Mutation of the High Affinity Calcium Binding Sites in Cardiac Troponin C*

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Fast skeletal and cardiac troponin C (TnC) contain two high affinity Ca\(^{2+}\)/Mg\(^{2+}\) binding sites within the C-terminal domain that are thought to be important for association of TnC with the troponin complex of the thin filament. To test directly the function of these high affinity sites in cardiac TnC they were systematically altered by mutagenesis to generate proteins with a single inactive site III or IV (CBM-III and CBM-IV, respectively), or with both sites III and IV inactive (CBM-III-IV). Equilibrium dialysis indicated that the mutated sites did not bind Ca\(^{2+}\) at pCa 4. Both CBM-III and CBM-IV were similar to the wild type protein in their ability to regulate Ca\(^{2+}\)-dependent contraction in slow skeletal muscle fibers, and Ca\(^{2+}\)-dependent ATPase activity in fast skeletal and cardiac muscle myofibrils. The mutant CBM-III-IV is capable of regulating contraction in permeabilized slow muscle fibers but only if the fibers are maintained in a contraction solution containing a high concentration of the mutant protein. CBM-III-IV also regulates myofibril ATPase activity in fast skeletal and cardiac myofibrils but only at concentrations 10–100-fold greater than the normal protein. The pCa\(_{50}\) and Hill coefficient values for Ca\(^{2+}\)-dependent activation of fast skeletal muscle myofibril ATPase activity by the normal protein and all three mutants are essentially the same. Competition between active and inactive forms of cardiac and slow TnC in a functional assay demonstrates that mutation of both sites III and IV greatly reduces the affinity of cardiac and slow TnC for its functionally relevant binding site in the myofibrils. The data indicate that although neither high affinity site is absolutely essential for regulation of muscle contraction in vitro, at least one active C-terminal site is required for tight association of cardiac troponin C with myofibrils. This requirement can be satisfied by either site III or IV.

Contraction in striated muscle is regulated by the binding of Ca\(^{2+}\) to the troponin C (TnC)\(^{1}\) subunit of the troponin complex. Troponin C is a member of a growing family of Ca\(^{2+}\)-binding proteins which contain at least one EF-hand or helix-loop-helix Ca\(^{2+}\)-binding motif originally observed in parvalbumin (Kretsinger et al., 1973). Within this large family of proteins, TnC and calmodulin are the best-characterized members with defined Ca\(^{2+}\)-dependent regulatory functions. Both calmodulin and fast skeletal TnC have very similar crystal structures which resemble a dumbbell in shape (Herzberg and James, 1985; Sundaralingam et al., 1985; Babu et al., 1985). It is reasonable to postulate that this tertiary motif may be shared by other EF-hand proteins with regulatory activities. Functional distinction may be conferred by the nature of the amino acid side chains and the affinity or functionality of the individual Ca\(^{2+}\) binding sites.

Tissue and species divergence of TnC suggests that evolutionary mutation of its Ca\(^{2+}\) binding sites has provided a mechanism to modify function. Similar to calmodulin, vertebrate fast skeletal TnC (sTnC) binds four Ca\(^{2+}\) ions/mol of protein (Potter et al., 1975), while in the cardiac isoform (cTnC) Ca\(^{2+}\) binding site I is inactive (Collins et al., 1977; Van Eerd et al., 1976; Van Eerd et al., 1975). Mutagenesis has been used to demonstrate directly the functional role of the N-terminal Ca\(^{2+}\) binding sites in cardiac and fast skeletal TnC. A functional site II in cTnC is essential for regulating contraction in slow muscle fibers, and activation of the dormant site I modifies the characteristics of contraction in both fast and slow muscle fibers (Putkey et al., 1989, 1991). Binding of Ca\(^{2+}\) to site I alone cannot generate force in slow muscle fibers (Sweeney et al., 1990) but can initiate partial force generation in fast muscle fiber (Putkey et al., 1991). Similar studies on sTnC have shown that the N-terminal Ca\(^{2+}\) binding sites regulate muscle contraction in fast muscle fibers and show that both sites are needed for normal function (Sheng et al., 1990). These data are consistent with previous studies on sTnC which have implicated sites I and II as responsible for regulating muscle contraction (Johnson et al., 1979; Robertson et al., 1981; Zot and Potter, 1982).

Metal binding sites III and IV in vertebrate cardiac and fast skeletal TnC appear to bind both Mg\(^{2+}\) and Ca\(^{2+}\) (Potter et al., 1975; Leavis et al., 1978). Potter and Gergely (1975) demonstrated large conformational changes upon Ca\(^{2+}\) or Mg\(^{2+}\) binding to these sites, and suggested that these sites were responsible for maintaining the proper protein confor-

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1 The abbreviations used are: TnC, both forms of troponin C; sTnC, fast skeletal troponin C; cTnC, cardiac and slow skeletal troponin C; cTnC3, bacterially synthesized chicken cTnC (des M1, D2A); CBM-III, cTnC3 (D65A); CBM-IV, cTnC3 (D105A); CBM-I-III, IV, cTnC3 (D105A, D141A); CBM-II-III-IV, cTnC3 (D65A, D105A, D141A); MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethyleneglycolbis(oxyethylenedinitrilo)tetracetetic acid; SDS, sodium dodecyl sulfate.
mation for activation by Ca\textsuperscript{2+}. The majority of circular dichroism spectral changes occur upon binding Ca\textsuperscript{2+} to sites III and IV (Hinke et al., 1978; Nagy et al., 1979). Titration of sTnC with Mg\textsuperscript{2+} induced 80% of the circular dichroism spectral change produced by Ca\textsuperscript{2+} (Van Eerd et al., 1972). In a complementary study using solution x-ray scattering it was shown that slow protonation of the N-terminal tryptic fragment of sTnC occurs when it binds Ca\textsuperscript{2+} but that the C-terminal tryptic fragment shrinks (Fujisawa et al., 1989). These data, together with the crystal structures of calmodulin and sTnC, suggest the Ca\textsuperscript{2+} bound states of the N- and C-terminal halves of sTnC have a similar conformation but that the apo forms may be quite different with the N-terminal half having a higher percentage of \(\alpha\)-helix and the C-terminal half having a higher percentage of random coil.

Calcium binding sites III and IV are thought to perform a structural role in allowing TnC to properly associate with the tropinin complex. Tropinin C can be extracted from myofibrils at low ionic strength in the presence of EDTA (Cox et al., 1981). Extraction can be prevented by the presence of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (Zot and Potter, 1982). This suggests that firm association of sTnC with the myofibril is dependent on occupation of sites III and IV with Ca\textsuperscript{2+} or Mg\textsuperscript{2+} and not to binding of Ca\textsuperscript{2+} at site I or II. Photo cross-linking studies support a role for the C-terminal metal binding sites in the formation of interactions between sTnC and TnI and TnT and suggest that these interactions are qualitatively different in the reconstituted tropinin complex as compared to the thin filament. Addition of Ca\textsuperscript{2+} to reconstituted fast skeletal muscle troponin complexes showed no effect on the extent of cross-linking detected between sTnC and TnI, or between sTnC and sTnT. However, in the thin filament, cross-linking of the troponin subunits was significantly affected by the presence of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (Sutoh, 1980) and maximum cross-linking between sTnC and sTnI required the presence of both Ca\textsuperscript{2+} and Mg\textsuperscript{2+}. In the presence of only Mg\textsuperscript{2+} or in the absence of divalent cations, cross-linking between sTnC and sTnl was significantly reduced. In contrast to the interactions between sTnC and TnI, cross-linking between sTnC and sTnT in the thin filament remained relatively low in the presence or absence of divalent cations (Sutoh, 1980). Van Eky and Hodges (1988) have suggested that the Ca\textsuperscript{2+}/Mg\textsuperscript{2+} sites are responsible for the increased interaction between the inhibitory region of TnI and tropomyosin-actin during muscle relaxation.

Here we have investigated the functional significance of Ca\textsuperscript{2+} binding sites III and IV in cTnC by site-directed mutagenesis. We show that inactivation of either site III or IV does not greatly alter the functional characteristiscs of cTnC or its affinity for the thin filament. Inactivation of both Ca\textsuperscript{2+} binding sites III and IV greatly decreases the affinity of cTnC for functionally relevant binding sites in both cardiac and fast skeletal muscle myofibrils. However, even a protein with both sites III and IV mutated remains functional and exhibits Ca\textsuperscript{2+}-dependent activation properties which are similar to the normal protein.

**MATERIALS AND METHODS**

*Mutation of cTnC—*Four mutant proteins, CBM-III, CBM-IV, CBM-III-IV, and CBM-II-III-IV were generated (see Fig. 1). In all proteins, Ca\textsuperscript{2+} binding sites were inactivated by changing the first coordinating ligand of the Ca\textsuperscript{2+} binding loop from Asp to Ala. Site-directed mutagenesis was performed by the method of Kunkel (1985) using a commercially available kit (Bio-Rad). All mutations were confirmed by DNA sequence analysis. The oligonucleotide primer used for mutating site III (Asp-105 to Ala) had the sequence 5'-GCCATCAGTTTITGACCCCCATCTTTC-3'. The oligonucleotide primer used for mutating site IV, (Asp-141 to Ala) had the sequence 5'-GCCATCAGATTCTTCTGACAACATCTG-3'.

**Fig. 1. Summary of mutant proteins.** The proteins used in this study are summarized above with a diagrammatic representation of the primary structure and predicted secondary structure of chicken cTnC. The structure is based on the three-dimensional structures for calmodulin and sTnC (Babu et al., 1965; Herzberg et al., 1985). Squares represent \(\alpha\)-helical regions, and circles represent \(\beta\) sheets and random coils. Coordination bonds are represented by lines radiating from Ca\textsuperscript{2+}. To avoid lengthy conventional terminology the mutated proteins used here are called CBMs for Calcium Binding Mutants. The site that is altered is indicated by a roman numeral. The nomenclature used here differs slightly from the previous report (Putkey et al., 1989) in that + and - symbols are used to designate an active or inactive site, respectively. Using this convention, CBM-III refers to a calcium-binding mutant in which Ca\textsuperscript{2+} binding site III is inactive.
established in relaxing solution to which 2 mg/ml of bacterially synthesized TnC was added. Reconstitution took place at 20 °C for 1 h. After reconstitution, maximal calcium activation (pCa 4.3) was elicited.

Following force measurements SDS-polyacrylamide gel electrophoresis was used to assess reconstitution in all fibers and with all forms of bacterially synthesized TnC. It had previously been determined using SDS-polyacrylamide gel electrophoresis that the extraction conditions resulted in loss of TnC from the fibers.

Myofibril ATPase Assays—Fast skeletal and cardiac myofibrils were prepared as described by Solara et al. (1971) from rabbit back and leg muscle or bovine left ventricle. Endogenous TnC was extracted as described by Morimoto and Ohitsuki (1987) with the exception that the extraction buffers contained 1% Triton X-100 and 0.5 mM trifluoperazine. The relative protein concentrations of different preparations of extracted myofibrils were determined by first dissolving an aliquot of myofibrils in a solution of 10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.1% SDS. Protein concentration was calculated using an ε of 280 nm of 6.5. Assays were performed in microtiter plates at 25 °C. The reaction mixture contained 120 mM MOPS, pH 7.0, 90 mM KCl, 9.45 mM MgCl₂, 0.1 mM dithiothreitol, 2 mM EGTA, 5 mM Na₃ATP, 2.6 mM CaCl₂, 0.5 mg/ml of extracted myofibrils, and varying concentrations of recombinant TnC or mutated derivatives in a total volume of 0.2 ml. The reaction was started by the addition of 5 mM ATP, and stopped after 5 or 7 min by the addition of 0.05 ml of ice-cold 12.5% trichloroacetic acid. After centrifugation, 0.2 ml of the reactions were transferred to a fresh microtiter plate for phosphate determination using the method of Fiske & Subbarow (1925).

RESULTS

Mutation of cTnC3—Fig. 1 illustrates the nature of the mutations and the nomenclature selected for the proteins characterized in this report. All mutated proteins described here were derivatives of bacterially synthesized chicken cTnC referred to as cTnC3 (Putkey et al., 1989). Inactivation of Ca²⁺ binding sites III and IV was accomplished by changing the first amino acid in the Ca²⁺ binding loop from Asp to Ala. An analogous mutation at position 65 was shown previously to effectively inactivate Ca²⁺ binding to site II (Putkey et al., 1989). The proteins with altered Ca²⁺-binding characteristics were designated CBM for Calcium Binding Mutants followed by a minus sign and roman numeral to designate the inactive site. Using this convention CBM-III defines a recombinant cTnC protein in which site III has been inactivated by changing Asp-105 to Ala.

Ca²⁺ Binding Properties of the Mutant Proteins—Fig. 2 compares the Ca²⁺ binding capacities of CBM-III, CBM-IV, and CBM-III-IV with normal cTnC3 and a mutant, CBM-IIA, which has an inactive site II. All mutated proteins displayed the predicted Ca²⁺ binding capacities. At pCa 6, both cTnC3 and CBM-IIA bound 2 mol of Ca²⁺/mol of protein, consistent with the presence of two functional high affinity sites. Both CBM-III and CBM-IV were found to bind approximately 0.75 mol of Ca²⁺/mol of protein at pCa 6, consistent with only one active high affinity site. In contrast, CBM-III-IV was found to bind less than a stoichiometric amount of Ca²⁺ at pCa 6, suggesting that both high affinity Ca²⁺ binding sites are inactive. At pCa 4, CBM-III, CBM-IV, and CBM-IIA all bound 2 mol of Ca²⁺/mol of protein although each have different combinations of two active Ca²⁺ binding sites. CBM-III-IV and cTnC3 bound 1 and 3 mol of Ca²⁺/mol of protein, respectively, at pCa 4.

Activity of the Mutant Proteins in Skinned Slow Skeletal Muscle Fibers—The functional characteristics of the mutant proteins were first examined using cTnC-depleted skinned slow skeletal muscle fibers. Initial experiments (see Fig. 3A) showed that both CBM-III and CBM-IV were functional but that CBM-III-IV appeared inactive. The typical sequence of manipulation of these fibers included: 1) extraction of the endogenous cTnC; 2) reconstitution of the fibers by soaking them in a relaxing solution containing exogenous cTnC; 3) transfer of the reconstituted fibers to a relaxing solution without exogenous cTnC; and 4) measurement of the force of contraction as the fibers were exposed to a continuous flow of contraction buffer with or without Ca²⁺. These manipulations could allow CBM-III-IV to be washed from the reconstituted fibers during the measurement of contraction. To prevent this, experiments using CBM-III-IV were repeated with CBM-III-IV present in the contraction solution. As shown in Fig. 3B, under these conditions CBM-III-IV was also able to restore Ca²⁺-dependent contraction to the extracted skinned muscle fibers to a level equivalent to cTnC3.

Activity of the Mutant Proteins in Fast Skeletal and Cardiac Myofibrils—The effectiveness of the extraction procedure for removal of TnC from fast skeletal and cardiac myofibrils was assessed by SDS-gel electrophoresis of myofibrillar proteins and by the level of Ca²⁺-dependent ATPase activity of extracted myofibrils without the addition of exogenous TnC. By both criteria the procedure was effective for the consistent removal of essentially all cTnC from fast skeletal muscle fibers. Removal of cTnC from cardiac muscle fibers was more variable and the extracted cardiac myofibrils generally retained about 10–20% of the Ca²⁺-dependent ATPase activity of native myofibrils. The maximal Ca²⁺-dependent ATPase activity for

**Fig. 2.** Ca²⁺ binding characteristics of wild type and mutant proteins. Experiments were performed using equilibrium dialysis as described under "Materials and Methods" with 0.11 mM cTnC proteins and pCa concentrations from 8 to 4. An EGTA-buffered system was used to obtain free Ca²⁺ concentrations ranging from pCa 8 to 6. The results from two experiments are shown expressed as moles of Ca²⁺/mol of protein. Error bars represent the standard deviation of triplicates performed at each pCa concentration.

**Fig. 3.** Force generation in skinned muscle fibers reconstituted with wild type and mutant cTnC proteins. Tension measurements were performed on skinned rabbit soleus muscle fibers at pCa 4.3 as described under "Materials and Methods." Activity was reported as the percentage of maximal force of native fibers. A, assays performed without activator proteins present in the contraction solution; B, assays performed with CBM-III-IV present in the contraction solution.
both types of myofibrils was similar to that reported by Morimoto and Ohtsuki (1988). Both extracted fast skeletal and cardiac myofibrils had Ca\(^{2+}\)-independent ATPase activities of approximately 15–20 nmol of P\(_i\)/min/mg of myofibrillar protein. Since the specific activity of Ca\(^{2+}\)-dependent ATPase activity is about 10-fold greater for fast skeletal as compared to cardiac myofibrils, the signal-to-noise ratio is much better in the fast skeletal myofibril system. In all myofibril ATPase assays the activator proteins were present in the assay buffer at the indicated concentrations.

Fig. 4A shows the relative ATPase activity of extracted fast skeletal muscle fibers reconstituted with increasing concentrations of sTnC, cTnC, CBM-III, and CBM-IV. All proteins were effective regulators of ATPase activity. Cardiac TnC and CBM-IV, which has an inactive site IV, were essentially identical in their activation characteristics. The dose-response curve for CBM-III, with an inactive site III, is shifted to the right relative to cTnC. This difference is not striking and may be due to a greater structural perturbation induced by changing Asp-105 to Ala in site III which is located at the end of the central helix of cTnC. Increasing the concentration of all proteins to 150 \(\mu\)g/ml did not significantly increase the level of ATPase activity. Fig. 4B, shows the ATPase activity of extracted fast skeletal muscle fibers reconstituted with increasing concentrations of CBM-III-IV. A much higher concentration of CBM-III-IV is required to achieve maximal activity relative to that required for the proteins shown in A.

Fig. 4C compares the maximal ATPase activity of the native and extracted fast skeletal muscle myofibrils and extracted myofibrils reconstituted with sTnC, cTnC, CBM-III, CBM-IV, or CBM-III-IV. The maximal level of Ca\(^{2+}\)-dependent ATPase activity seen for CBM-III and CBM-IV was equivalent to cTnC. The level of myofibril Ca\(^{2+}\)-dependent ATPase activity in the presence of 15 \(\mu\)g/ml CBM-III-IV is minimal, however, at a concentration of 150 \(\mu\)g/ml the ATPase activity is essentially identical to cTnC. Although reconstitution with sTnC recovers native levels of Ca\(^{2+}\)-dependent ATPase activity, reconstitution with cTnC or its mutated derivatives recovers only about 50% of the native levels. This phenomenon has also been observed previously in skinned skeletal muscle fibers (Babu et al., 1987). Higher concentrations of cTnC did not further increase the activity nor did preincubation of cTnC with fast skeletal muscle fibers in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) for up to 3 h prior to initiating the assay. The difference between maximal activation by sTnC and cTnC is not due to peculiar structural features of bacterially synthesized cTnC since it is also observed with bovine cTnC.

Fig. 5 shows the activation characteristics of normal and modified cTnC proteins assessed in cardiac muscle myofibrils. In contrast to the fast skeletal fibers, sTnC and cTnC confer equivalent maximal levels of Ca\(^{2+}\)-dependent ATPase activity to cardiac muscle fibers. The activation characteristics of CBM-III and CBM-IV are very similar to the unmodified proteins. Much higher concentrations of CBM-III-IV are required to achieve significant levels of activation. Maximal activity is apparently not achieved even at a concentration of CBM-III-IV of 500 \(\mu\)g/ml.

**Fig. 4.** ATPase activity of extracted fast skeletal muscle myofibrils reconstituted with wild type and mutant cTnC proteins. In A and B increasing concentrations of wild type and mutant cTnC proteins were added to the ATPase assay reaction mixture which included assay buffer, 0.5 mg/ml myofibrillar protein, and 5 mM free Mg\(^{2+}\) at pCa 4. The assay mix was allowed to equilibrate at 25 °C for 20 min prior to the addition of ATP. To directly compare the curves, activity is expressed as percentage of maximal response for each protein. In C the maximal Ca\(^{2+}\)-dependent ATPase activity of the reconstituted fibers was compared to the native and extracted fibers. Activity is given in units of nmol of inorganic phosphate released per min per mg of myofibrillar protein. Ca\(^{2+}\)-dependent ATPase is defined as the difference in activity at pCa 4.0 and 8.0. The Ca\(^{2+}\)-independent activity was about 15–20 nmol of P\(_i\)/min/mg of protein. The values in parentheses indicate the number of separate experiments at a given protein concentration, and the error bars indicate the standard error of the mean.

**Fig. 5.** Ca\(^{2+}\)-ATPase activity of extracted cardiac muscle myofibrils reconstituted with wild type and mutant cTnC proteins. Increasing concentrations of wild type and mutant cTnC proteins were added to the ATPase assay reaction mixture which included assay buffer, 1 mg/ml myofibrillar protein, and 5 mM free Mg\(^{2+}\) at pCa 4. The assay mix was allowed to equilibrate at 25 °C for 20 min prior to the addition of ATP. Activity is given in units of nmol of inorganic phosphate released per min per mg of myofibrillar protein. Ca\(^{2+}\)-dependent ATPase is defined as the difference in activity at pCa 4.0 and 8.0. The Ca\(^{2+}\)-independent activity was about 15–20 nmol of P\(_i\)/min/mg of protein. The data show a representative experiment in which each data point is the average of triplicate determinations.
**Binding Sites III and IV**—The fast skeletal muscle myofibril system was used to determine the Ca\(^{2+}\) dependence of the various mutants for several reasons. First, although the extraction procedure used here is very effective in removing sTnC from fast skeletal fibers, cardiac myofibrils are only partially extracted as evidence by the residual Ca\(^{2+}\)-dependent ATPase activity between 10 and 20% of the native fibers. Second, the specific activity of the fast skeletal muscle myofibrils is about 10-fold greater than that of cardiac myofibrils. Finally, the pCa\(_{50}\) and Hill coefficient values for extracted skeletal muscle myofibrils reconstituted with cTnC are very similar to the values seen for native cardiac myofibrils (Morigo et al., 1988). Fig. 6 shows representative activity versus Ca\(^{2+}\) curves for fast skeletal muscle myofibrils reconstituted with either cTnC3, CBM-III, CBM-IV, or CBM-III-IV. Table I summarizes the pCa\(_{50}\) and Hill coefficients determined for a number of experiments. It is clear that inactivation of even both high affinity Ca\(^{2+}\) binding sites in cTnC does not greatly alter either the pCa\(_{50}\) or Hill coefficient for Ca\(^{2+}\)-dependent activation of ATPase activity in skeletal muscle myofibrils.

**Preliminary experiments** showed this approach to be impractical since a large amount of nonspecific binding to the extracted myofibrils was observed. As an alternate approach for assessing the relative contribution of sites III and IV to the affinity of cTnC for the contractile apparatus, the myofibril ATPase assay was used under conditions where inactive cTnC derivatives compete with cTnC or sTnC for functional binding sites thereby inhibiting ATPase activity.

In a previous study, site II in cTnC was inactivated by conversion of Asp-65 to Ala (Putkey et al., 1989). The mutant protein, CBM-IIA, was inactive but bound to TnC-extracted skinned slow skeletal muscle fibers as determined by SDS-gel electrophoresis. Preliminary experiments showed CBM-IIA to be an effective inhibitor of myofibril ATPase activity if it was mixed with active cTnC prior to addition of extracted myofibrils, but it would not readily displace TnC from native fibers (data not shown). To determine if this inhibition could be affected by inactivation of Ca\(^{2+}\) binding sites III and IV, the Ca\(^{2+}\)-binding mutant CBM-II-III-IV was generated (see Fig. 1). The relative abilities of CBM-IIA and CBM-II-III-IV to compete with cTnC or sTnC for functionally relevant binding sites in myofibrils would be a direct measure of the contribution of sites III and IV to tight binding.

Fig. 7 shows that both CBM-IIA and CBM-II-III-IV are incapable of recovering Ca\(^{2+}\)-dependent ATPase activity in extracted fast skeletal (Fig. 7A) or cardiac (Fig. 7B) muscle myofibrils. CBM-II-III-IV is inactive even when present at concentrations of 150 \(\mu g/ml\), the concentration at which CBM-III-IV shows maximal activity. To compare the ability of these inactive proteins to compete with sTnC and cTnC3 for functionally relevant binding sites in myofibrils, the active and inactive proteins were first combined in the presence of Ca\(^{2+}\) and Mg\(^{2+}\). Myofibrils were then added and after a 20-min preincubation time, the assay was initiated by the addition of ATP. Fig. 8 shows that increasing concentrations of CBM-IIA effectively prevents sTnC and cTnC from associating with fast skeletal (Fig. 8A) or cardiac (Fig. 8B) myofibrils. At a ratio of CBM-IIA to cTnC or sTnC of 2:1, ATPase activity is maximally inhibited. This suggests that CBM-IIA not only competes effectively with the active proteins but also inhibits cooperativity by disrupting communication between troponin subunits. CBM-II-III-IV does not inhibit ATPase

**TABLE I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hill coefficient</th>
<th>pCa(_{50})</th>
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</thead>
<tbody>
<tr>
<td>Native</td>
<td>3.81 ± 0.08</td>
<td>6.90 ± 0.01</td>
</tr>
<tr>
<td>cTnC3</td>
<td>1.6 ± 0.00</td>
<td>6.07 ± 0.01</td>
</tr>
<tr>
<td>CBM-III</td>
<td>1.7 ± 0.01</td>
<td>6.11 ± 0.01</td>
</tr>
<tr>
<td>CBM-IV</td>
<td>1.5 ± 0.02</td>
<td>6.17 ± 0.02</td>
</tr>
<tr>
<td>CBM-III-IV</td>
<td>1.7 ± 0.04</td>
<td>6.15 ± 0.01</td>
</tr>
</tbody>
</table>

**Fig. 6. Ca\(^{2+}\) dependence of wild type and mutant proteins determined in fast skeletal muscle myofibrils.** The percentage of maximal response in ATPase activity is shown as a function of the Ca\(^{2+}\) concentration from pCa 7.5 to 4.5 using reconstituted fast skeletal myofibrils.

**Fig. 7. Comparative activities of proteins with an active or inactive Ca\(^{2+}\) binding site II.** The activities of sTnC and cTnC3 were compared in both fast skeletal and cardiac myofibrils to the activities of CBM-IIA and CBM-II-III-IV (see Fig. 1). All proteins were assayed at a concentration of 15 \(\mu g/ml\). CBM-II-III-IV was assayed at concentration of both 15 and 150 \(\mu g/ml\) as indicated.
activity of fast skeletal or cardiac myofibrils even when present at an 8-fold molar excess over cTnC3 or sTnC. Thus, unlike CBM-IIA, CBM-II-III-IV cannot effectively compete with sTnC or cTnC to prevent them from integrating into myofibrils and regulating ATPase activity.

Fig. 8 shows that CBM-II-III-IV appears to increase Ca$^{2+}$-dependent cardiac myofibrillar ATPase activity in the presence of 10 μg/ml cTnC3. A likely explanation for this observation is that CBM-II-III-IV binds to and saturates the nonspecific sites that were detected with radiolabeled cTnC3 and CBM-III-IV as described above. This would essentially increase the effective concentration of cTnC since the active protein would not partition into the pool of nonspecific binding sites. This effect is not seen for fast skeletal myofibrils (Fig. 8A) since activity is maximal at a TnC concentration of 10 mg/ml (see Fig. 4A). If this is true, then enhanced activity in response to CBM-II-III-IV should not be seen in the presence of higher concentrations of the active protein since there would be sufficient cTnC3 to saturate the functionally relevant binding sites. As shown in Fig. 8B, when the concentration of cTnC3 is increased from 10 to 20 μg/ml, increasing concentrations of CBM-II-III-IV do not enhance activity.

**DISCUSSION**

Cardiac TnC contains two high affinity Ca$^{2+}$/Mg$^{2+}$ binding sites (sites III and IV) located in the C-terminal domain and one low affinity Ca$^{2+}$-specific binding site (site II) located in the N-terminal domain. The purpose of the present study was to use mutagenesis to directly demonstrate the necessity of the high affinity Ca$^{2+}$/Mg$^{2+}$ binding sites. The EF-hand metal binding sites in bacterially synthesized cTnC3 were altered both individually and in combination to yield the four mutant proteins summarized in Fig. 1. The predicted Ca$^{2+}$-binding properties of these proteins was confirmed and their functional properties characterized. The results are consistent with a molecular model for TnC proposed by Zot and Potter (1982) in which the C-terminal domain is primarily responsible for the tight association of TnC with the tropinin complex on the thin filament.

Aspartate residues are found in the first ligand position of all functional Ca$^{2+}$ binding sites in cTnC, sTnC, and vertebrate calmodulin. The Asp side chain at this position is thought to be critical not only because it contributes one of the seven ligands for Ca$^{2+}$ binding, but also because it is predicted to form a strong hydrogen bond with the amide proton of the invariant Gly found at the sixth position of the loop (Strynadka and James, 1989). Using NMR techniques, this hydrogen bond was shown to be present in solution and to be absent in the mutant CBM-IIA which has site II inactivated by conversion of Asp-65 to Ala (Krudy et al., 1992). Given the positional significance of these particular Asp residues it is not surprising that changing Asp-65, Asp-105, or Asp-141 to Ala was effective in preventing the binding of Ca$^{2+}$ to sites II, III, and IV.

Any modification to the primary sequence of a protein in an effort to achieve a desired functional change also carries the risk of inducing undesired structural changes which can complicate the interpretation of observed functional characteristics. A comparative NMR study of cTnC3 and CBM-IIA, which has site II inactivated by conversion of Asp-65 to Ala, showed the aromatic region of the NMR spectra for the two proteins to be very similar in the apo form but different in the Ca$^{2+}$-bound form (Brito et al., 1991). This suggests that similar modifications to sites III and IV may impose limited structural changes. However, both circular dichroism and x-ray scattering studies suggest that the α-helical content of the N-terminal domain is greater than for the C-terminal domain in the apo form of TnC (Nagy et al., 1979; Hincke et al., 1978; Van Eerd et al., 1972; Fujisawa et al., 1989). This could influence the degree of structural perturbation induced by inactivation of site II as compared to inactivation of sites III and IV. Nevertheless, both CBM-III and CBM-IV have essentially normal functional characteristics. This might not be expected if the structures of these proteins were significantly altered relative to cTnC3. If the lowered affinity of CBM-III-IV for the myofibrils is due to undesired structural perturbations it must result from the synergistic effects of modification of both Asp-105 and Asp-141.

The high affinity metal binding sites of sTnC and cTnC bind both Ca$^{2+}$ and Mg$^{2+}$. It has been reported that TnC may bind 5–10 mol of Mg$^{2+}$/mol of protein (Potter and Gergely, 1975; Tauda et al., 1990). These additional binding sites may be the clusters of acidic residues found in both TnC and calmodulin. The presence of numerous Mg$^{2+}$ binding sites coupled with their low affinity would make it difficult to distinguish differences in Mg$^{2+}$ binding capacities between the mutant and normal proteins using the relatively insensitive techniques for direct determination of Mg$^{2+}$ binding. The presence of Mg$^{2+}$ will prevent the extraction of sTnC from muscle fibers, presumably by binding to the Ca$^{2+}$/Mg$^{2+}$ sites thereby inducing a high affinity conformation in the C-terminal half of the protein (Zot and Potter, 1982). Since CBM-III-IV has a greatly reduced affinity for the myofibrils even in the presence of Mg$^{2+}$, it is likely that changing Asp-105 and Asp-141 to Ala inhibits the binding of both Ca$^{2+}$ and Mg$^{2+}$.
Activation of the thin filament is a cooperative process that involves communication between troponin subunits. The $\text{Ca}^{2+}$ response curves for both cardiac fibers and myofibrils is sigmoidal, and their Hill coefficients approach 2.0 (Kerrick et al., 1980; Morimoto and Ohtsuki, 1988) even though cTnC has only one functional regulatory $\text{Ca}^{2+}$ binding site. Partial extraction of sTnC from skinned muscle fibers results in a disproportionately large decrease in tension as well as a decrease in the Hill coefficient (Brandt et al., 1984; Moss et al., 1985). This suggests that communication between troponin subunits within the thin filament is disrupted by partial and selective removal of TnC from individual troponin complexes. The fact that CBM-III-IV has essentially the same Hill coefficient as cTnC3 when incorporated into fast skeletal muscle myofibrils suggests that tight association of the C-terminal domain with the troponin complex is not necessary for communication between complexes. In contrast, the data in Fig. 8 suggest that an active $\text{Ca}^{2+}$ binding site II is necessary for the cooperativity that results from communication between troponin subunits. Even when combined at a ratio of 1:1, competition between CBM-IIA and cardiac or skeletal TnC resulted in a striking decrease of the maximal ATPase activity in both skeletal and cardiac myofibrils. Although this result could be explained by an increase in affinity of CBM-IIA for the troponin complex it may also result from the ability of CBM-IIA to disrupt cooperativity within the thin filament.

The TnC proteins associated with ascidial smooth muscle (Takagi and Konishi, 1983), the $\gamma$ and $\beta$ isoforms of crayfish striated muscle (Wnuk, 1989; Kobayashi et al., 1989), and the $\beta_1$ and $\beta_2$ isoforms of barnacle TnC (Collins et al., 1991) all appear to bind $\text{Ca}^{2+}$ specifically, while sites I and III are inactive (for review see Collins, 1991). The $\text{Ca}^{2+}$ binding properties of site IV in both the $\alpha$ and $\gamma$ isoforms of crayfish TnC (Wnuk, 1989) pose an interesting challenge to the structural role for metal binding sites I, II, and III can also confer apparently normal functional characteristics in vitro assays.

REFERENCES


Mutation of the High Affinity Sites in Cardiac Troponin C