Structural Determinants of Ca^{2+} Exchange and Affinity in the C Terminal of Cardiac Troponin C†

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Received June 19, 1998; Revised Manuscript Received August 6, 1998

ABSTRACT: The C terminal of cardiac troponin C (TnC) has two Ca^{2+}−Mg^{2+} sites which exhibit ∼20-fold higher Ca^{2+} affinity than the two C-terminal Ca^{2+} specific sites in calmodulin (CaM). Substitution of the third EF-hand of TnC for the corresponding EF-hand of CaM produced a mutant (CaM[3TnC]) with a 10-fold higher C-terminal Ca^{2+} and Mg^{2+} affinity. Substitution of loop 3 of TnC for loop 3 of CaM produced a mutant (CaM[loop3TnC]) with a 10-fold faster Ca^{2+} on rate and a 5-fold faster Ca^{2+} off rate than CaM. A mutant CaM (CaM[loop3*X*,*Z*]) which contained the identical coordinating amino acids and *X* and *Z* acid pairs of TnC loop 3 had a 3-fold higher C-terminal Ca^{2+} affinity without the increased Ca^{2+} exchange rates exhibited by CaM[loop3TnC]. Thus, loop factors other than the acid pairs must be responsible for the rapid Ca^{2+} exchange rates of CaM[loop3TnC]. Helix 6 and helix 5 in the third EF-hand of TnC support the rapid Ca^{2+} on rate of TnC's loop 3 and produce an ∼4-fold reduction in its Ca^{2+} off rate, explaining the high Ca^{2+} affinity of the third EF-hand of TnC. Exchanging loop 3 or helix 5 of TnC into CaM increased the Mg^{2+} affinity by decreasing the Mg^{2+} off rate. Our results are consistent with the high Ca^{2+} and Mg^{2+} affinity of the third EF-hand of TnC resulting from the two (*X* and *Z*) acid pairs in loop 3, coupled with the greater hydrophobicity of helix 6 and helix 5 compared to that of the third EF-hand of CaM.

Cardiac troponin C (TnC)[1] and calmodulin (CaM) are ∼51% homologous in amino acid sequence and consist of N- and C-terminal globular lobes connected by a long central helix (*1*, *2*). The C-terminal half of each protein contains two EF-hands which have dramatically different affinities and specificities for Ca^{2+}. The C-terminal domain of TnC contains two Ca−Mg sites which bind Ca^{2+} with high affinity (∼1×10^{-7} M) and Mg^{2+} with lower affinity (∼1×10^{-3} M) (*3*). The C-terminal EF-hands of CaM are Ca^{2+}-specific (at physiological Mg^{2+} concentrations) and have an ∼20-fold lower Ca^{2+} affinity and an ∼10-fold lower Mg^{2+} affinity than the C-terminal sites of TnC (*4*−*6*). These differences in C-terminal Ca^{2+} affinity and specificity between TnC and CaM allow TnC to remain anchored to the thin filament at resting Ca^{2+} and Mg^{2+} concentrations (*7*, *8*), while CaM is thought to exist as a soluble cytosolic protein.

The EF-hand motif, consisting of a helix−loop−helix structure, was first described by Kretsinger and Nickolds (*9*) as the liganding structure for Ca^{2+} and Mg^{2+} in parvalbumin. Since that time, hundreds of EF-hands have been identified in numerous Ca^{2+} binding proteins, and many theories have been advanced to correlate EF-hand structure to cation affinity and specificity (for reviews, see refs *10*−*14*). The factors controlling cation affinity in a particular EF-hand remain quite controversial, but appear to involve both helix and loop factors.

Reid and co-workers (*15*, *16*) and Marsden et al. (*17*) have suggested that the number and location of acidic amino acid residues in chelating positions (*X*, *Y*, *Z*, −*Y*, −*X*, and −*Z*) of the Ca^{2+} binding loop play a primary role in determining Ca^{2+} affinity. Studies using synthetic EF-hand peptides, modeled after the third EF-hand of CaM, have shown that increasing the number of acid pairs (acidic side chains paired on the vertices of the regular octahedral arrangement of liganding atoms) from zero to one and from one to two increased the Ca^{2+} affinity (*18*). Synthetic EF-hands which contained a *Z*-axis acid pair exhibitied Mg^{2+} binding (*19*). Wu and Reid (*20*) made a mutant CaM in which they blocked Ca^{2+} binding to the fourth EF-hand of CaM which caused a 24-fold decrease in the Ca^{2+} affinity for the third EF-hand. They then increased the number of acid pairs in the third EF hand from zero to two, producing an ∼160-fold increase in its Ca^{2+} affinity. Thus, studies with model peptides and with mutant CaMs indicate that increasing the number of

† 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. is an Established Investigator of the American Heart Association.

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; CaM[3TnC], CaM with the third EF-hand of TnC; CaM[loop3TnC], CaM with the third Ca^{2+} binding loop of TnC; CaM[helix5TnC], CaM with helix 5 of TnC; CaM[helix6TnC], CaM with helix 6 of TnC; CaM[loop3*X*,*Z*], CaM with the D95N, N97D, and S101D mutations in its third Ca^{2+} binding loop; TnC[3CaM], TnC with the third EF-hand of CaM; TnC-[4CaM], TnC with the fourth EF-hand of CaM; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

S0006-2960(98)01464-0 CCC: $15.00
Published on Web 09/25/1998

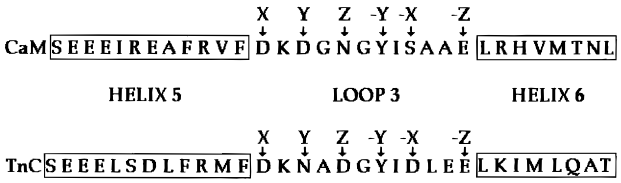

FIGURE 1: Comparison of the amino acid sequence of the third EF-hand of CaM and TnC. The helices surrounding each Ca^{2+} binding loop are boxed, and the coordinating amino acids are labeled *X*, *Y*, *Z*, $-Y$, $-X$, and $-Z$.

acid pairs in an EF-hand can dramatically increase Ca^{2+} affinity. In addition to the number of acid pairs, numerous studies have indicated that the hydrophobicity of the α-helices in the EF-hand can have dramatic effects on Ca^{2+} affinity (*10*).

By exchanging entire EF-hand motifs or EF-hand components (helix, loop, or helix) between the C-terminal EF-hands of TnC and CaM, we have attempted to define the structural requirements and mechanisms which give the C-terminal Ca^{2+} binding sites of TnC a 20-fold higher Ca^{2+} affinity. In Figure 1, the amino acid sequences of the third EF-hand of CaM and TnC are compared.

George et al. (*6*) have shown that when both the third and fourth EF-hands of TnC were substituted into CaM (CaM-[3,4TnC]), Ca^{2+} binding to the C-terminal half of this chimeric protein was equal to that observed in TnC. Furthermore, substitution of the third EF-hand of TnC into CaM (CaM[3TnC]) produced an ∼10-fold increase in C-terminal Ca^{2+} affinity, suggesting that it was primarily responsible for the higher C-terminal Ca^{2+} affinity of TnC (*6*). In this paper, we substitute the entire third EF-hand of TnC or its individual components (helix 5, helix 6, or Ca^{2+} binding loop 3) into CaM. We examine the effect of these substitutions on Ca^{2+} and Mg^{2+} affinity and exchange rates with the C-terminal domain of these proteins. We find that loop 3 of TnC when substituted into CaM allows a faster rate of Ca^{2+} exchange but a slower rate of Mg^{2+} exchange. The increase in the number of acid pairs appears to be responsible for the reduced Mg^{2+} off rate but not for the increased Ca^{2+} off rate observed in CaM[loop3TnC]. The helices which surround this loop in TnC serve to increase both Ca^{2+} and Mg^{2+} affinity.

MATERIALS AND METHODS

Construction, expression, purification, and sequence analysis of chicken CaM, TnC, and their chimeras were carried out as previously described (*6*, *21*, *22*). CaM-D95N/N97D/S101D (CaM[loop3*X*,*Z*]) was constructed from rat wild-type CaM plasmid using primer-selected site-directed mutagenesis following the protocol provided by Stratagene's (La Jolla, CA) QuikChange Site-Directed Muatagenesis Kit. DNA sequence analysis confirmed the sequence of the CaM-[loop3*X*,*Z*] mutant. The purified CaM, TnC, or their chimeric proteins were all dialyzed against two exchanges of 4 L of 10 mM MOPS (pH 7.0) and 90 mM KCL at 4 °C. Fluorescence titrations and spectra were recorded on a Perkin-Elmer LS5 spectrofluorometer at 22 °C by following Tyr fluorescence with the excitation wavelength at 275 nm and emission wavelength at 305 nm. Ca^{2+} titrations of CaM, TnC, and their chimeric proteins were conducted by addition of microliter amounts of $CaCl_2$ to 1 mL of buffer containing

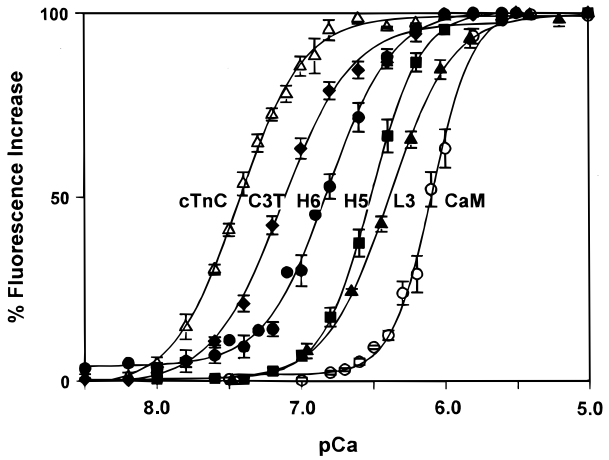

FIGURE 2: Ca^{2+}-induced increase in CaM, TnC, CaM[3TnC], CaM-[helix5TnC], CaM[loop3TnC], or CaM[helix6TnC] tyrosine fluorescence. Increasing concentrations of Ca^{2+} were added to a 1 mL solution of 1 μM CaM, TnC, CaM[3TnC] (C3T curve), CaM-[helix5TnC] (H5 curve), CaM[loop3TnC] (L3 curve), or CaM-[helix6TnC] (H6 curve) in a buffer of 200 mM MOPS, 90 mM KCl, and 2 mM EGTA (pH 7.0). The pCa was determined as described in Materials and Methods. One hundred percent fluorescence corresponds to a 2.8-, 1.2-, 1.7-, 1.8-, 2.6-, or 1.3-fold fluorescence increase for CaM, TnC, CaM[3TnC], CaM[helix5TnC], CaM[loop3TnC], or CaM[helix6TnC], respectively. Excitation was at 275 nm, and emission was monitored at 305 nm at 22 °C. Each data point represents the average ± standard error of three titrations.

200 mM MOPS (to prevent pH changes upon addition of Ca^{2+}), 90 mM KCl, and 2 mM EGTA. The free Ca^{2+} concentration was calculated and calibrated as previously described (*23*). Quin 2 was obtained from Molecular Probes (Eugene, OR), and EGTA was obtained from Sigma Chemical Co. (St. Louis, MO). Rates of Ca^{2+} dissociation from CaM, TnC, and their chimeric proteins were determined at 22 °C using an Applied Photophysics model SF-17 MV stopped-flow instrument with a mixing time of 1.6 ms. The Quin signal was calibrated and converted into moles of Ca^{2+} dissociating per mole of protein as previously described (*24*). Protein concentrations were determined by the Bradford method (*25*) using γ-globulin as the standard or by UV absorbance.

Changes in terbium (Tb) luminescence can be used to determine the rates of Ca^{2+} and Mg^{2+} dissociation from proteins (*26*). When Ca^{2+} is rapidly mixed with a protein with bound Ca^{2+} or Mg^{2+}, Tb fluorescence increases at the rate of cation dissociation. The rate of Mg^{2+} dissociation from our chimeric proteins was determined as described in the legend of Figure 6.

RESULTS

Effect of Substituting the Third EF-Hand of TnC or Its Subcomponents (Helix 5, Loop 3, or Helix 6) into CaM on C-Terminal Ca^{2+} Affinity and Ca^{2+} Exchange Rates. CaM and TnC have two tyrosine residues at the corresponding position of their third (Tyr99 in CaM and Tyr111 in TnC) and fourth (Tyr138 in CaM and Tyr150 in TnC) EF-hands. We and others have shown that Ca^{2+} binding to the C-terminal sites of TnC and CaM can be accurately and specifically followed by increases in their tyrosine fluorescence (*6*, *24*). Figure 2 shows the Ca^{2+}-dependent increase in tyrosine fluorescence that occurs with Ca^{2+} binding to the C terminal of TnC, CaM, and their chimeric proteins.

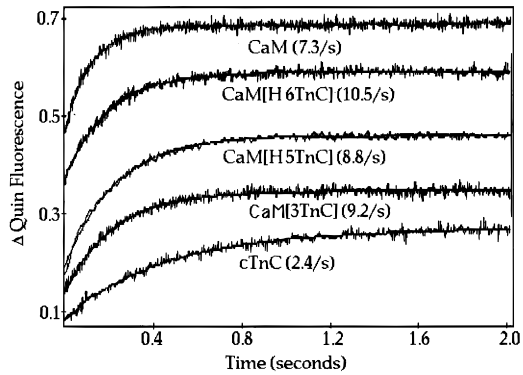

FIGURE 3: Rates of Ca^{2+} dissociation from the C-terminal Ca^{2+} binding sites of CaM, TnC, CaM[3TnC], CaM[helix5TnC], and CaM[helix6TnC] using Quin 2 fluorescence. CaM, TnC, CaM-[3TnC], CaM[helix5TnC], CaM[loop3TnC], or CaM[helix6TnC] (4 μM) in 10 mM MOPS, 90 mM KCl, and 60 μM Ca^{2+} (pH 7.0) was rapidly mixed with an equal volume of Quin 2 (150 μM) in 10 mM MOPS and 90 mM KCl (pH 7.0) at 22 °C. Quin 2 fluorescence was monitored through 510 nm a broad band-pass interference filter with excitation at 330 nm. Each trace represents an average of five to seven traces, and the data were fit with a single-exponential equation (variance $< 1.1 \times 10^{-4}$). For the EGTA-induced decrease in protein tyrosine fluorescence, each protein at 4 μM in 10 mM MOPS, 90 mM KCl, and 60 μM Ca^{2+} (pH 7.0) was mixed with an equal volume of EGTA (10 mM) in 10 mM MOPS and 90 mM KCl (pH 7.0) at 22 °C.

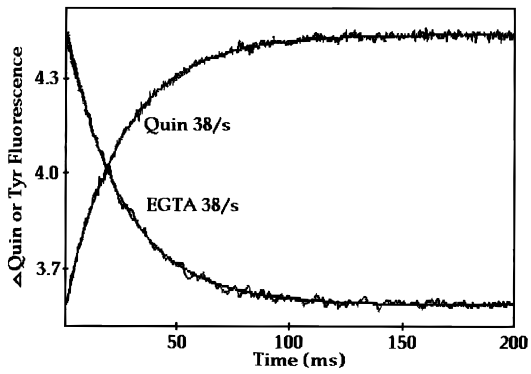

FIGURE 4: Rates of Ca^{2+} dissociation from CaM[loop3TnC] using Quin 2 or EGTA. The experimental conditions and protein concentration for Quin and tyrosine fluorescence were the same as those described in the legend of Figure 3. Each trace represents an average of five to seven traces, and the data were fit with a single-exponential equation (variance $< 2.3 \times 10^{-4}$).

Table 1: Ca^{2+} Affinity and Rates of Association and Dissociation from the C-Terminal Domain of CaM, cTnC, and CaM−TnC Chimeras

protein	K_d (M)	K_{off}[a] (s^{-1})	K_{on} (M^{-1} s^{-1})
CaM	7.9×10^{-7}	7.3	9.2×10^{6}
CaM[3TnC]	7.9×10^{-8}	9.2	1.2×10^{8}
CaM[helix5TnC]	3.2×10^{-7}	8.8	2.8×10^{7}
CaM[loop3TnC]	4.1×10^{-7}	38	9.3×10^{7}
CaM[loop3*X*,*Z*]	2.6×10^{-7}	6.2	2.4×10^{7}
CaM[helix6TnC]	1.6×10^{-7}	10.5	6.6×10^{7}
TnC	4.0×10^{-8}	2.4	6.0×10^{7}

[a] Ca^{2+} off rates were determined with Quin 2 fluorescence.

Cardiac TnC binds Ca^{2+} half-maximally at pCa 7.4, while CaM binds Ca^{2+} with an ~20-fold lower affinity (half-maximal at pCa 6.1). When the third EF-hand of CaM was replaced with the third EF-hand of TnC to produce CaM-[3TnC], it exhibited a half-maximal increase in tyrosine fluorescence at pCa 7.1, indicating a 10-fold increase in CaM's C-terminal Ca^{2+} affinity. Thus, the third EF-hand of TnC plays a predominant role in maintaining high-affinity Ca^{2+} binding to the C-terminal Ca^{2+} binding sites of TnC, in agreement with the results of George et al. (*6*).

To determine which subcomponents of TnC's third EF-hand were responsible for its high Ca^{2+} affinity, helix 5, loop 3, or helix 6 of TnC was substituted for the corresponding region of CaM's third EF-hand, producing CaM[helix5TnC], CaM[loop3TnC], and CaM[helix6TnC], respectively. Ca^{2+} concentration-dependent increases in tyrosine fluorescence for CaM[helix6TnC], CaM[helix5TnC], and CaM[loop3TnC] occurred half-maximally at pCa 6.8, 6.5, and 6.4, indicating a 4.9-, 2.5-, and 1.9-fold increase in Ca^{2+} affinity relative to that of CaM, respectively (Figure 2). Thus, while all subdomains of the third EF-hand of TnC contribute to its high Ca^{2+} affinity, helix 6 produces the most dramatic increase.

Ca^{2+} Binding Kinetics. Fluorescence stopped-flow measurements were conducted using Quin 2 and intrinsic tyrosine fluorescence to determine the rates of Ca^{2+} dissociation from the C-terminal sites of these proteins. Quin 2 dissociated ~2 mol of Ca^{2+} from the C-terminal sites of CaM, TnC, CaM[3TnC], CaM[helix6TnC], and CaM[helix5TnC] at rates of 7.3, 2.4, 9.2, l0.5, and 8.8 s^{-1}, respectively (Figure 3). EGTA dissociated Ca^{2+} from the C-terminal sites of CaM, TnC, CaM[3TnC], CaM[helix6TnC], and CaM[helix5TnC] and produced decreases in tyrosine fluorescence at rates of 7.4, 2.4, 11.4, 10.4, and 7.2 s^{-1}, respectively (data not shown). Thus, both methods yield nearly identical rates for Ca dissociation from the C-terminal domains of these proteins. Clearly, substitution of CaM's entire third EF-hand or of helix 6 or helix 5 with the corresponding region from TnC had little impact on Ca^{2+} dissociation rates.

The effect of substitution of loop 3 of TnC into CaM was far more striking than that of any other mutation. Ca^{2+} dissociated from the C-terminal domain of CaM[loop3TnC] at the rate of 38 s^{-1} as monitored by the decrease in tyrosine fluorescence and the increase in Quin 2 fluorescence (Figure 4). Thus, substitution of TnC loop 3 alone produced a dramatic ~5.2-fold increase in the rate of Ca^{2+} dissociation relative to that of CaM.

Knowing the K_d of Ca^{2+} for the C-terminal sites of these proteins from the Ca^{2+} titration of tyrosine fluorescence (Figure 1) and the rates of Ca^{2+} dissociation (K_{off}) from the stopped-flow studies above, we calculated the rates of Ca^{2+} association (Ca^{2+} on rate) with the C-terminal Ca^{2+} binding sites of these proteins. In Table 1, the Ca^{2+} affinity (K_d), on rate, and off rate for each of these proteins are compared. These data show the following.

(1) The Ca^{2+} affinity for the C-terminal domain of TnC is ~20 times higher than that of CaM, yet the Ca^{2+} off rate is only 3 times slower. Therefore, the Ca^{2+} on rate for the C-terminal domain of TnC is calculated to be ~6.5 times faster than that of CaM.

(2) The C-terminal Ca^{2+} affinity of CaM[3TnC] is 10.0 times higher than that of CaM, due solely to its 13.0-fold faster Ca^{2+} on rate.

(3) Putting helix 6 or helix 5 of TnC into CaM produced 4.9- and 2.5-fold increases in C-terminal Ca^{2+} affinity, which were due primarily to 7.2- and 3.0-fold increases in their Ca^{2+} on rates, respectively.

(4) When loop 3 of TnC was put into the loop 3 environment of CaM, the rates of Ca^{2+} exchange were dramatically enhanced. The Ca^{2+} on rate of CaM[loop3TnC] increased ~10-fold and the Ca^{2+} off rate increased ~5.2-fold, compared to those of CaM.

(5) Although loop 3 of TnC markedly accelerated Ca^{2+} exchange rates, it had little impact on Ca^{2+} affinity (1.9-fold increase) because it increased Ca^{2+} on and off rates to a similar extent.

(6) Substitution of helix 6 and helix 5 of TnC into CaM supported the increase in Ca^{2+} on rate; moreover, when combined with loop 3 of TnC (CaM[3TnC]), they further increased Ca^{2+} affinity by slowing the rapid Ca^{2+} off rate observed with loop 3 alone.

Effect of Increasing the Number of Acid Pairs in Loop 3 of CaM. Loop 3 of TnC has an *X* and *Z*, acid pair while loop 3 of CaM has no acid pairs. Our results with CaM and CaM[loop3TnC] could be explained if increasing the number of acid pairs in a Ca^{2+} binding loop from zero to two produced the 10-fold increase in on rate and the 5.2-fold increase in off rate observed with CaM[loop3TnC]. To test this, we made a CaM mutant (CaM[loop3*X*,*Z*], with D95N, N97D, and S101D substitutions) which had an *X* and *Z* acid pair and the identical coordinating amino acids at the *X* and $-X$, *Y* and $-Y$, and *Z* and $-Z$ positions as CaM-[loop3TnC]. The half-maximal increase in tyrosine fluorescence was shifted from pCa 6.1 for CaM to pCa 6.6 for CaM[loop3*X*,*Z*], indicating a 3-fold increase in C-terminal Ca^{2+} affinity (Table 1). Interestingly, CaM[loop3*X*,*Z*] shows an ~4-fold slower Ca^{2+} on rate and a 6-fold slower Ca^{2+} off rate than CaM[loop3TnC] (Table 1). Thus, increasing the number of acid pairs is not responsible for the increased Ca^{2+} on and off rates exhibited by CaM[loop3TnC]. This suggests that noncoordinating amino acids within the Ca^{2+} binding loop must be responsible for these dramatic effects on Ca^{2+} exchange.

Role of TnC's Third EF-Hand in Mg^{2+} Binding. Because the third EF-hand of TnC played a predominate role in maintaining high C-terminal Ca^{2+} affinity, we used these chimeric proteins to determine its role in facilitating Mg^{2+} binding. Mg^{2+} binding to the C terminal of TnC can be followed by its ability to increase C-terminal tyrosine fluorescence. Mg^{2+} produced half-maximal increases in TnC, CaM[3TnC], CaM[loop3TnC], CaM[helix5TnC], and CaM tyrosine fluorescence at 0.5, 0.34, 0.8, 1.3, and 3.1 mM, respectively (Figure 5). CaM[helix6TnC] showed no increase in tyrosine fluorescence, even at Mg^{2+} concentrations of ≥5 mM (data not shown). TnC[4CaM], like CaM[3TnC], contains the third EF-hand of TnC and the fourth EF-hand of CaM, and it exhibited the same (half-maximal binding at 0.3 mM Mg^{2+}) Mg^{2+}-induced increases in tyrosine fluorescence as CaM[3TnC] (data not shown). These data show that chimeras containing the entire third EF-hand of TnC have a relatively high Mg^{2+} affinity. This effect it appears can be largely attributed to the acid pairs in loop 3 since the incorporation of an *X* and *Z* acid pair in loop 3 of CaM (CaM[loop3*X*,*Z*]) produced ~66% of the increase in Mg affinity exhibited by CaM[loop3TnC] (Table 2). Incorporation of helix 5 of TnC into CaM increased Mg^{2+} affinity (~2.4-fold), but no Mg^{2+}-dependent increase in C-terminal tyrosine fluorescence was observed with CaM[helix6TnC].

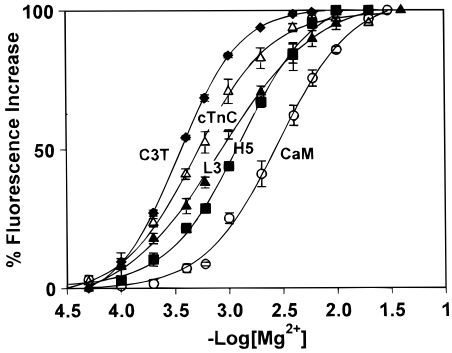

FIGURE 5: Mg^{2+} titrations of CaM, TnC, CaM[3TnC], CaM-[helix5TnC], and CaM[loop3TnC] tyrosine fluorescence. Increasing concentrations of Mg^{2+} were added to a 1 mL solution of 1 μM CaM, TnC, CaM[3TnC] (C3T curve), CaM[helix5TnC] (H5 curve), or CaM[loop3TnC] (L3 curve) in a buffer of 10 mM MOPS, 90 mM KCl, and 0.2 mM EGTA (pH 7.0). One hundred percent fluorescence corresponds to a 1.35-, 1.2-, 1.6-, 1.3-, or 1.7-fold fluorescence increase for CaM, TnC, CaM[3TnC], CaM[helix5TnC], or CaM[loop3TnC], respectively. Excitation was at 275 nm, and emission was monitored at 305 nm at 22 °C. Each data point represents the average ± standard error of three titrations.

Table 2: Mg^{2+} Affinity and Rates of Association and Dissociation from the C-Terminal Domain of CaM, cTnC, and CaM−cTnC Chimeras

protein	K_d (M)	K_{off}[a] (s^{-1})	K_{on} (M^{-1} s^{-1})
CaM	3.1×10^{-3}	158	5.1×10^4
CaM[3TnC]	3.4×10^{-4}	14	4.7×10^4
CaM[helix5TnC]	1.3×10^{-3}	21	1.6×10^4
CaM[loop3TnC]	8.0×10^{-4}	19	4.6×10^4
CaM[loop3*X*,*Z*]	1.2×10^{-3}	26	2.2×10^4
CaM[helix6TnC]	not observed	not observed	not observed
TnC	5.0×10^{-4}	20	4×10^4

[a] Mg^{2+} off rates were determined with Tb^{3+} fluorescence.

In contrast, the fourth EF-hand of TnC had relatively little impact on Mg^{2+} affinity. Mg^{2+} produced a half-maximal increase in CaM[4TnC] tyrosine fluorescence at 6.3 mM, and no increase in tyrosine fluorescence of TnC[3CaM] was observed even at 5 mM Mg^{2+}. Since both chimeras contain the third EF-hand of CaM and the fourth EF-hand of TnC, these data show that at most, TnC's fourth EF-hand minimally enhances Mg^{2+} affinity. Viewed as a whole, the Mg^{2+} tyrosine fluorescence studies suggest that the third EF-hand of TnC is primarily responsible for Mg^{2+} binding to the C terminal of TnC and that substitution of the third EF-hand of TnC, its loop 3, or its helix 5 into CaM facilitates higher-affinity Mg^{2+} binding.

Rates of Mg^{2+} Dissociation from CaM−TnC Chimeras. The rates of Mg^{2+} dissociation from proteins can be followed by the rate of increase in terbium (Tb) fluorescence when Tb is mixed with the Mg^{2+}−protein complex (*26*). Using this method, we found that Mg^{2+} dissociates from TnC at 20 s^{-1} and from TnC[4CaM] at 13 s^{-1}. The third EF-hand of CaM was engineered into TnC, and this chimeric protein (TnC[3CaM]) exhibited Tb binding during the mixing time of the instrument. This suggests either that Mg^{2+} does not bind to this protein or that it binds with such low affinity that its dissociation is too rapid to be observed. These studies suggest that the third EF-hand of TnC is sufficient for Mg^{2+} binding, and when it is replaced by the third EF-hand of CaM, Mg^{2+} no longer binds with physiologically relevant affinity.

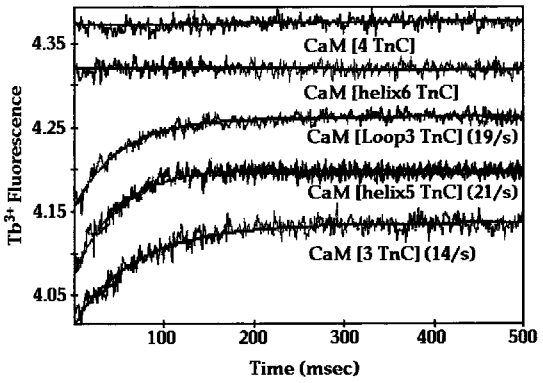

FIGURE 6: Rates of Mg^{2+} dissociation from CaM[3TnC], CaM-[helix5TnC], CaM[loop3TnC], CaM[helix6TnC], and CaM[4TnC] using terbium fluorescence. Each protein (2 μM) was saturated with 20 mM Mg^{2+} in 10 mM MOPS, 90 mM KCl, and 500 μM EGTA (pH 7.0) and the solution rapidly mixed with an equal volume of Tb (520 μM) in the same buffer without EGTA at 22 °C. Tb fluorescence was monitored through a 500 nm broad band-pass interference filter with excitation at 275 nm. Each trace represents an average of five to seven traces fit with a single-exponential equation or linear regression (variance $< 1.1 \times 10^{-4}$).

Consistent with its low C-terminal Mg^{2+} affinity, Mg^{2+} dissociates from CaM at $\sim$158 s^{-1} using Tb fluorescence (Table 2) and at $\sim$140 s^{-1} using the EDTA-induced decrease in Mg^{2+}−CaM tyrosine fluorescence (data not shown). Figure 6 shows Mg^{2+} dissociation from CaM chimeras which contain TnC's third EF-hand (CaM[3TnC]), TnC's fourth EF-hand (CaM[4TnC]), or components of TnC's third EF-hand (CaM[loop3TnC], CaM[helix6TnC], or CaM-[helix5TnC]). Substitution of the third EF-hand of TnC into CaM increases Mg^{2+} affinity and results in an $\sim$11-fold slower rate of Mg^{2+} dissociation from CaM[3TnC] at 14 s^{-1}. Thus, Mg^{2+} dissociates from TnC[4CaM] at essentially the same rate as CaM[3TnC]. Substitution of helix 5 or loop 3 of TnC into CaM also facilitates Mg^{2+} binding; we observed Tb-induced Mg^{2+} dissociation from these proteins at 21 and 19 s^{-1}, respectively. The acid pairs of TnC's loop 3 are apparently responsible for slowing Mg^{2+} dissociation since Mg^{2+} dissociates from CaM[loop3*X*,*Z*] at 26 s^{-1}, $\sim$6 times slower than Mg^{2+} dissociation from CaM (Table 2). These kinetic results are consistent with the Mg^{2+}-induced increases in tyrosine fluorescence in Figure 5 and suggest that the third (but not the fourth) EF-hand of TnC can increase Mg^{2+} binding to CaM. Furthermore, loop 3 and helix 5 of the third EF-hand of TnC are responsible for the increase in Mg^{2+} affinity.

DISCUSSION

Ca^{2+} or Mg^{2+} binding to the C-terminal domain of TnC allows it to bind the thin filament, presumably through an interaction with troponin I. When TnC is anchored to the thin filament by its C-terminal domain, the rapid association of Ca^{2+} with the N-terminal Ca^{2+}-specific regulatory site(s) of skeletal or cardiac TnC initiates striated muscle contraction (*27*−*30*).

Previous studies have shown that Ca^{2+} binding to the third EF-hand of TnC is primarily responsible for anchoring TnC to the thin filament (*8*, *31*) and results in structural changes larger than those associated with Ca^{2+} binding to the fourth EF-hand (*32*, *33*). Consistent with this, our studies indicate that the third EF-hand of TnC is primarily responsible for the higher Ca^{2+} and Mg^{2+} affinity of the C-terminal domain of TnC relative to that of CaM. Thus, the third EF-hand of TnC appears to play a major role in maintaining TnC in the Ca^{2+}- or Mg^{2+}-dependent tertiary structure necessary for its thin filament interaction.

To determine which subcomponents of the third EF-hand of TnC were responsible for the 10-fold increase in Ca^{2+} affinity observed in CaM[3TnC], helix 6, helix 5, or loop 3 of the third EF-hand of TnC was engineered into the corresponding positions of CaM. CaM[helix6TnC], CaM-[helix5TnC], and CaM[loop3TnC] increased the C-terminal Ca^{2+} affinity 4.9-, 2.5-, and 1.9-fold, respectively. The additive affect of these substitutions resulted in a 9.3-fold increase in C-terminal Ca^{2+} affinity, similar to the 10-fold increase resulting from substitution of the entire third EF-hand of TnC into CaM. The increases in Ca^{2+} affinity induced by helix 6 and helix 5 of TnC may result from their greater hydrophobicity relative to the corresponding helices of CaM. Helix 6 of TnC is 4.3 times more hydrophobic than helix 6 of CaM (hydrophobicity index = 7.7 and 1.8 kcal/mol, respectively); similarly, helix 5 of TnC is 1.5 times more hydrophobic than helix 5 of CaM (−5.0 and −7.7 kcal/mol, respectively). Similar direct relationships between helical hydrophobicity and Ca^{2+} affinity have been observed by others; for example, Corson et al. (*34*) found that the proteolytic removal of hydrophobic amino acids from the C-terminal α-helix of parvalbumin decreased both helical hydrophobicity and Ca^{2+} affinity. In addition, two-dimensional NMR studies have shown that Ca^{2+} binding to the third EF-hand forms a stable hydrophobic cluster in the C-terminal domain of TnC (*33*). Helices 5 and 6 of TnC presumably stabilize this hydrophobic core more than the less hydrophobic CaM helices, thereby promoting increased Ca^{2+} affinity.

The subcomponents of TnC's third EF-hand, helix 5, loop 3, and helix 6, have significant effects on both C-terminal Ca^{2+} affinity and Ca^{2+} on and off rates. Placing the entire third EF-hand of TnC into CaM produced a 10-fold increase in Ca^{2+} affinity, which could be attributed to a 13-fold increase in Ca^{2+} on rate and a slight ($\sim$1.3-fold) increase in off rate. Thus, the enhanced Ca^{2+} affinity of the third EF-hand of TnC follows from its ability to rapidly bind Ca^{2+}. Loop 3 of TnC was partly responsible for this effect, since it enhanced the Ca^{2+} on rate by $\sim$10-fold when substituted for loop 3 in CaM. Since the loop 3 substitution also caused a 5.2-fold increase in Ca^{2+} off rate, it increased Ca^{2+} affinity only modestly (1.9-fold).

CaM[loop3TnC] and CaM[3TnC] both have the same loop 3, yet they exhibit very different Ca^{2+} exchange rates and affinities. Clearly, something other than the loop must be responsible for the increased Ca^{2+} affinity and slower Ca^{2+} off rate of CaM[3TnC]. Some insight into this interaction may be gained by comparing kinetic results; both CaM-[loop3TnC] and CaM[3 TnC] have rapid on rates (9.3×10^7 and 1.2×10^8 M^{-1} s^{-1}, respectively), but the Ca^{2+} off rate of CaM[3TnC] is 4-fold slower than that of CaM-[loop3TnC] (9.2 and 38 s^{-1}, respectively). Possibly, placing the more hydrophobic helix 5 and helix 6 of TnC around its loop 3 dramatically slows the Ca^{2+} off rate, resulting in the observed increase in Ca^{2+} affinity. Although these latter inferences await experimental confirmation, they are consistent with the observations of Corson et al. (*34*), who found

that proteolytic removal of hydrophobic residues from the C-terminal α-helix of parvalbumin enhanced the Ca^{2+} off rate. Thus, factors other than the Ca^{2+} binding loop can have dramatic effects on Ca^{2+} affinity.

The 10-fold increase in on rate and the 5.2-fold increase in off rate observed with CaM[loop3TnC] relative to those of CaM must reflect fundamental structural differences between loop 3 of TnC and loop 3 of CaM. An obvious difference is that TnC has *X* and *Z* acid pairs (aspartic acid residues at the *X* and $-X$ coordinates and aspartic and glutamic acid at the *Z* and $-Z$ positions, respectively), while loop 3 of CaM has three acidic amino acids but no acid pairs. Our CaM[loop3*X*,*Z*] mutant has an *X* and *Z* acid pair and the identical coordinating amino acids at the *X* and $-X$, *Y* and $-Y$, and *Z* and $-Z$ positions as CaM[loop3TnC], yet it exhibits an ~4-fold slower Ca^{2+} on rate and a 6-fold slower Ca^{2+} off rate than CaM[loop3TnC]. Thus, increasing the number of acid pairs is apparently not responsible for the increased Ca^{2+} on and off rates exhibited by CaM-[loop3TnC]. These studies suggest that noncoordinating residues (G96A, A102L, or A103E) within the Ca^{2+} binding loop can have dramatic effects on Ca^{2+} exchange.

Our CaM[loop3*X*,*Z*] mutant is similar to the 4*XZ*CaM mutant produced by Wu and Reid (*20*) except that ours has a functional fourth EF-hand. The modest 3-fold increase in Ca^{2+} affinity that we observe in CaM[loop3*X*,*Z*] relative to that of CaM, compared to the 160-fold increase observed with the CaM mutant of Wu and Reid, probably results from the fact that our mutant had a functional fourth EF-hand and a much higher initial C-terminal Ca^{2+} affinity. Thus, our results are consistent with those of Wu and Reid in that increasing the number of acid pairs does indeed increase Ca^{2+} affinity, even within a pair of EF-hands. However, the increase in Ca^{2+} on and off rates observed in CaM-[loop3TnC] must result from differences in the noncoordinating residues within the Ca^{2+} binding loop. Clearly, the nature of the Ca^{2+} binding loop and the noncoordinating amino acids within the loop can dramatically affect Ca^{2+} exchange.

In addition to its role in enhancing Ca^{2+} affinity, several lines of evidence suggest that the third EF-hand of TnC is also primarily responsible for Mg^{2+} binding to the C-terminal domain. Its substitution into CaM (CaM[3TnC]) produces a 9.1-fold increase in Mg^{2+} affinity. Substitution of the third EF-hand of CaM into TnC dramatically decreases its Mg^{2+} binding, and substitution of the fourth EF-hand of CaM into TnC produced no change in Mg^{2+} affinity. Thus, the third EF-hand of TnC appears to be largely responsible for its C-terminal Mg^{2+} binding.

Loop 3 and helix 5 were primarily responsible for Mg^{2+} binding, since their individual introduction into CaM nearly added to the full 9.1-fold increase in Mg^{2+} affinity exhibited by CaM[3TnC]. Loop 3 produced this increase in Mg^{2+} affinity by inducing an ~8-fold slower Mg^{2+} dissociation rate. CaM[loop3*X*,*Z*] increased Mg^{2+} affinity 2.6-fold, primarily by decreasing the Mg^{2+} dissociation rate ~6-fold. Thus, for CaM[loop3*X*,*Z*], increasing the number of acid pairs produced an ~3-fold increase in both Ca^{2+} and Mg^{2+} affinity.

Our studies, with a whole Ca^{2+} binding protein, clearly demonstrate that increasing the number of acid pairs in one EF-hand can increase the Ca^{2+} affinity of an EF-hand pair. In addition to the number of acid pairs within an EF-hand, the helices of the EF-hand and the nonchelating residues within the loop can also play a significant role in modulating Ca^{2+} and Mg^{2+} affinity and in producing dramatic changes in the rate of cation exchange.

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BI9814641