGTTCTGC	CATGTGCTAACAAATCTTGGGGAG
T110Q	ATTCTGCATCACATGACGAAGTTC	GTGATGCAGAATCTTGGGGAGAAG
N111A	AAGTGCTGTCATCACATGACGAAG	ATGACAGCACTTGGGGAGAAGCTA
L112T	CCCAGTATTTGTCATCACATGACG	ACAAATACTGGGGAGAAGCTAACA
K115T	TGTTAGCGTCTCCCCAAGATTTGTCAT	GGGGAGACGCTAACAGATGAAGAAGTC
T110Q-L112T	CCCAGTATTTTGGCATCACATGACG	ATGCAAAATACTGGGGAGAAGCTA
T110Q-L112T-K115T	CCCAGTATTTTGGCATCACATGACG	ATGCAAAATACTGGGGAGACGCTA
C3T [T110L-Q112T -T115K]	CTTCTCTCCAAGTGCTGTTAGCATGATC	ACAGCACTTGGAGAGAAGATCACTGAGG
CH6T [T110L-Q112T]	AGCTGTCAACATGATACGAAGTTC	ATGTTGACAGCTCTTGGGGAGAAG

FIG. 1. **Construction of domain 3 CaM mutants using overlap extension PCR.** Oligonucleotides are indicated by *numbered arrows*. Mutated regions, in oligonucleotides 2 and 3, are indicated by a *carat*. The table at the *bottom* shows sequences of oligonucleotides used to construct domain 3 CaM mutants (see "Experimental Procedures" for discussion).

[helix 5-TnC], the template DNA was pCaM[*Sac*I, *Sal*I] (13); for C3T[Q110T/T112L/T115K], the template DNA was pCaM[3 TnC]; and for CH6T[Q110T/T112L], the template DNA was CaM[helix 6-TnC].

The products of the first-stage reactions incorporate the desired mutations and overlap in the mutated region. The first-stage products are purified by agarose gel electrophoresis and combined with oligos 1 and 4 in a second-stage PCR. The second-stage product is treated with proteinase K (17), purified by agarose gel electrophoresis, digested with *Nco*I and *Sal*I, purified again, and subcloned into a vector obtained by digesting pCaM[*Sal*I] (13) with *Nco*I and *Sal*I. DNA sequences obtained by PCR are fully sequenced to ensure that no undesired mutations are present. The proteins are expressed and purified as described by George *et al.* (12, 13).

Enzyme Activation Assays—Rat brain phosphodiesterase activity was assayed as described by Putkey *et al.* (18). Smooth muscle myosin light chain kinase assays were performed as described by VanBerkum *et al.* (23). Evaluation of chimeras as smMLCK inhibitors was performed as described by George *et al.* (12) and VanBerkum and Means (10).

Fluorescence Enhancement—A 20-residue peptide, based on the CaM-binding domain of smMLCK (19, 20), was obtained from American Peptide Co., Sunnyvale CA. The peptide sequence is Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Leu-Ser-Ser and is hereafter referred to as smMLCK peptide. Its purity was 99.7% as determined by reversed-phase high performance liquid chromatography. Fluorescence measurements were made at ambient temperature on a Shimadzu RF-5000 spectrofluorimeter, with an excitation wavelength of 295 nm and a bandwidth of 5 nm. Three microliters of a 2 mg/ml protein solution (containing CaM or the mutant CaM proteins, as indicated) were added in successive aliquots to 3 ml of a solution containing 50 mM MOPS, pH 7.0, 150 mM KCl, 0.4 mM EGTA, 1.0 mM $CaCl_2$, 1.8 μM smMLCK peptide. After each addition of protein solution, the fluorescence emission at 328 nm was recorded (emission bandwidth, 5 nm). After every five additions of protein solution, the fluorescence spectrum from 305 to 410 nm was recorded. Three successive titrations were performed for each protein, each yielding virtually identical data. The observed fluorescence enhancement and shift in maximal enhancement did not occur when Ca^{2+} was omitted from the buffer.

Inhibition of PDE Activity by smMLCK Peptide—Each of the domain 3 mutant CaM proteins can produce maximal PDE activation. Therefore, the ability of the smMLCK peptide to inhibit PDE activation by the mutant CaM proteins may be used to determine dissociation constants (K_d) (10, 24, 25). For each mutant CaM protein, we determined K_{act}

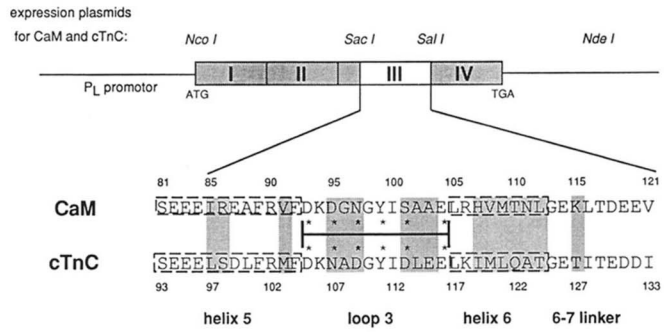


FIG. 2. **Map of domain 3 mutations.** *Top*, partial map of expression vectors for CaM and cTnC. The coding region is *boxed*. Domains *I–IV* and the restriction sites used to construct the mutants are indicated (for details, see "Experimental Procedures"). *Bottom*, amino acid sequence comparison of domain 3 from CaM and cTnC. Residue numbers are indicated *above* and *below* the sequences. Helical residues are *boxed* within *broken lines*. The Ca^{2+} binding loop is indicated by the *bracket* between the sequences; residues involved in Ca^{2+} coordination are marked with an *asterisk*. The *shaded boxes* show CaM residues that were mutated, individually and in groups, to the corresponding residue of cTnC.

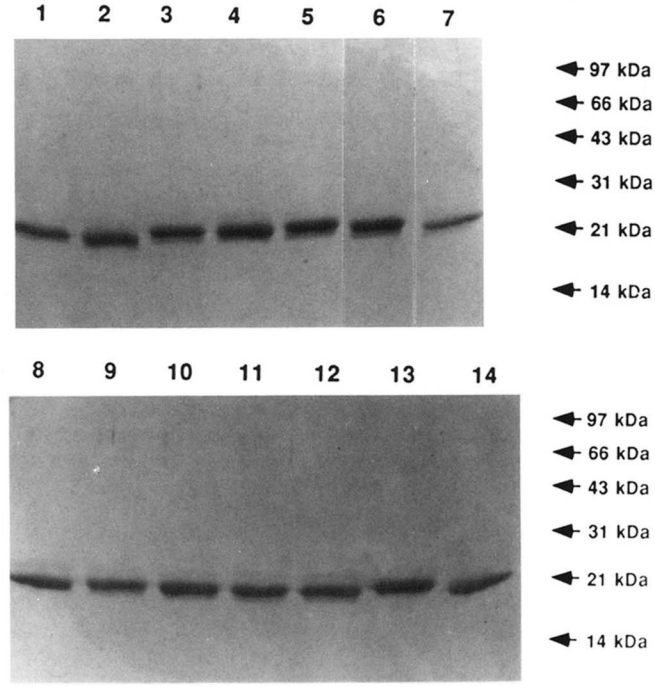


FIG. 3. **SDS-polyacrylamide gels of domain 3 CaM mutants.** Mobilities of molecular weight markers are indicated by *arrows*. *1*, CaM-[helix 5-TnC]; *2*, CaM[loop 3-TnC]; *3*, CaM[helix 6-TnC]; *4*, T110Q/L112T; *5*, T110Q/L112T/K115T; *6*, H107I; *7*, V108M; *8*, M109L; *9*, T110Q; *10*, N111A; *11*, L112T; *12*, K115T; *13*, CH6T[Q110T-T112L]; *14*, C3T[Q110T/T112L/T115K].

(concentration producing half-maximal activation) under previously described assay conditions (12, 18). We then assayed each mutant CaM protein for PDE activation at five different concentrations selected to produce a range of PDE activation from 25 to 100% of maximum. The assays were repeated in the presence of four different amounts of smMLCK peptide (for CaM, T110Q, and K115T, 5–40 nM smMLCK peptide; for L112T and T110Q/L112T, 25–200 nM smMLCK peptide; for T110Q/L112T/K115T and CaM[helix 6-TnC], 40–300 nM smMLCK peptide). K_d was determined by the method of Cox *et al.* (24). The data presented are the mean of four determinations at four different concentrations of smMLCK peptide, plus or minus the standard error.

RESULTS AND DISCUSSION

We constructed a total of 14 domain 3 CaM mutants for this study (Fig. 2). Three of these are approximate subdomain ex-

TABLE I
Activation of PDE by domain 3 CaM mutants

$S_{0.5}$ is the concentration of activation producing half-maximal activation. Maximal activation is defined as PDE activity observed in the presence of 100 nM CaM. Standard errors are less than 5%.

	$S_{0.5}$	Maximal activation
	nM	%
CaM	2.2	100
CaM[helix 5-TnC]	7.6	97
CaM[loop 3-TnC]	1.3	93
CaM[helix 6-TnC]	58	96
H107I	1.8	100
V108M	3.8	93
M109L	1.3	99
T110Q	4.4	94
N111A	2.2	100
L112T	45	99
K115T	1.2	100
T110Q/L112T/K115T	41	100

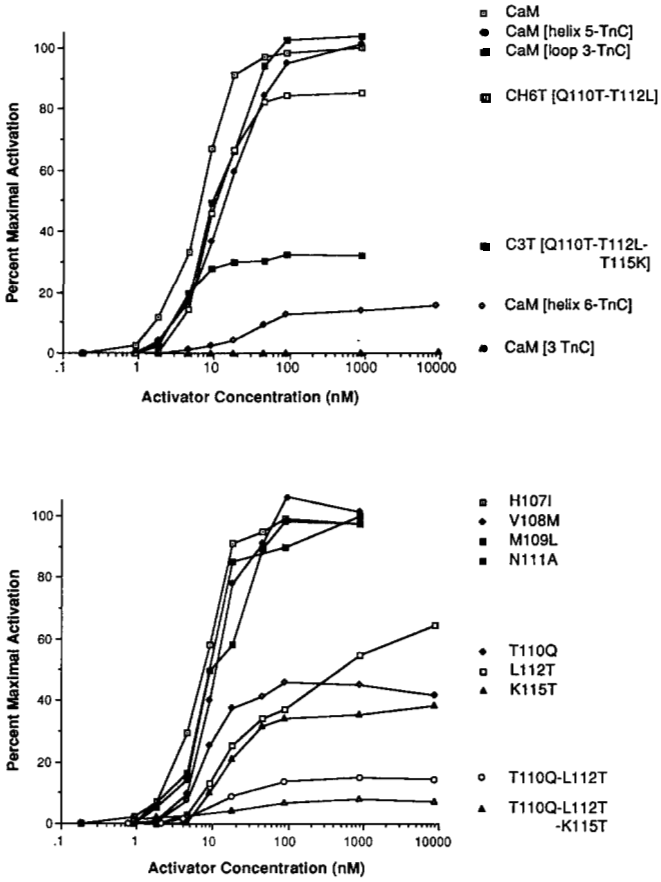


FIG. 4. **Activation of smMLCK by domain 3 CaM mutants.** Maximal activation is defined as smMLCK activity observed in the presence of 100 nM CaM. Results are from a representative experiment, done in triplicate. Standard errors were generally less than 5% and in no case greater than 10% of the absolute value of the determination.

changes between corresponding residues of the helix-loop-helix structure that constitutes domain 3 of CaM and cTnC (*i.e.* CaM[helix 5-TnC], CaM[loop 3-TnC], and CaM[helix 6-TnC]). CaM[helix 5-TnC] differs from a full helix 5 exchange in that Glu-87 and Ala-88 remain unmutated (the corresponding residues are Asp and Leu, respectively, in cTnC). CaM[helix 6-TnC] differs from a full helix 6 exchange in that Arg-106 (Lys in cTnC) remains unmutated.

Nine mutants involve amino acid substitutions in helix 6 and the helix 6-helix 7 linker region of CaM. In each, we replaced

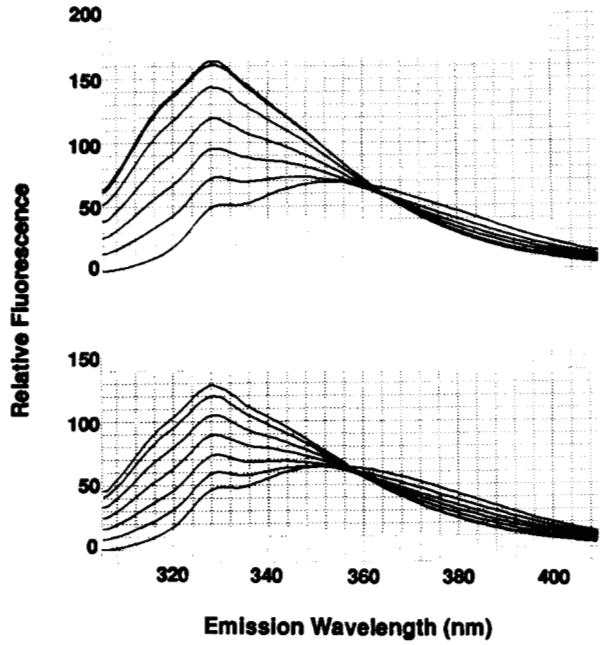


FIG. 5. **Fluorescence spectra of smMLCK peptide titrated with CaM and T110Q/L112T/K115T.** *Top*, smMLCK peptide titrated with T110Q/L112T/K115T; *bottom*, smMLCK peptide titrated with CaM. The *lowest curve* in each titration is the fluorescence spectra of smMLCK peptide in the absence of protein. Each successive higher spectrum represents a protein concentration increment of 300 nM (*i.e.* 0, 300, 600, 900, 1200, 1500, and 1800 nM).

the CaM amino acid residue with the corresponding residue from cTnC, naming the mutant protein according to the substitution(s) produced: H107I, V108M, M109L, T110Q, N111A, L112T, K115T, the double mutant T110Q/L112T, and the triple mutant T110Q/L112T/K115T.

The final two constructs are back mutations. C3T[Q110T/T112L/T115K] is derived from CaM[3 TnC], a CaM-cTnC chimera that contains the entire third domain of cTnC (13). C3T[Q110T/T112L/T115K] is identical to CaM[3 TnC], except that 3 cTnC residues have been mutated back to the corresponding CaM residue (Q110T/T112L/T115K). Similarly, CH6T[Q110T/T112L] is identical to CaM[helix 6-TnC], except that 2 cTnC residues have been mutated back to the corresponding CaM residue (Q110T/T112L).

The domain 3 CaM mutants were expressed in *Escherichia coli* and purified using hydrophobic affinity and ion-exchange chromatography. An SDS-polyacrylamide gel of the mutants is shown in Fig. 3.

Enzyme Activation—To localize more precisely the molecular basis of the K_{act} increase observed with CaM[3 TnC] (13), we assayed the domain 3 CaM mutants for the ability to stimulate PDE (Table I). All domain 3 mutants activate PDE maximally with variable K_{act} shifts. CaM[loop 3-TnC] was not substantially different from CaM, whereas CaM[helix 5-TnC] had about a 4-fold increase in K_{act}. In contrast, the K_{act} for CaM[helix 6-TnC] was over 30-fold higher than the K_{act} for CaM. The bulk of this shift could be traced to a single substitution, L112T, which has a 20-fold increase in K_{act} relative to CaM. The remaining single mutations in helix 6 produced relatively small K_{act} shifts. The mutation K115T resulted in a small reduction in K_{act} (1.2 nM, *versus* 2.2 nM for CaM).

We also used the domain 3 CaM mutants to determine why CaM[3 TnC] could not activate smMLCK. CaM[helix 5-TnC] and CaM[loop 3-TnC] retain the full ability to activate smMLCK (Fig. 4, *top panel*). In contrast, CaM[helix 6-TnC] shows markedly impaired smMLCK activation, reaching only 15% of the maximum observed with CaM. The critical helix 6

residues appear to be Thr-110 and Leu-112, since the single mutants T110Q and L112T demonstrate marked activation impairment (45 and 64% of maximum activation; Fig. 4, *bottom panel*). In contrast, the remaining four point mutations in helix 6 did not materially affect smMLCK activation.

The single mutation K115T, in the 6–7 linker, also impairs smMLCK activation (38% of maximum activation; Fig. 4, *bottom panel*). Lysine 115 is trimethylated in native CaM but unmodified in bacterially expressed CaM (21). Since vertebrate CaM and bacterially expressed CaM activate smMLCK identically (Ref. 22 and data not shown), the role of Lys-115 in smMLCK activation does not appear to be influenced by the presence or absence of trimethylation.

The data obtained with combinations of mutations provide further evidence that Thr-110, Leu-112, and Lys-115 play an essential role in smMLCK activation (Fig. 4, *bottom panel*). The double mutant T110Q/L112T reaches only 17% of the maximal activation observed with CaM and, in fact, shows smMLCK activation that is virtually identical to that observed with CaM-[helix 6-TnC]. Activation of smMLCK by the triple mutant T110Q/L112T/K115T is reduced to 6% of maximum.

Finally, the two back mutation constructs also indicate that Thr-110, Leu-112, and Lys-115 are important smMLCK-activating residues. CaM[3 TnC] shows no detectable activation of smMLCK and is a potent competitive inhibitor of smMLCK activation by CaM (13). The restoration of Thr-110, Leu-112, and Lys-115 to CaM[3 TnC] abolishes its competitive inhibitor phenotype and restores 33% of maximal activation (C3T[Q110T/T112L/T115K]; Fig. 4, *top panel*). In a similar manner, CaM[helix 6-TnC] is virtually inactive toward smMLCK, but back mutation of Q110T/T112L restores 85% of the activation observed with CaM (CH6T[Q110T/T112L]; Fig. 4, *top panel*).

TABLE II
Summary of smMLCK peptide binding data

The domain 3 CaM mutants were added in aliquots to a solution containing 1.8 μM smMLCK peptide, and fluorescence intensity (FI) at 328 nm was monitored as described under "Experimental Procedures." Magnitude of enhancement was determined when fluorescence at 328 nm reached a maximum (magnitude of enhancement = ($FI_{328\ nm}$ at maximum)/($FI_{328\ nm}$ prior to addition of protein)). The lowest concentration of protein required for maximal enhancement was noted and divided by the concentration of peptide to determine the molar ratio yielding maximal enhancement. K_d were determined from the ability of the smMLCK peptide to inhibit PDE activation by CaM or the domain 3 CaM mutant proteins (see "Experimental Procedures" for details).

Protein	Magnitude of enhancement	Molar ratio yielding maximal enhancement	K_d
	-fold		*nM*
CaM	2.7	0.95:1	2.4 ± 0.5
T110Q	2.8	0.89:1	3.2 ± 0.4
L112T	3.2	0.92:1	8.1 ± 2.0
K115T	2.9	0.96:1	2.9 ± 0.5
T110Q/L112T	3.2	0.93:1	14.0 ± 0.8
T110Q/L112T/K115T	3.2	0.90:1	18.1 ± 1.3
CaM[helix 6-TnC]	4.4	1.02:1	22.3 ± 5.2

smMLCK Peptide Binding—The triple mutant T110Q/L112T/K115T does not activate smMLCK, but it does not reproduce the strong competitive inhibitor phenotype of CaM[3 TnC] (K_i > 2 μM (data not shown) *versus* 42 nM for CaM[3 TnC] (13)). We and others have previously interpreted competitive inhibition by mutant CaMs to reflect the binding of smMLCK but an inability to activate the enzyme (10, 12, 13). Since T110Q/L112T/K115T neither activates nor inhibits smMLCK, we questioned whether it lacked the ability to bind the enzyme. The crystal structure of the CaM-smMLCK peptide suggested that a lack of binding by T110Q/L112T/K115T was plausible. Although Thr-110 and Lys-115 have no demonstrable interaction with the CaM-binding domain of smMLCK, Leu-112 interacts closely with Ala-11 of the smMLCK peptide (8). In the triple mutant, a threonine residue at position 112 might disrupt binding to the CaM-binding domain of smMLCK and, thus, result in an inactive phenotype.

As an initial approach to this question, we assessed the abil-

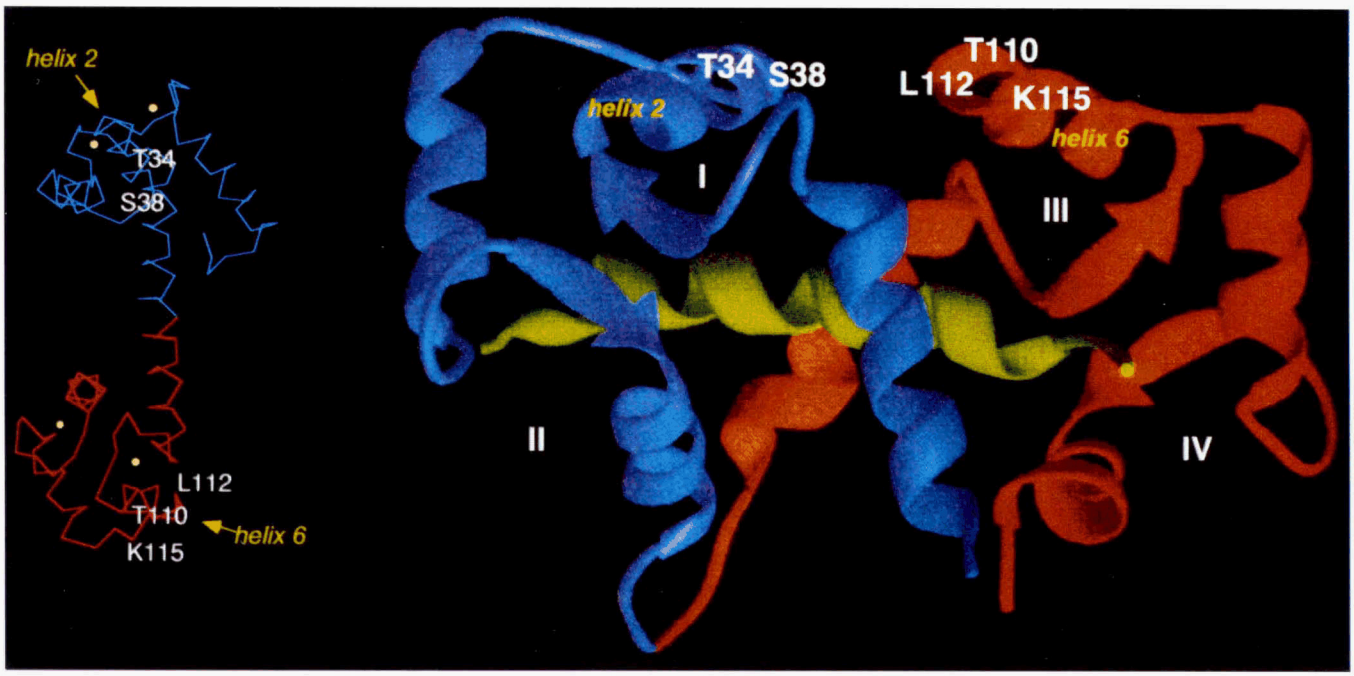


FIG. 6. **Possible role of helices 2 and 6 in smMLCK activation.** *Left*, α-carbon crystal structure of CaM. The amino-terminal half of CaM is shown in *blue*, and the carboxyl-terminal half is shown in *red*. The helix 2 smMLCK activating residues *T34* and *S38* (10) are separated by over 50 Å from the smMLCK-activating residues *T110*, *L112*, and *K115* in helix 6 and the helix 6-helix 7 linker. *Right*, ribbon representation of the nuclear magnetic resonance structure of the skeletal isoform of myosin light chain kinase bound to a peptide known as M13 (*green*). The sequence of M13 is based on the CaM binding region of skeletal isoform of MLCK. Peptide binding induces a 100° bend and 120° twist in the central helix, enveloping the smMLCK peptide and forming a latch between helices 2 and 6 (8). The peptide-induced rearrangement closely approximates the proposed activating residues. The NMR structure of the CaM-M13 complex (9) is very similar to CaM-smMLCK peptide complex (8).

ity of T110Q/L112T/K115T to bind to the smMLCK peptide. Peptide binding by CaM or the mutant protein was monitored by a blue shift and an enhancement in maximal fluorescence of the tryptophan-containing peptide as an increasing amount of protein was added. Spectra obtained with CaM and T110Q/L112T/K115T are shown in Fig. 5. Both CaM and T110Q/L112T/K115T produced a similar shift in wavelength for maximal enhancement, from 352 nm in the absence of protein to 328 nm when the peptide was fully bound by protein. The shift likely reflects sequestration of the smMLCK peptide's tryptophan residue within the hydrophobic pockets of CaM (19, 26). The magnitude of enhancement at 328 nm was less for CaM than for T110Q/L112T/K115T, possibly reflecting a greater degree of fluorescence quenching by the CaM-smMLCK peptide complex (Table II) (26). For both CaM and T110Q/L112T/K115T, the fluorescence intensity was maximal at a molar ratio near 1:1. Similar spectra were obtained for T110Q, L112T, K115T, T110Q/L112T, and CaM[helix 6-TnC], showing significant variation only in the magnitude of fluorescence enhancement at 328 nm (Table II).

Additional fluorescence studies performed at an smMLCK peptide concentration of 200 nM (data not shown) produced data similar to those presented in Table II. Thus, the K_d for each of the peptide-protein complexes was 100 nM or less. Since the K_d for the CaM-smMLCK peptide (about 1 nM, Ref. 19) lies below detection sensitivity of techniques that rely on intrinsic tryptophan fluorescence (27), we used competitive phosphodiesterase assays to determine dissociation constants for the mutant CaM-smMLCK peptide complexes (10, 24, 25). In contrast to their marked effect on smMLCK activation, the domain 3 mutations generally produced relatively small effects on smMLCK peptide binding (Table II). These data suggest that the domain 3 CaM mutants' impaired smMLCK activating ability cannot be explained by altered interactions with residues 796–815 of smMLCK, from which the smMLCK peptide was derived (19, 20).

Relationship of Proposed Activating Residues—When cTnC's domain 1 or domain 3 replaces the corresponding CaM domain, the resulting chimera cannot activate MLCK (12, 13). In domain 1, the impairment is largely attributable to two helix 2 mutations, T34K and S38M (10). In domain 3, the impairment is largely attributable to three mutations in the region of helix 6, T110Q, L112T, and K115T. In crystal structure, helices 2 and 6 are about 50 Å apart, and there is no clear relationship between the proposed activating residues (Fig. 6) (4). However, peptide binding rearranges the CaM molecule about its central helix, bringing helices 2 and 6 into very close approximation (less than 5 Å at their closest point) (8). This close approximation is so striking that the helices 2 and 6 have been described as forming a "latch" between the amino- and carboxyl-terminal halves of CaM (8).

The clustering of 5 important activating residues in this "latch" region leads us to hypothesize that it may function as an activating domain for smMLCK. This proposed functional domain of CaM exists only in the peptide-bound form; the peptide-induced bend and twist of the central helix brings helices 2 and 6 together in the proper antiparallel orientation for smMLCK activation. The surface residues of this domain then bind to smMLCK and help to stabilize the active conformation of the enzyme. We propose that these additional CaM-smMLCK interactions involve solvent-exposed predominantly polar residues of CaM helices 2 and 6 and immediately adjacent regions and involve regions of smMLCK that are not identical with the CaM-binding domain of the enzyme.

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