The Fourth EF-Hand of Calmodulin and Its Helix−Loop−Helix Components: Impact on Calcium Binding and Enzyme Activation†

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Received February 28, 1996; Revised Manuscript Received April 29, 1996⊗

ABSTRACT: CaM (4 cTnC) is a calmodulin−cardiac troponin C chimeric protein containing the first, second, and third calcium-binding EF-hands of calmodulin (CaM) and the fourth EF-hand of cardiac troponin C (cTnC) [George, S. E., Su, Z., Fan, D., & Means, A. R. (1993) *J. Biol. Chem. 268*, 25213−25220]. CaM (4 cTnC) showed 2-fold-enhanced carboxy-terminal Ca^{2+} affinity relative to CaM and also exhibited impaired activation of the CaM-regulated enzymes smooth muscle myosin light chain kinase (smMLCK), neuronal nitric oxide synthase (nNOS), and phosphodiesterase (PDE). To investigate the molecular basis for these effects, we constructed (1) additional chimeras, replacing most of CaM helix 7, Ca^{2+}-binding loop 4, and helix 8 with the corresponding helices and loops of cTnC; and (2) point mutants in the fourth EF-hand of CaM. Replacement of CaM's fourth loop with the corresponding loop of cTnC enhanced Ca^{2+} affinity by over 3-fold through an increase in the Ca^{2+} on rate and also reduced cooperativity of Ca^{2+} binding. In contrast, substitution of CaM helix 7 or 8 modestly decreased Ca^{2+} affinity by increasing the Ca^{2+} off rate, without impairment of cooperativity. All three of the helix and loop chimeras fully activated PDE, with minor shifts in K_{act}. CaM (helix 7 cTnC) showed a significantly impaired ability to activate smMLCK and nNOS, whereas the other two chimeras retained about 80% of the maximal smMLCK and nNOS activation observed with CaM.

Calmodulin (CaM)[1] is a 17 kDa cytosolic protein that transduces intracellular calcium signals (Means et al., 1991; Crivici & Ikura, 1995). The structural basis of its high-affinity Ca^{2+} binding and the nature of its target protein interactions are important keys to understanding the diverse regulatory functions of CaM. Targeted mutagenesis continues to be a useful tool for defining these relationships [see, e.g., VanBerkum et al. (1990), George et al. (1993), and Ohya and Botstein (1994)].

CaM and troponin C (TnC) are homologous proteins, with 50% direct amino acid indentity and remarkably similar crystal structures (Strynadka & James, 1989; Herzberg & James, 1988; Chattopadhyaya et al., 1992). The N- and C-terminal domains of both CaM and TnC are comprised of a pair of high-affinity, helix−loop−helix calcium-binding motifs, termed EF-hands (Kretsinger, 1987). In both proteins, Ca^{2+} binding to both EF-hand pairs is highly cooperative, with Hill coefficients approaching the theoretical maximum of 2 (Cox, 1988; Linse et al., 1991; Holroyde et al., 1980; Grabarek et al., 1992). In spite of their structural similarity, there are important functional differences between CaM and TnC. Neither isoform of TnC (skeletal muscle, sTnC, or cardiac, cTnC) effectively activates most CaM target enzymes (George et al., 1990; Walsh et al., 1980), nor does CaM substitute well in skinned skeletal muscle fibers (Brandt et al., 1994). Moreover, there are notable differences in cation affinity. The carboxy-terminal domain of cTnC has over 1 order of magnitude higher affinity for Ca^{2+} than the carboxy-terminal domain of CaM (Leavis & Kraft, 1978; Crouch & Klee, 1980; George et al., 1993); moreover, the carboxy-terminal domain of cTnC binds both Ca^{2+} and Mg^{2+}, whereas the corresponding domain of CaM is relatively Ca^{2+}-specific (Leavis & Kraft, 1978; Crouch & Klee, 1980; Holroyde et al., 1980; Johnson et al., 1980). By exchanging domains or elements of the EF-hand motif between CaM and cTnC, we can define regions of CaM that affect cation binding and target enzyme activation (George et al., 1990, 1993; Su et al., 1994, 1995).

The chimera CaM (4 cTnC) (residues 135−161 of cTnC substituted for 123−148 of CaM) had enhanced Ca^{2+} affinity relative to CaM and impaired ability to activate target enzymes (George et al., 1993; Su et al., 1995). In evaluating the fourth EF-hand substitution, we noted structural differences between CaM and cTnC that might account for the observed functional divergence. First, the 12-residue Ca^{2+}-binding loop of CaM has only one acid pair (negatively charged Ca^{2+}-coordinating residues at opposition to each other), whereas the cTnC loop has two; such acid pairs are believed to enhance Ca^{2+} affinity (Reid & Hodges, 1980; Strynadka & James, 1989). Second, helix 7 and helix 8 of CaM were more hydrophobic than the corresponding helices

† This work was supported by Public Health Service Grants HL48662 (S.E.G.) and DK33727 (J.D.J.) and a Grant-in-Aid from the American Heart Association (S.E.G.). S.E.G. is an Established Investigator of the American Heart Association.

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; PCR, polymerase chain reaction; CaM, calmodulin; TnC, troponin C; cTnC, cardiac isoform of troponin C; PDE, calmodulin-stimulated cyclic nucleotide phosphodiesterase; smMLCK, smooth muscle myosin light chain kinase; nNOS, neuronal isoform of nitric oxide synthase; RS20, a 20-residue peptide (ARRKWQKTGHAVRAIGRKSS) modeled after the CaM-binding domain of smMLCK.

S0006-2960(96)00495-3 CCC: $12.00

of cTnC. Since hydrophobic interactions provide the primary driving force for CaM target enzyme binding (Meador et al., 1992), their disruption or alteration could account for the observed differences in activation. In the present study, we sought to investigate these hypotheses by substituting the helix−loop−helix components of CaM's fourth EF-hand with the corresponding helix−loop−helix components of cTnC. We show that each helix or loop substitution has significant effects on Ca^{2+} affinity, with a striking enhancement of affinity associated with loop 4 of cTnC. We also observe that each component substitution had negative effects on enzyme activation, with particularly marked impairment associated with helix 7 of cTnC.

EXPERIMENTAL PROCEDURES

Construction and Purification of Mutant Proteins. We used PCR mutagenesis techniques to construct the bacterial expression plasmids encoding the fourth EF-hand CaM mutants (George et al., 1993; Su et al., 1994). The proteins are expressed in *Escherichia coli* and purified as previously described (George et al., 1990, 1993).

Tyrosine Fluorescence. Fluorescence enhancements of CaM, cTnC, and the chimeras were determined as previously described (George et al., 1993). Briefly, the excitation wavelength was 278 nm and the emission wavelength 307 nm, and the band widths were 5 nm. Protein concentrations were adjusted to 6 μM in 100 mM MOPS (pH 7.0), 150 mM KCl, and 0.4 mM EGTA. Three microliters of a 10 mM $CaCl_2$ solution was added in successive aliquots to 3 mL of protein solution. Data reported are the mean of three successive titrations, ± standard errors. Free calcium concentration was determined as described by VanBerkum et al. (1990). Buffers were calibrated, and free calcium concentration was verified, by titrating a 3 mL sample of 6 μM CaM, 1 μM indo-1, in 100 mM MOPS, 150 mM KCl, and 0.4 mM EGTA, with successive 3 μL aliquots of 10 mM $CaCl_2$. The calibration sample was excited at 355 nM, and emission was scanned from 380 to 550 nM. The calculated free calcium concentrations were then confirmed by ratiometric evaluation of indo-1-emission fluorescence as described in the supplier's instructions (Molecular Probes, Eugene, OR). Data reported are the mean of three successive titrations, ± standard errors as indicated in the figure. As previously described (George et al., 1993), data were fit to the nonlinear Hill equation

$$y = y_{max}/[1 + 10^{[n(pCa_{50} - pCa)]}]$$

where y = the percent of maximal enhancement observed at a particular pCa, y_{max} = the maximal percentage enhancement, n = the Hill coefficient, pCa_{50} = the negative logarithm of free Ca^{2+} concentration producing half-maximal enhancement, and pCa = the negative logarithm of free Ca^{2+} concentration producing y percent of maximal enhancement. Fitting to a two-site model of the nonlinear Hill equation did not improve any of the curve fits.

Ca^{2+} Dissociation Rates. Kinetic measurements were carried out on an Applied Photophysics model SF.17 stopped-flow instrument with a dead time of 1.6 ms. Rates of Ca^{2+} dissociation from the carboxy terminal of CaM and CaM−cTnC chimeras were measured by the decrease in tyrosine fluorescence or by the increase in Quin 2 fluorescence, as described by Johnson et al. (1994), and as noted in the figure legends.

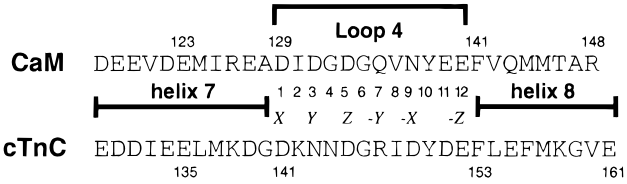

FIGURE 1: Map of the fourth EF-hand mutations. Amino acid sequence comparison of EF-hand 4 from CaM and cTNC, with residue numbers indicated above and below the sequences. Helix 7, loop 4, and helix 8 residues are indicated by brackets; loop residues are numbered 1−12, and those involved in Ca^{2+} coordination are indicated by the letters *X*, *Y*, *Z*, −*Y*, −*X*, and −*Z*. The helix 7 substitution extends from CaM residue 123 to 128, the loop 4 substitution from 128 to 140, and the helix 8 substitution from 141 to 148.

Enzyme Assays. Phosphodiesterase activity was assayed as previously described (George et al., 1993). Myosin light chain kinase assays were performed as described by VanBerkum et al. (1990). [^{3}H]Arginine to [^{3}H]citrulline conversion, a measure of nitric oxide synthase activity, was performed as described by Bredt and Snyder (1989), with minor modifications (Su et al., 1995). K_{act} values were determined by double reciprocal analysis of the activation data. Enzyme activation curves were fit using a logistic sigmoid function, $y = y_{min} + [(y_{max} - y_{min})/[1 + \exp[-k(x - x_{50})]]]$, where y_{min} and y_{max} are the minimum and maximum values of enzyme activation, respectively, k is the logarithim of the Hill coefficient, x is the logarithim of the activator concentration, and x_{50} is the logarithim of the activator concentration producing half-maximal activation.

RESULTS

Construction of Mutants. We constructed nine mutants involving the fourth EF-hand of calmodulin using standard mutagenesis techniques as previously described (George et al., 1993). Three of these involve exchanges between corresponding residues of the helix−loop−helix structure that makes up the fourth EF-hand of CaM and cTnC (Figure 1). We named these chimeras as follows: CaM (helix 7 cTnC) [residues 135−140 of cTnC substituted for residues 123−128 of CaM; splice point chosen to correspond to the splice point used in constructing CaM (4 cTnC); George et al., 1993], CaM (loop 4 cTnC) (residues 141−152 of cTnC substituted for residues 129−140 of CaM), and CaM (helix 8 cTnC) (residues 153−161 of cTnC substituted for residues 141−148 of CaM). A fourth chimera substituted both of the cTnC carboxy-terminal loops for the corresponding loops of CaM; we named this construct CaM (loop 3, loop 4 cTnC) (residues 105−116 and 141−152 of cTnC substituted for residues 93−104 and 129−140 of CaM, respectively). The remaining five mutants involved amino acid substitutions in helix 7 of CaM. In each, we replaced the CaM amino acid residue with the corresponding residue from cTnC and named the mutant protein according to the substitution produced: M124L (i.e., Met 124 of CaM replaced by Leu), I125M, R126K, E127D, and A128G. Expression plasmids encoding the mutants were used to transform *E. coli*, and the expressed protein was purified using phenyl-Sepharose affinity chromatography (George et al., 1993). The yield was 25−50 mg of purified protein per liter of bacterial culture.

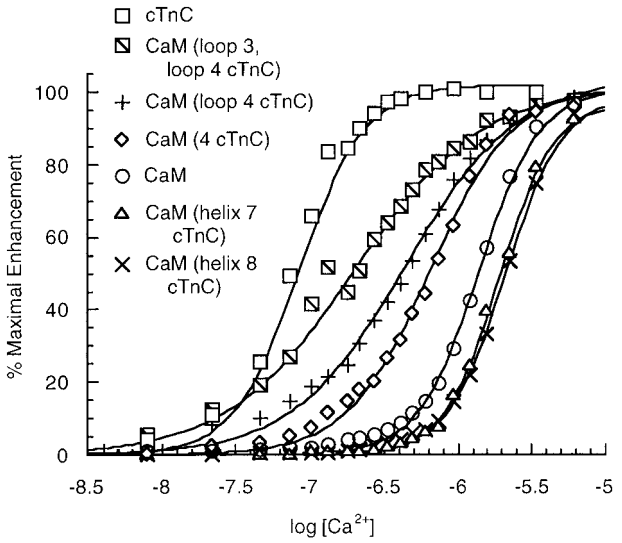

FIGURE 2: Fluorescence enhancement. Changes in peak fluorescence were monitored as a function of the log of free Ca^{2+} concentration. Data are expressed as percent of maximal fluorescence enhancement obtained between pCa 8.5 and 4.5, for that protein. Proteins were adjusted to a concentration of 6 μM in 100 mM MOPS, 150 mM KCl, and 0.4 mM EGTA; 3 μL of a 10 mM $CaCl_2$ solution was added in successive aliquots to yield the free calcium concentration indicated on the *x* axis.

Table 1: Fluorescence Enhancement[a]

	pCa_{50}	Hill coefficient
cTnC	7.11 ± 0.01	1.99 ± 0.11
CaM (loop 3, loop 4 cTnC)	6.73 ± 0.01	1.01 ± 0.03
CaM (loop 4 cTnC)	6.39 ± 0.02	1.15 ± 0.02
CaM (4 cTnC)	6.21 ± 0.02	1.56 ± 0.07
CaM	5.90 ± 0.01	2.18 ± 0.09
CaM (helix 7 cTnC)	5.72 ± 0.01	2.35 ± 0.08
CaM (helix 8 cTnC)	5.68 ± 0.01	2.22 ± 0.07

[a] Concentrations of Ca^{2+} at which fluorescence enhancement was half-maximal (pCa_{50}) and Hill coefficients are presented. Parameters were determined by analyzing the data in Figure 2 using the nonlinear Hill equation.

Ca^{2+} Binding. To evaluate Ca^{2+} binding, we determined the carboxy-terminal fluorescence enhancement in response to incremental additions of Ca^{2+} (Figure 2). Both CaM and cTnC contain a tyrosine residue at corresponding positions in Ca^{2+} binding loops 3 and 4; titration with Ca^{2+} produces fluorescence enhancement which reflects Ca^{2+}-dependent alterations in tertiary structure. The carboxy-terminal domain of cTnC has greater than 1 order of magnitude higher affinity for Ca^{2+} than the corresponding domain of CaM, and therefore, the Ca^{2+}-dependent increase in tyrosine fluorescence with cTnC occurs at a higher pCa range than with CaM (Holroyde et al., 1980; George et al., 1993). Half-maximal fluorescence enhancement (pCa_{50}) occurs at pCa 7.11 for cTnC, pCa 6.21 for CaM (4 cTnC), and pCa 5.90 for CaM (Table 1). CaM (loop 4 cTnC) demonstrates a 3.1-fold-enhanced Ca^{2+} affinity relative to CaM, as shown by the leftward shift in its fluorescence titration curve and an increase in pCa_{50}. When both loops 3 and 4 are replaced by the corresponding cTnC loops [i.e., CaM (loop 3, loop 4)], a further increase in pCa_{50} is observed, reflecting a 6.8-fold increase in Ca^{2+} affinity. In contrast, the Ca^{2+} titrations of CaM (helix 7 cTnC) and CaM (helix 8 cTnC) are modestly right-shifted relative to CaM. These results were confirmed by direct determination of ^{45}Ca binding (data not shown) and indicate that CaM (loop 4 cTnC) binds more Ca^{2+} at pCa 6.75−6.25, whereas the helix 7 and helix 8 substitutions significantly reduce Ca^{2+} affinity at pCa 6.0−5.0.

CaM (4 cTnC) has a reduced Hill coefficient relative to CaM, indicating that the substitution of the fourth EF-hand impairs cooperativity of Ca^{2+} binding. CaM (loop 4 cTnC) demonstrates a marked reduction in the Hill coefficient, whereas CaM (helix 7 cTnC) and CaM (helix 8 cTnC) both maintained high Hill coefficients. Thus, CaM (4 cTnC)'s reduced Hill coefficient appears to be a consequence of the loop substitution. It seemed possible that the loss of cooperativity might be attributable to the loss of favorable loop−loop interactions within the EF-hand pair (Sekharudu & Sundaralingam, 1988; Strynadka & James, 1989), and CaM (loop 3, loop 4 cTnC) was constructed to evaluate this possibility. However, CaM (loop 3, loop 4 cTnC) also had a low Hill coefficient.

The observed alterations in Ca^{2+} affinity may result from changes in either Ca^{2+} on or Ca^{2+} off rates ($K_d = k_{off}/k_{on}$). We determined the rates of Ca^{2+} dissociation from the carboxy-terminal half of the molecule by following the rates of the EGTA-induced decrease in tyrosine fluorescence (Figure 4A) and the rates of increase in fluorescence of the Ca^{2+} chelator Quin 2 (Figure 4B). On the basis of changes in tyrosine fluorescence, Ca^{2+} dissociates from the carboxy terminal of CaM, CaM (loop 4 cTnC), CaM (helix 7 cTnC), and CaM (helix 8 cTnC) at rates of 9, 11, 32, and 17 s^{-1}, respectively (Figure 4A). Very similar results were obtained when 2 mol of Ca^{2+} was dissociated from the carboxy terminal with the fluorescent Ca^{2+} chelator Quin 2 (rates of 9, 14, 32, and 23 s^{-1}, respectively; Figure 4B). Thus, although substitution of CaM's loop 4 with that of cTnC produces a 3.1-fold increase in carboxy-terminal Ca^{2+} affinity, it does this in spite of a modest increase in the rate of Ca^{2+} dissociation. This indicates that cTnC's loop 4, placed in the carboxy terminal of CaM, provides about a 5-fold faster Ca^{2+} on rate than loop 4 of CaM. In contrast, substitution of cTnC helix 7 or helix 8 for the corresponding CaM helices serves to decrease Ca^{2+} affinity, a consequence of about 3−4-fold increases in the rate of Ca^{2+} dissociation.

Enzyme Activation. All three of the helix and loop chimeras fully activate PDE, with modest increases in K_{act} (Table 2). Substitution of loop 4, helix 7, and helix 8 of cTnC into CaM produced 1.8-, 1.9-, and 3.5-fold increases in K_{act}, respectively. Thus, each helix, loop, and helix contributes in a virtually additive manner to the 9.5-fold K_{act} shift observed with CaM (4 cTnC) (George et al., 1993). In contrast to its ability to maximally activate PDE, CaM (4 cTnC) showed impaired activation of nNOS and smMLCK (48 and 12%, respectively, of the maximal activation observed with CaM; Su et al., 1995; George et al., 1993). CaM (helix 8 cTnC) and CaM (loop 4 cTnC) retained about 80% of the maximal ability to activate nNOS and smMLCK, but CaM (helix 7 cTnC) was an impaired activator of both enzymes (Figure 4). Thus, all three helix−loop−helix substitutions impair the activation of smMLCK and nNOS, but the effect was most marked with the helix 7 substitution. In general, the helix 7 point mutations produced small to moderate reductions in enzyme activation; the effect was most marked for the Met 124 to Leu and Glu 127 to Asp mutations (Table 3).

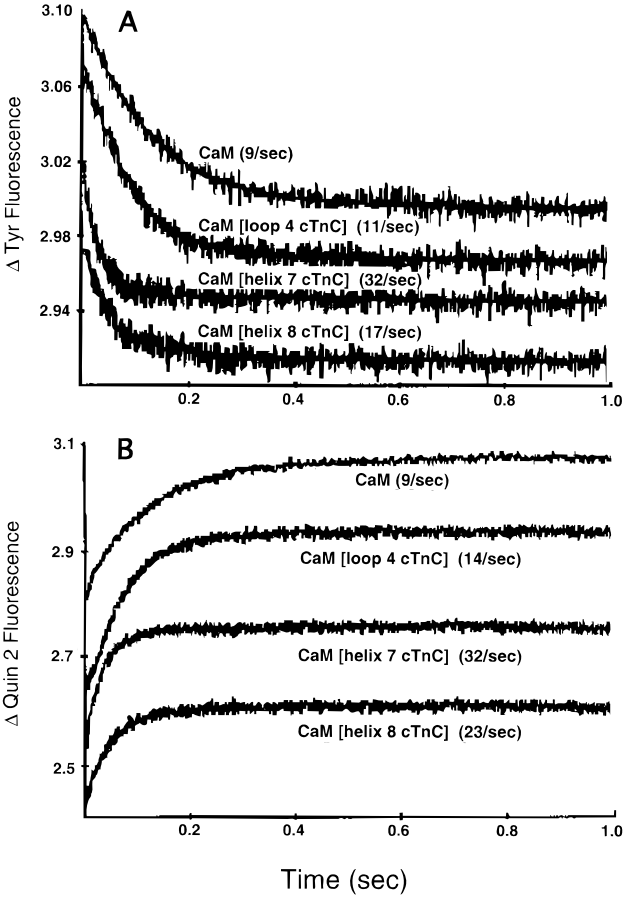

FIGURE 3: (A) Rates of Ca^{2+} dissociation from the C-terminal of CaM and CaM−cTnC chimeras as monitored by tyrosine fluorescence. The time course of the decrease in C-terminal tyrosine fluorescence is shown when CaM, CaM (loop 4 cTnC), CaM (helix 7 cTnC), and CaM (helix 8 cTnC) are rapidly mixed with EGTA-containing buffer. CaM and each chimera (4 μM) + Ca^{2+} (60 μM) in 10 mM MOPS and 90 mM KCl (pH 7.0) were rapidly mixed with an equal volume of EGTA (10 mM) in the same buffer at 22 °C. Tyrosine fluorescence was measured through a 360 nm broad band-pass with excitation at 275 nm. Each trace represents an average of five to seven determinations and was fit with a single exponential curve (variance $<2.0 \times 10^{-5}$). (B) Rates of Ca^{2+} dissociation monitored by Quin 2 fluorescence. The time course of the increase in Quin 2 fluorescence is shown as Ca^{2+} dissociates from CaM, CaM (loop 4 cTnC), CaM (helix 7 cTnC), and CaM (helix 8 cTnC). CaM and each chimera (4 μM) + Ca^{2+} (60 μM) in 10 mM MOPS and 90 mM KCl (pH 7.0) were rapidly mixed with an equal volume of Quin 2 (150 μM) in the same buffer at 22 °C. Quin 2 fluorescence was monitored with a 510 nm broad band-pass filter with excitation at 330 nm. Each trace is an average of five to seven determinations fit with a double exponential (variance $< 3.4 \times 10^{-5}$). All kinetic traces were triggered at time zero; the first 1.6 ms of premixing is shown, and all traces were fit after mixing was complete.

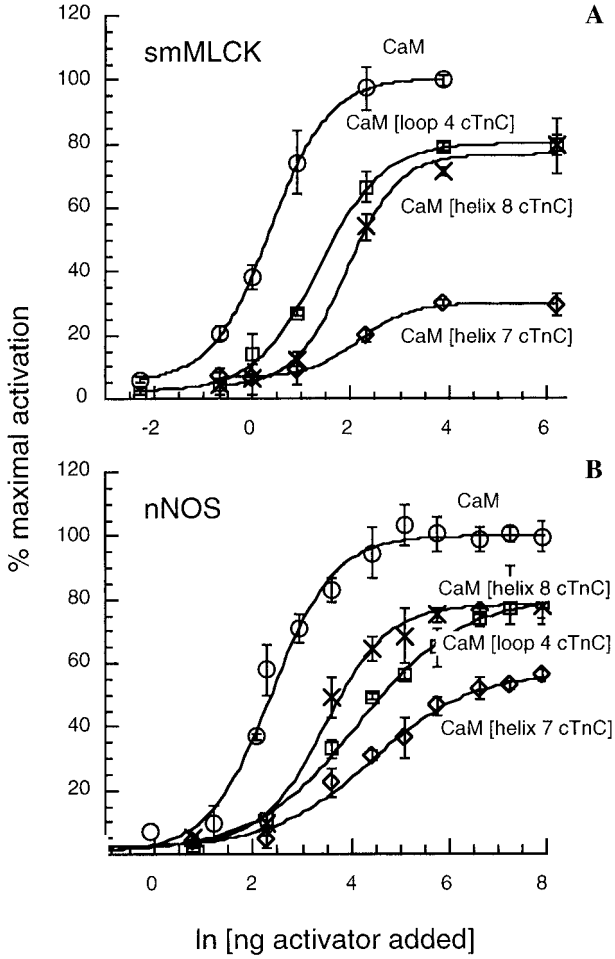

FIGURE 4: Activation of smMLCK and nNOS. Graphs of smMLCK (A) and nNOS (B) activation by chimeras. Assays were done in triplicate, with each point representing the mean and the error bars indicating the standard error. "Percent maximal activation" is the observed maximal activation of the chimera divided by the activation observed with 100 nM CaM and multiplied by 100.

Table 2: Phosphodiesterase Activation by CaM−cTnC Chimeras Involving the Fourth EF-Hand[a]

	K_{act} (nM)	% maximal activation
CaM	2.1 ± 0.4	100 ± 2
CaM (loop 4 cTnC)	3.8 ± 0.1	97 ± 5
CaM (loop 3, loop 4 cTnC)	7.9 ± 1.5	104 ± 3
CaM (helix 7 cTnC)	3.9 ± 0.4	103 ± 2
CaM (helix 8 cTnC)	7.3 ± 0.4	103 ± 5

[a] Maximal activation is defined as the phosphodiesterase activity observed in the presence of 100 nM CaM. Results presented here are from a representative experiment done in triplicate, ± standard errors.

DISCUSSION

In EF-hand superfamily members, the 12-residue loop coordinates Ca^{2+} through seven oxygens at an average distance of 2.4 Å from the Ca^{2+} atom [reviewed in Strynadka and James (1989)]. The coordinating arrangement is pentagonal bipyramidal; five coordinating residues form an approximately coplanar pentagon (typically the side chains at *Y*, *Z*, and $-Z$, where $-Z$ provides two coordinating oxygens through an invariant Glu; and the main chain carbonyl from the residue at $-Y$), and the remaining two are arrayed perpendicular to the pentagonal plane (the side chains of *X* and $-X$, with coordination at $-X$ usually through an intervening H_2O molecule). Several loop structural features have been proposed to enhance Ca^{2+} affinity, such as acid pairs (Asp or Glu) at loop positions *X*,$-X$ and *Z*,$-Z$ (Reid, 1990; Procyshyn & Reid, 1994); the total number of acid residues in chelating positions (Sekharudu & Sundaralingam, 1988); Lys at the nonliganding loop position 2 (Strynadka & James, 1989; Shaw et al., 1991); Asp versus Asn at *Y*, when associated with an uncharged residue at $-X$ (Waltersson et al., 1993); and Glu versus Asp at $-X$, which allows direct Ca^{2+} coordination rather than through an intervening H_2O molecule (Palmisano et al., 1990; Renner et al., 1993). These divergent observations illustrate that

Table 3: Activation of smMLCK and nNOS[a]

protein	smMLCK K_{act} (nM)	smMLCK % maximal activation	nNOS K_{act} (nM)	nNOS % maximal activation
CaM	0.8	100 ± 4	2.3	100 ± 2
CaM (loop 4 cTnC)	2.0	80 ± 2	19	81 ± 2
CaM (helix 8 cTnC)	2.2	81 ± 2	22	78 ± 2
CaM (helix 7 cTnC)	2.7	31 ± 1	42	58 ± 1
M124L	1.9	81 ± 2	3	65 ± 1
I125M	1.7	103 ± 1	4	84 ± 1
R126K	1.6	98 ± 2	4	88 ± 1
E127D	2.3	83 ± 1	6	80 ± 2
A128G	1.3	93 ± 2	3	79 ± 1

[a] K_{act} and percent maximal activation, ± standard error for the subdomain chimeras and for the helix 7 point mutants.

Ca^{2+} affinity is affected by a range of complex interactions, even when only loop factors are considered.

CaM and cTnC both have corresponding tyrosine residues, the fifth residue ($-Y$ position) of Ca binding loop 3 and the tenth residue of Ca binding loop 4. The observed Ca^{2+}-induced changes in tyrosine fluorescence therefore report the macroscopic binding constant for the entire carboxy-terminal domain of these proteins. Our data demonstrate that the substitution of cTnC's loop 4 into CaM enhances Ca^{2+} affinity by 3.1-fold. The presence of two acid pairs in the cTnC loop ($X,-X$ and $Z,-Z$), versus only one in the CaM loop ($Z,-Z$), may account for the observed affinity difference; sequence comparisons and direct experimental evidence using synthetic peptides indicate that acid pairs in the $X,-X$ and $Z,-Z$ positions promote high-affinity Ca^{2+} binding (Reid & Hodges, 1980; Reid, 1990; Procyshyn & Reid, 1994). Other factors may also contribute to the observed affinity difference. Adjacent Asp residues in the X and Y position of CaM's fourth loop may create dentate−dentate electrostatic repulsion that reduces Ca affinity (Marsden et al., 1988); Lys at loop position 2 may enhance the Ca^{2+} affinity of cTnC's fourth loop, by stabilizing the helix on the NH_2-terminal side of the loop (Strynadka & James, 1989; Shaw et al., 1991).

The helices also play a major role in promoting high-affinity Ca^{2+} binding in EF-hand proteins (Strynadka & James, 1989; Sekharudu & Sundaralingam, 1988). Synthetic peptides containing only a loop, or a loop plus one helix, bind Ca^{2+} with low affinity (Marsden et al., 1988; Reid et al., 1981); the addition of both helices is necessary to approach the micromolar Ca^{2+} affinity typical of this superfamily (Reid et al., 1981). The clearest positive correlation between helical structure and Ca^{2+} affinity is helical hydrophobicity (Sekharudu & Sundaralingam, 1988), although other helic factors also enhance affinity [*e.g.*, increased NH_2-terminal helix length and nature of its cap residue (Reid et al., 1981; Shaw et al., 1991; Trigo-Gonzalez et al., 1993) and the presence of negative charges on carboxyl groups near the loop (Linse et al., 1988)]. In our studies, the presence of either helix 7 or 8 from cTnC's fourth EF-hand reduces Ca^{2+} affinity, in spite of the fact that cTnC's fourth EF-hand has overall higher Ca^{2+} affinity than that of CaM (George et al., 1993). This may occur because substitution of helix 7 or helix 8 may disrupt the interactions that are known to occur between these helices and helix 6 and helix 5 of the third EF-hand, respectively (Strynadka & James, 1989).

Regardless of their precise nature, the affinity-enhancing structural feature(s) in cTnC loop 4 promote Ca^{2+} binding through a 5-fold more rapid Ca^{2+} on rate, relative to CaM loop 4. In contrast, the reduced Ca^{2+} affinity of CaM (helix 7 cTnC) and CaM (helix 8 cTnC) follows from 3−4-fold increases in the Ca^{2+} dissociation rate relative to CaM. These findings are consistent with the results obtained by proteolysis of parvalbumin, in which deletion of hydrophobic residues from the carboxy-terminal helix resulted in a marked acceleration in Ca^{2+} dissociation rate and a decrease in Ca^{2+} affinity (Corson et al., 1986).

The Hill coefficient is a useful, although nonquantitative estimate of cooperative interactions that influence multiple ligand binding in a macropmolecule (Forsen & Linse, 1995). Calcium binding to the carboxy-terminal domain of CaM and cTnC is highly cooperative, with Hill coefficients approaching the theoretical maximum of 2 (Holroyde et al., 1980; Linse et al., 1991; George et al., 1993). In an earlier study, we found that cooperativity was fully retained in half-CaM, half-cTnC chimeras but significantly impaired in all chimeras in which EF-hand 3 and 4 were derived from different sources [i.e., CaM (3 cTnC), CaM)4 cTnC), cTnC (3 CaM), and cTnC (4 CaM) (George et al., 1993)]. This result suggested that essential interactions between the third and fourth EF-hands responsible for cooperative Ca^{2+} binding were not reproduced in single-EF-hand chimeras, e.g., in CaM (4 cTnC). The helix and loop chimeras in this study implicate loop 4 as a significant source of such cooperative interactions. Loop−loop interactions may be critical for cooperativity, since the two loops of an EF-hand pair interact extensively to form a β-sheet structure in the Ca^{2+}-bound state (Strynadka & James, 1989). It was possible that the loss of such interactions might account for the reduced Hill coefficients of CaM (4 cTnC) and CaM (loop 4 cTnC). However, CaM (loop 3, loop 4 cTnC) also failed to exhibit evidence of cooperative Ca^{2+} binding, showing that loop−loop interactions are not in themselves sufficient for cooperativity. For CaM and the two helix chimeras, the Hill coefficient modestly exceeded the theoretical maximum of 2, which we believe reflects the imperfect relationship between Hill coefficients and true quantitation of cooperativity (Forsen & Linse, 1995).

Although all three components of cTnC's fourth EF-hand contribute to CaM (4 cTnC)'s impaired ability to activate smMLCK and nNOS, the detrimental impact of helix 7 is most apparent. Helix 7 residues interact extensively with the first five amino-terminal residues of peptides based on various CaM binding domains. Structural studies show that helices 7 and 8 form one end of a hydrophobic tunnel surrounding the CaM-binding peptides (Meador et al., 1992, 1993; Ikura et al., 1992). The limited ability of CaM (helix 7 cTnC) to activate the enzymes suggests that the cTnC helix 7 disturbs the structure of this important functional domain.

In the point mutation study of helix 7, mutations of Met 124 (Leu), Glu 127 (Asp), and Ala 128 (Gly) resulted in significant detrimental effects on smMLCK and nNOS activation. Met 124 plays a central role in forming the CaM's carboxy-terminal hydrophobic pocket, an essential structure for drug and enzyme binding. Met 124 interacts closely with Trp 5 of the smMLCK CaM-binding domain peptide (RS20) (Meador et al., 1992); replacement of Met 124 with the shorter and less flexible Leu side chain may significantly impair this critical interaction [cf. Bagchi et al.

(1992)]. In the CaM binding domain of nNOS, Phe is present in place of Trp (Vorherr et al., 1993; Zhang & Vogel, 1994); our data would suggest that interaction of Met 124 with Phe is also quite significant for nNOS activation. Like Met 124, Ala 128 interacts with Trp 5 of the RS20 peptide (Meador et al., 1992), an observation that may explain the modest detrimental effect of the Ala 128 to Gly mutation. The conservative Glu 127 to Asp mutation also reduced activation of both enzymes. Glu 127 forms an important salt bridge with Lys 4 of the RS20 and CaM kinase II peptides and Arg 3 of the skeletal muscle MLCK peptide (Meador et al., 1992, 1993; Ikura et al., 1992); it may be that the shorter Asp side chain substitutes poorly in this interaction. Interestingly, a human calmodulin-like protein with a Glu 127 to Ala mutation was a poor activator of most CaM target enzymes (Edman et al., 1994) and could not support growth in CaM-deficient yeast (Harris et al., 1995). Mutagenesis studies showed that restoration of Glu 127 was critical for promoting yeast growth. Thus, the interactions of Glu 127 are essential for at least some intracellular signaling functions of CaM.

The loop 4 and helix 8 substitutions reduce smMLCK and nNOS activation by about 20%, a considerably more modest effect than the helix 7 substitution. No loop 4 residues have discernable interactions with CaM binding peptides, so the detrimental effect of loop 4 substitution must lie elsewhere. It is possible that the substitution of Lys for Ile in position 130, or Asp for Glu in position 139, may contribute; both are solvent-exposed side chains not directly involved in Ca coordination (Strynadka & James, 1989) and thus are available for activating interactions with target enzymes. In the case of helix 8, Met 144 has important interactions with Trp 5 and Gln 6 of the RS20 peptide; these interactions may be weakened when the less flexible Phe side chain is present.

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BI960495Y