Effects of Cardiac Thin Filament Ca^{2+}: Statistical Mechanical Analysis of a Troponin C Site II Mutant

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ABSTRACT Cardiac thin filaments contain many troponin C (TnC) molecules, each with one regulatory Ca^{2+} binding site. A statistical mechanical model for the effects of these sites is presented and investigated. The ternary troponin complex was reconstituted with either TnC or the TnC mutant CBMII, in which the regulatory site in cardiac TnC (site II) is inactivated. Regardless of whether Ca^{2+} was present, CBMII-troponin was inhibitory in a thin filament-myosin subfragment 1 MgATPase assay. The competitive binding of [^{3}H]troponin and [^{14}C]CBMII-troponin to actin·tropomyosin was measured. In the presence of Mg^{2+} and low free Ca^{2+} they had equal affinities for the thin filament. When Ca^{2+} was added, however, troponin's affinity for the thin filament was 2.2-fold larger for the mutant than for the wild type troponin. This quantitatively describes the effect of regulatory site Ca^{2+} on troponin's affinity for actin·tropomyosin; the decrease in troponin-thin filament binding energy is small. Application of the theoretical model to the competitive binding data indicated that troponin molecules bind to interdependent rather than independent sites on the thin filament. Ca^{2+} binding to the regulatory site of TnC has a long-range rather than a merely local effect. However, these indirect TnC-TnC interactions are weak, indicating that the cooperativity of muscle activation by Ca^{2+} requires other sources of cooperativity.

INTRODUCTION

Ligand-induced alterations in protein-protein interactions are crucial for the regulation of allosteric systems with multiple subunits, including the thin filament. For example, cooperative oxygen binding to hemoglobin has been explained by detailed studies of hemoglobin assembly. The ligand-ligand interactions that cause hemoglobin's cooperative behavior can be measured by determining tetramer assembly free energies, using subunits that are homogeneous and heterogeneous with respect to ligand binding (Ackers et al., 1992). In the case of the thin filament, there are two important ligands that alter protein-protein interactions within the system, Ca^{2+} and myosin (Leavis and Gergely, 1984; Zot and Potter, 1987; Chalovich, 1992; Tobacman, 1996). In the present study the allosteric effects of regulatory site Ca^{2+} binding are assessed by investigating the assembly of thin filaments with mixtures of normal and regulatory site-mutant troponin C (TnC).

Striated muscle contraction is controlled by the reversible binding of Ca^{2+} to TnC, which typically has three or four Ca^{2+} binding sites. Regulation primarily involves sites I and II in fast skeletal muscle TnC and site II in cardiac muscle TnC (Potter and Gergely, 1975; Holroyde et al., 1980; Pan and Solaro, 1987), which lacks the required amino acid sequence for Ca^{2+} binding at site I (van Eerd and Takahashi, 1975). Inactivation of the regulatory site(s) by mutagenesis of residues required for Ca^{2+} coordination results in constitutively inhibitory forms of TnC, forms that prevent the activation by Ca^{2+} of muscle contraction or myofibrillar ATPase activity (Putkey et al., 1989; Sheng et al., 1990; Babu et al., 1992). These inhibitory TnCs retain normal Ca^{2+}/Mg^{2+} binding at sites III and IV, normal C-terminal domain structure by nuclear magnetic resonance (Brito et al., 1991; Lin et al., 1994), and bind readily to TnC-depleted muscle fibers or myofibrils.

The present study employs inhibitory cardiac TnC (recombinant murine troponin C with the mutation D65A/E66A and defective site II Ca^{2+} binding; designated CBMII by Putkey et al., 1989) to test and measure several aspects of regulation. Ca^{2+} binding to TnC is believed to weaken troponin-thin filament binding (Hitchcock et al., 1973; Potter and Gergely, 1974; Margossian and Cohen, 1973; Mak and Smillie, 1981; Pearlstone and Smillie, 1983; Ishii and Lehrer, 1991; Zot and Potter, 1987), which is at least partially due to the reversal of a specific interaction between troponin I (TnI) and actin that is required for the inhibitory state (Syska et al., 1976; Talbot and Hodges, 1981; Van Eyk et al., 1991). However, it is difficult to determine whether binding is significantly weakened by Ca^{2+} because the affinity of troponin for the thin filament is sufficiently tight that its measurement is problematic. An indirect measurement of this process was recently reported by calculation from equilibrium linkage relationships involved in the associations of actin, tropomyosin, and troponin (Dahiya et al., 1994; Fisher et al., 1995). Both in cardiac muscle and in skeletal muscle Ca^{2+} had only a twofold effect on troponin-thin filament binding, quite small for a major regulatory switch. This unexpected result has not been confirmed by an independent, sensitive, and more direct method. We now report such a method. It involves competitive binding to the

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thin filament of troponin containing CBMII versus troponin containing wild type (wt) TnC, examined in the presence and in the absence of Ca^{2+}. The readily measurable relative affinities of these two forms of troponin for actin·tropomyosin (in the presence of Ca^{2+}) provide a direct measure of the effect of regulatory site Ca^{2+} binding on troponin-thin filament affinity. Furthermore, this competition method should be of general value. It permits a quantitative analysis of the effect of any troponin mutation on thin filament assembly, both in the presence and in the absence of Ca^{2+}.

Finally, because each thin filament binds multiple troponin molecules, the competition between the two forms of troponin is influenced by any interactions that may exist between neighboring troponins on the thin filament. Although troponin molecules do not contact each other directly, the affinity of troponin for a particular site on the thin filament could be influenced by Ca^{2+} binding at a neighboring troponin, if there is a Ca^{2+}-dependent conformational change transmitted via actin and/or tropomyosin. The effect of such interactions on the competitive binding can be analyzed with a linear lattice, statistical mechanical model. The model suggests that by experimentally varying the ratio of the two troponins over a wide range, it may be possible to determine i) in the absence of myosin, whether neighboring troponin molecules interact despite their separation along the thin filament; ii) whether these interactions are specifically perturbed by Ca^{2+} binding to the TnC regulatory sites; and iii) whether the strength of these perturbations implies a significant contribution to cooperative muscle activation by Ca^{2+}.

MATERIALS AND METHODS

Cloning strategy

The murine cardiac TnC cDNA (Parmacek and Leiden, 1989) in pGEM3z was altered using gapped heteroduplex oligonucleotide-mediated site-directed mutagenesis (Karpinski et al., 1989). Nucleotides GACGAG encoding Asp-Glu (amino acids 65 and 66), were altered to GCCGCG, encoding Ala-Ala. The D65A mutation abolishes Ca^{2+} binding (Putkey et al., 1989), even in the presence of the other troponin subunits (Dotson and Putkey, 1993). Although the mutagenesis also caused the change E66A, this mutation does not eliminate a Ca^{2+} coordination site and has too small an effect on TnC structure to inactivate site II (Babu et al., 1992). The wt and mutant cDNAs were subcloned into the pSP72 *Bam*HI/*Eco*RI and *Eco*RI sites, respectively, and the complete nucleotide sequences were confirmed by dideoxynucleotide sequencing (Sanger et al., 1977). The coding sequences were then removed from pSP72 by digestion with *Nco*I and *Bgl*II and inserted into the *Nco*I/*Bam*HI site of the expression plasmid pET3d (Studier et al., 1990).

Protein expression and purification

DE-3 (BL21) cells were transformed with pET3d encoding either wt TnC or CBMII, defined here as TnC with the above-described Ca^{2+} binding site II mutation. Satisfactory yields (25 mg TnC/liter of culture) were obtained by innoculating 500 ml LB-50 mg/liter ampicillin with a single colony from a fresh transformation, growing the cells overnight, and then adding 0.4 mM isopropyl β-D-thiogalactopyranoside for 4 h. Pelleted cells were resuspended in 13 ml iced 25 mM Tris HCl (pH 7.5), 20% sucrose, 1 mM EDTA, 1 mg/ml lysozyme, 5 mg/liter Nα-*p*-tosyl-L-lysine chloromethyl ketone, 5 mg/L *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.3 mM phenylmethylsulfonyl fluoride. After 1 h the cells were stored frozen overnight. The cells were thawed, 5 M NaCl was added to give a concentration of 1 M, and the cells were lysed either by a French press or by a probe sonicator. After a 1-h centrifugation at 46,000 rpm, the pellet was re-extracted by a similar buffer that also contained 5 M urea and 0.1% Triton X-100. The wt TnC or CBMII was extracted, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and did not pellet in 1 h at 46,000 rpm. Unlike chicken cardiac TnC (Putkey et al., 1989), the recombinant murine TnC did not bind to phenyl Sepharose, regardless of whether the CBMII mutation was present and regardless of whether divalent metal was present. Therefore, the recombinant TnC was purified by an alternative scheme, which involved successive DE52 (Whatman) and FPLC Resource Q chromatography. When necessary, an additional Sephadex G-100 step was added to achieve a homogeneous preparation.

On SDS-PAGE the wt TnC comigrated with bovine cardiac TnC (data not shown). Amino-terminal sequencing of the recombinant protein (19 cycles) at the University of Iowa protein structure facility using an Applied Biosystems 475A protein Sequencer was consistent with the expected amino acid sequence, except for the presence of 4–5% des-Met TnC. An amino acid analysis of CBMII with a Beckman 6300 analyzer was also consistent with the expected sequence. However, although the mutant protein was expected to differ by 102 Da in molecular mass in comparison to the wt TnC, it appeared by SDS-PAGE to be 1000 Da smaller than either the wt TnC or TnC purified from bovine ventricle. Therefore, laser desorption mass spectrometry was performed on the wt TnC and on CBMII, using an HP G2025A mass spectrometer. The measured molecular weights of wt TnC and CBMII differed by 124 Da, in agreement (within experimental error) with the value that the mutation would be expected to produce.

Bovine cardiac whole troponin, TnC, troponin T (TnT), TnI, and tropomyosin were purified as previously described (Tobacman and Adelstein, 1986; Tobacman, 1988), except for an additional purification step using an FPLC Resource S column (Pharmacia) for troponin I, with a 30 mM sodium citrate (pH 6), 5 M urea buffer and a 0 to 1 M NaCl gradient. Rabbit fast skeletal muscle actin (Spudich and Watt, 1971) and myosin subfragment 1 (Weeds and Taylor, 1975) were isolated as done previously.

Troponin reconstitution and labeling

Troponin T was reduced and then stoichiometrically labeled (Mehegan and Tobacman, 1991) on Cys-39 with either [2-^{14}C]iodoacetic acid (52 mCi/mmol; Amersham) or [2-^{3}H]iodoacetic acid (150 mCi/mmol; Amersham). A ternary troponin complex containing CBMII, bovine TnI, and ^{14}C-labeled bovine TnT was obtained by mixing the three subunits under denaturing conditions, performing successive dialysis steps, and perfoming Sephadex G-100 chromatography (Tobacman and Lee, 1987). Similar reconstitutions were performed to obtain troponin containing [^{3}H]bovine TnT, bovine TnI, and either murine wt TnC or bovine TnC, which differ by two conservative substitutions: Glu-115/Ile-119 bovine (van Eerd and Takahashi, 1975) versus Asp-115/Met-119 murine (Parmacek and Leiden, 1989). The murine and bovine cardiac TnCs were indistinguishable in all of the functional assays in this study. Figures showing composite results of several experiments (Figs. 4 and 6) include data points with both bovine TnC and wt murine TnC.

MgATPase assays

The actin-activated MgATPase rates were measured by extraction into isobutanol/benzene of [^{32}P]P_i molybdate (Pollard and Korn, 1973). Six successive aliquots were extracted, and rates were linear for 8 min under the conditions studied: 25°C, 5 mM imidazole HCl (pH 7.5), 3.5 mM

$MgCl_2$, 1 mM ATP, 6.5 mM KCl, 0.3 μM myosin S-1, 2.8 μM tropomyosin, and 7 μM F-actin.

Binding of radiolabeled troponin to actin·tropomyosin

Actin, tropomyosin, and troponin were incubated in 200-μl samples for 30 min at 25°C, and then centrifuged for 30 min in a TLA100 rotor at 35,000 rpm and 25°C. For one experiment the incubation was extended to 16 h before centrifugation, with no detectable effect on the results. Bound troponin was assessed by comparing the total and supernatant radioactivity in duplicate aliquots counted for 5 min. For the competitive binding of two forms of troponin to actin·tropomyosin, the fraction of each that was bound to the thin filament was assessed in comparison to binding in the absence of the competitor. Correction for overlap of ^{14}C counts into the ^{3}H channel was made before calculation of free and bound ^{14}C and ^{3}H troponin. Previous work has shown that there is no exchange of subunits between differently labeled troponin complexes after incubation at 25°C for 1 h (Mehegan and Tobacman, 1991).

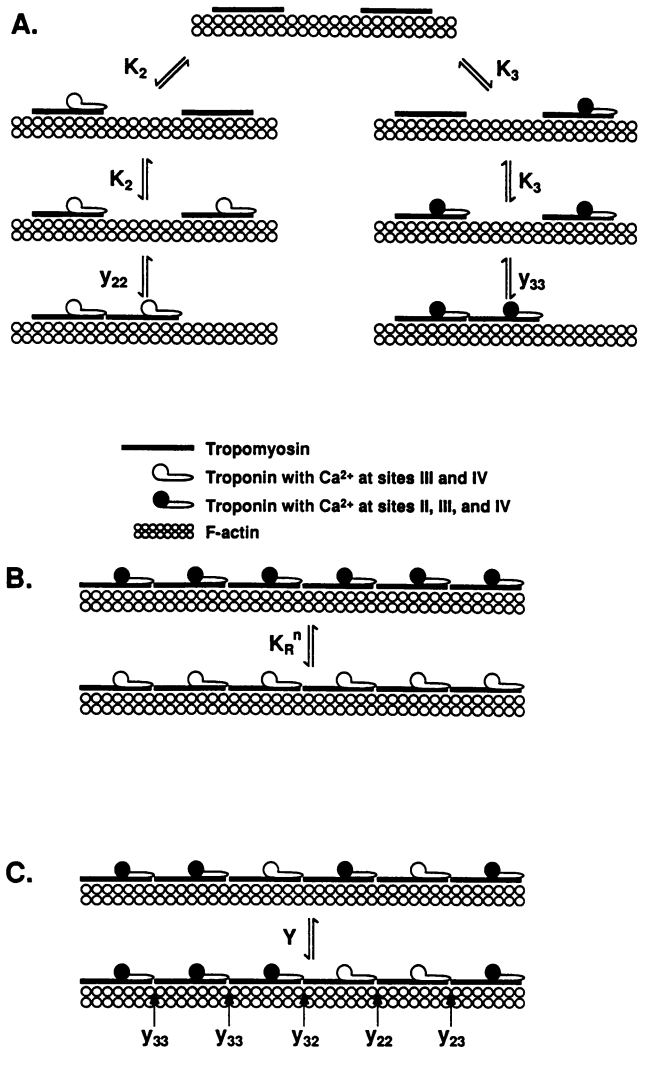

FIGURE 1 Schematic illustration of equilibrium constants governing the assembly onto the thin filament of troponin that does or does not have Ca^{2+} bound at TnC site II. (*A*) Troponin binds to an isolated tropomyosin on F-actin with an affinity constant that can be influenced by the occupancy of the TnC metal-binding sites. K_2 is the affinity constant when Ca^{2+} is bound to structural sites III and IV, but not to regulatory site II. This is accomplished experimentally by adding saturating Ca^{2+} to CBMII-troponin. K_3 is the affinity constant when all three Ca^{2+} sites are occupied (sites II, III, and IV). (Unlike skeletal muscle TnC, cardiac TnC does not bind Ca^{2+} at site I.) In other words, the subscript 2 refers to troponin with two Ca^{2+} ions bound (at sites III and IV), and the subscript 3 refers to troponin with three bound Ca^{2+} ions. The free energy of the thin filament is also influenced by unitless equilibrium constants defining the strength of the interactions between adjacent troponin·tropomyosin complexes. These constants are designated y_{22} or y_{33}, depending upon whether there are two or three Ca^{2+} ions bound to each TnC, respectively. Because troponin binding to the thin filament is relatively tight and difficult to measure, the four equilibrium constants in *A* are not individually determined in this study. (*B*) When troponin and CBMII-troponin are both present, they will compete for binding sites. The (measurable) ratio of their overall affinities for actin·tropomyosin is given by $K_R = y_{22}K_2/y_{33}K_3$. K_R is the fold change in troponin's affinity for the thin filament when Ca^{2+} is removed from the regulatory site of TnC. As shown, K_R is also the ratio, per troponin, between an all–CBMII-troponin thin filament and an all-troponin thin filament (if the free concentrations of both troponins are equal). Each thin filament has n troponins and n tropomyosins, but only part of each filament is shown (i.e., n is large). (*C*) When both forms of troponin are present, most filaments will be heterogeneous, with some of both forms bound. Therefore, the free energy (and the probability) of any thin filament configuration is influenced by four possible nearest-neighbor interaction equilibrium constants: y_{22}, y_{23}, y_{32}, and y_{33}, defined as in *A*. These parameters are not measured individually in the present experiments, but the competitive binding experiments allow determination of an important combination of all four of them: $Y = y_{22}y_{33}/y_{23}y_{32}$. As shown, Y is an equilibrium constant indicating the statistical tendency of regulatory site Ca^{2+} to be located on neighboring tropomyosins, rather than on random positions along the thin filament. Note that the upper and lower filaments in the panel differ in the numbers of each type of nearest neighbor boundary. Y is a measure of the site-site interactions that determine not only the competition between troponin and CBMII-troponin, but also the cooperativity of Ca^{2+} binding to the regulatory sites on the thin filament (Tobacman and Sawyer, 1990).

In the absence of actin and tropomyosin, no sedimentation of radioactive troponin was detected. In the absence of actin, but with the presence of tropomyosin, troponin sedimentation depended upon the troponin and tropomyosin concentrations and the ionic strength. Under the conditions used to study troponin binding to actin-tropomyosin, the fraction of troponin sedimenting in the absence of actin was 6% or less.

Theoretical analysis of competitive binding data

The thin filament resembles a one-dimensional latttice, with direct interactions between adjacent actin monomers and between neighboring troponin·tropomyosin complexes. Current atomic models of the two-stranded actin filament suggest that interstrand interactions exist but are less prominent than actin-actin contacts along each strand (Holmes et al., 1990; Lorenz et al., 1993). Neighboring troponin molecules do not directly contact each other but have the potential to indirectly influence each other via connections through tropomyosin and/or actin. These interactions can be understood in terms of various steps in thin filament assembly, as shown in Fig. 1 *A*. The quasi-equilibrium constants y_{22} and y_{33} define the strength of the interactions between adjacent troponin·tropomyosin complexes when Ca^{2+} is bound only to the Ca^{2+}/Mg^{2+} sites on each TnC, or when it is bound to all three Ca^{2+} sites on each TnC, respectively. The affinity of troponin for actin·tropomyosin will be altered by Ca^{2+} binding to the TnC regulatory site if K_2 differs from K_3 (effect of Ca^{2+} on binding to an isolated tropomyosin on actin) or if y_{22} differs from y_{33}. It is worth noting that the ratio y_{33}/y_{22} also influences the affinity of Ca^{2+} for the regulatory

sites on the thin filament (Mehegan and Tobacman, 1991; Tobacman and Sawyer, 1990). Note that the model treats the two strands of the actin filament as independent of each other with respect to troponin binding. Furthermore, it is assumed that actin-bound troponin·tropomyosin complexes do not interact unless they are positioned exactly adjacent to each other. These assumptions may be incorrect, because tropomyosin alters the actin monomer conformation (Lorenz et al., 1995) and adjacent tropomyosins do not interact solely by end-to-end contacts, but also via conformational changes in actin (Butters et al., 1993). Finally, the model includes the simplifying assumption of a strict correspondence between the local conformational state and the local presence of Ca^{2+} on TnC site II.

The competitive binding of two forms of troponin to actin·tropomyosin, under conditions where the thin filament is saturated with troponin, can be shown (Mehegan and Tobacman, 1991) to depend upon the two equilibrium constants illustrated in Fig. 1, *B* and *C*. K_R is the ratio of overall affinities of the two troponins for the thin filament, including nearest-neighbor effects as well as isolated site comparison. In terms of Fig. 1 *A*, $K_R = K_2y_{22}/K_3y_{33}$. When the free concentrations of the two troponins are equal, K_R^n equals the ratio of filaments containing only one form of troponin to filaments of the same length (n) containing only the other form of troponin. However, there will be very few such filaments. Instead, both forms of troponin will be found on individual actin filaments, with statistical distributions governed by the four different y_{ij}'s shown in Fig. 1 *C*. The definitions of y_{23} and y_{32} are analagous, in an obvious way, to those of y_{22} and y_{33} in Fig. 1 *A*. The equilibrium constant Y is dependent upon the relative values of all four of the y_{ij}'s: $Y = y_{22}y_{33}/y_{23}y_{32}$. If and only if $Y \neq 1$, does the competition between troponins display a complex pattern implying cooperativity (Mehegan and Tobacman, 1991). The value of Y is particularly significant because it not only influences these competition experiments, but also determines any cooperativity in Ca^{2+} binding to the regulatory sites on the thin filament. If $Y > 1$, this can be expected to result in cooperative thin filament activation by Ca^{2+}, independently of any additional source(s) of cooperativity (Tobacman and Sawyer, 1990).

Under conditions where the thin filament is saturated by the regulatory proteins and the two troponin forms are competing for binding sites, the partition function for the thin filament is obtained from the statistical weight matrix:

$$\begin{pmatrix} y_{22}K_2\text{Tn} & y_{23}K_2\text{Tn} \\ y_{32}K_3\text{CBMII-Tn} & y_{33}K_3\text{CBMII-Tn} \end{pmatrix}$$

where Tn and CBMII-Tn represent the free concentrations of troponin and troponin containing CBMII, respectively.

It is important to note that, although the statistical weight matrix contains eight distinct terms, the fractional saturation (θ) of the thin filament by CBMII-troponin depends only upon three specific combinations of these terms: K_R, Y, and R, which is defined as the ratio of the free CBMII-Tn to free Tn. The binding equation is obtained by differentiating the larger eigenvalue of the matrix, leading to the result (Mehegan and Tobacman, 1991)

$$\theta = \frac{2K_RR/Y}{\left[(1-K_RR)^2+\frac{4K_RR}{Y}\right]^{1/2}\left[1-K_RR+\left[(1-K_RR)^2+\frac{4K_RR}{Y}\right]^{1/2}\right]}. \quad (1)$$

RESULTS

Effect of CBMII on Ca^{2+}-sensitive regulation of the thin filament

Inactivation of the regulatory binding site of cardiac TnC results in a molecule that is constitutively inhibitory in myofibrils (Negele et al., 1992) and in muscle fibers (Putkey et al., 1989). Similarly, Fig. 2 shows that when troponin contains CBMII instead of TnC, this complex is inhibitory in an ATPase assay including tropomyosin, actin,

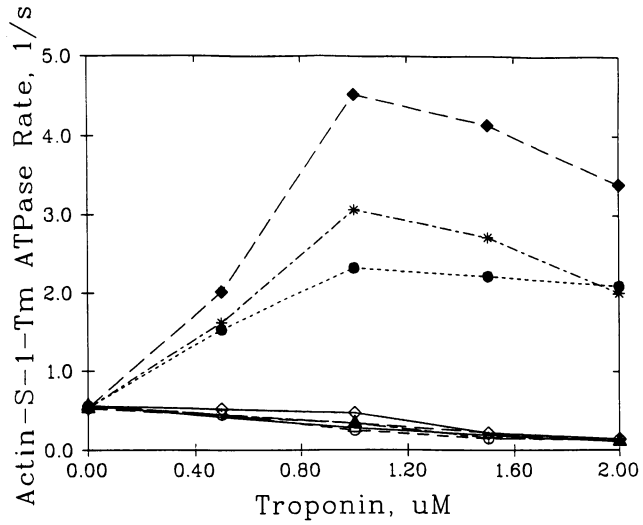

FIGURE 2 Effect of TnC mutation and troponin reconstitution on Ca^{2+}-sensitive regulation of the myosin subfragment 1-thin filament MgATPase rate. The figure shows representative measurements of the effect of various troponin preparations on the MgATPase rate of 0.3 μM myosin subfragment 1, 7 μM F-actin, and 2.8 μM tropomyosin. ◆, ●, ▲, *, 50 μM $CaCl_2$. ◇, ○, △, 0.5 mM EGTA. ◆, ◇, Native whole troponin. ●, ○, Troponin reconstituted with wild type (wt) TnC, bovine cardiac TnI, and bovine cardiac [^{3}H]TnT. *, troponin reconstituted with wt TnC, bovine cardiac TnI, and unlabeled bovine cardiac TnT. ▲, △, Troponin reconstituted with CBMII, bovine cardiac TnI, and bovine cardiac [^{14}C]TnT. The MgATPase rate inhibition by troponin in the absence of Ca^{2+} is indistinguishable from the inhibition by CBMII-troponin, whether Ca^{2+} is present or not. Note that reconstituted troponin does not activate the Mg^{2+} ATPase rate as well as native whole troponin does.

and myosin subfragment 1. As increasing concentrations of purified whole troponin are added, the MgATPase rate falls or rises, depending upon the presence of either 0.5 mM EGTA (open diamonds) or 50 μM $CaCl_2$ (filled diamonds), respectively. These results for cardiac troponin are similar to those reported previously (Tobacman and Adelstein, 1986). (Although the activating effect of troponin plus $CaCl_2$ seems large, this is partially attributable to reversal of an inhibitory effect of tropomyosin alone under these conditions.) In contrast to these results with normal troponin, the MgATPase rate is inhibited by troponin that has been reconstituted from TnI, [^{3}H]TnT, and CBMII, regardless of whether $CaCl_2$ or EGTA is present (filled and open triangles, which are superimposed in the data). This failure of activation by Ca^{2+} is primarily attributable to the TnC mutation, because reconstituted troponin including wt TnC (open circles, filled circles) confers Ca^{2+}-sensitive regulation. However, note that the MgATPase rate in the presence of Ca^{2+} and reconstituted troponin containing wt TnC (filled circles) is only about 50% the rate seen with troponin that was purified as a ternary complex and never subjected to denaturation (filled diamonds). This was a reproducible observation for all but one of 12 reconstituted troponin preparations, regardless of whether the TnT was radiolabeled on Cys-39 (filled circles) or not (*), and regardless of whether the TnC was isolated from bovine heart (not

shown) or from prokaryotes expressing murine cardiac TnC (filled circles, *). The explanation for this is not clear, but it may signify a defect in the refolding of the troponin complex from denatured subunits.

These MgATPase results serve primarily to characterize the reconstituted, radiolabeled troponins to be used (below) for studying thin filament assembly. Troponin containing TnI, CBMII, and ^{14}C-TnT is inhibitory, even in the presence of Ca^{2+}. Troponin containing TnI, TnC, and [^{3}H]TnT functions to regulate the thin filament in a Ca^{2+}-sensitive manner but does not produce as high an ATPase rate as is seen with native troponin.

Competitive binding of troponin and CBMII-troponin to actin·tropomyosin

To analyze troponin binding to the thin filament, and to determine the equilibrium constants K_R and Y, several requirements must be met. Nonspecific binding of troponin to actin (i.e., binding in excess of the amount of tropomyosin bound to actin, or binding independently of tropomyosin) can be minimized by performing experiments in the presence of high ionic strength (Hill et al., 1992). Furthermore, high ionic strength inhibits cardiac troponin binding to tropomyosin (Dahiya et al., 1994). This is important because the analysis in Eq. 1 involves the free concentrations of troponin, i.e., troponin bound neither to actin nor to tropomyosin. This can be approximated by the (measurable) non-thin filament-bound troponin concentration if the affinity of troponin for non-thin filament-bound tropomyosin is such that most of the nonsedimenting troponin is dissociated from tropomyosin. (An estimated correction for the formation of troponin·tropomyosin was calculated, using affinity constants obtained under similar conditions (Dahiya et al., 1994). This correction did not significantly alter calculations of K_R and Y, below.) Finally, to measure the interactions shown in Fig. 1 *B* the thin filament must be saturated with troponin and tropomyosin. Because high ionic strength inhibits tropomyosin binding to troponin and to actin (Hill et al., 1992; Willadsen et al., 1992; Dahiya et al., 1994), experiments were performed to determine the concentrations of these proteins required to saturate the thin filament. Fig. 3 shows that in the presence of 350 mM KCl, the binding of 2.6 μM troponin to thin filaments containing 7 μM actin reached saturation when the tropomyosin concentration was 3 μM or greater. Raising the troponin concentration above 2.6 μM did not increase its binding (data not shown).

The conditions and concentrations identified in Fig. 3 were used to study the competitive binding of troponin and CBMII-troponin to the thin filament (Fig. 4). All of the data points were obtained with the same (saturating) total concentration of the two troponins, but with the ratio of CBMII-troponin to troponin varying 100-fold, from 0.1 to 10. Composite results from several different preparations are shown in the figure, which includes data obtained both in the presence of 50 μM $CaCl_2$ (open circle) and in the presence of 0.5 mM EGTA (filled circle). As would be

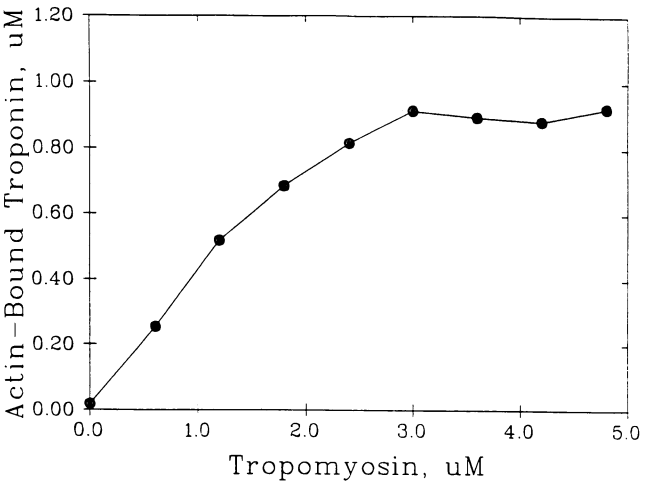

FIGURE 3 Determination of the protein concentrations required to saturate the thin filament with troponin and tropomyosin in the presence of 350 mM KCl. Troponin (2.6 μM) that had been reconstituted from wt TnC, bovine TnI, and [^{3}H]TnT was incubated with 7 μM F-actin, 350 mM KCl, 3 mM $MgCl_2$, 0.1 mM dithiothreitol, 10 mM Tris HCl (pH 7.5), 0.5 mM EGTA, and varying total concentrations of tropomyosin. The results suggest that, when the tropomyosin concentration was 3 μM or greater, the thin filament was saturated with the regulatory proteins, despite the weakening effect of high ionic strength on the interactions of actin, tropomyosin, and troponin (Dahiya et al., 1994; Willadsen et al., 1992; Butters et al., 1993).

expected, the fraction of sites occupied by CBMII-troponin increased as the CBMII-troponin:troponin ratio increased. In the absence of Ca^{2+} 50% of the sites were occupied by CBMII-troponin when the ratio was $10^0 = 1$; when the free concentrations of CBMII-troponin and troponin were equal they occupied equal fractions of the sites on the thin filament. This implies, not surprisingly, that the site II mutation has negligible effects on troponin-thin filament binding in the absence of Ca^{2+}. However, the figure also shows that, in the presence of Ca^{2+}, CBMII-troponin binds more tightly to the thin filament than does troponin. The two troponins are equally bound to actin-tropomyosin when the free CBMII-troponin concentration is less than the free troponin concentration. The degree to which the curve is shifted by Ca^{2+} is determined by K_R, the ratio of the two troponins' affinities for actin·tropomyosin. More significantly, K_R is the factor by which troponin's affinity for the thin filament is increased by the removal of Ca^{2+} from site II and has a value of 2.2 $\pm$ 0.1 from these data.

The solid lines in Fig. 4 represent the best fit of the data to Eq. 1. The slopes of these transitions are directly related to Y (Fig. 1). In the absence of Ca^{2+}, $Y = 0.9 \pm 0.1$, signifying no cooperativity, which is what would be expected for competition between two troponins that are both inhibitory and indistinguishable under these conditons. The curve in the presence of Ca^{2+} is only slightly steeper, $Y = 1.25 \pm 0.11$. This has marginal significance statistically and would have a minimal effect on the cooperativity of muscle activation.

For the reasons discussed above, the experiment shown in Fig. 4 was performed in the presence of high ionic strength.

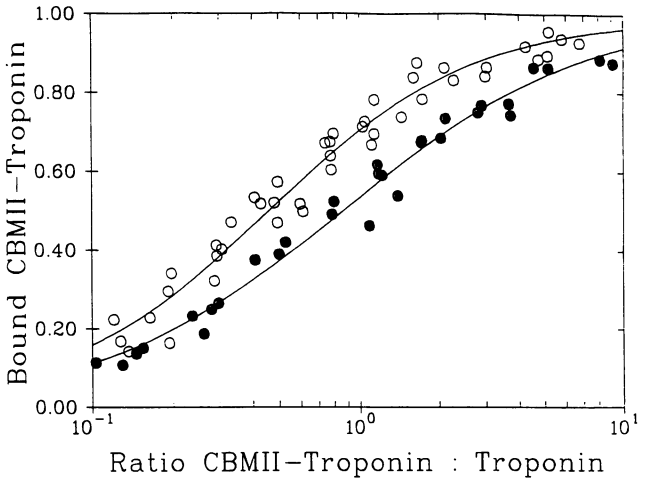

FIGURE 4 Ca^{2+} binding to TnC site II alters the binding of troponin to actin·tropomyosin. Analysis by competition. Same conditions as Fig. 3, except for the presence of either 0.5 mM EGTA (λ) or 50 μM $CaCl_2$ (μ). The total concentration of troponin plus CBMII-troponin was 2.5 μM for all points, but their ratio was varied. The abscissa shows the measured ratio of the free concentrations of the two forms of troponin. Note the logarithmic scale, showing that this ratio varied 100-fold. The ordinate shows the fraction of the sites occupied by CBMII-troponin. The solid lines represent the best fits of Eq. 1 to the data and indicate the values of Y and K_R listed in Table 1. In the presence of EGTA, both troponin and CBMII-troponin have Mg^{2+} bound to sites III and IV, and the two troponins compete equally well for thin filament binding. In the presence of $CaCl_2$ the curve is shifted to the left, indicating that CBMII troponin binds more tightly than troponin to the thin filament. Both forms of troponins have Ca^{2+} at sites III and IV, so the leftward shift measures the specific effect of site II Ca^{2+} on troponin binding to actin·tropomyosin. This effect is 2.2-fold (Table 1).

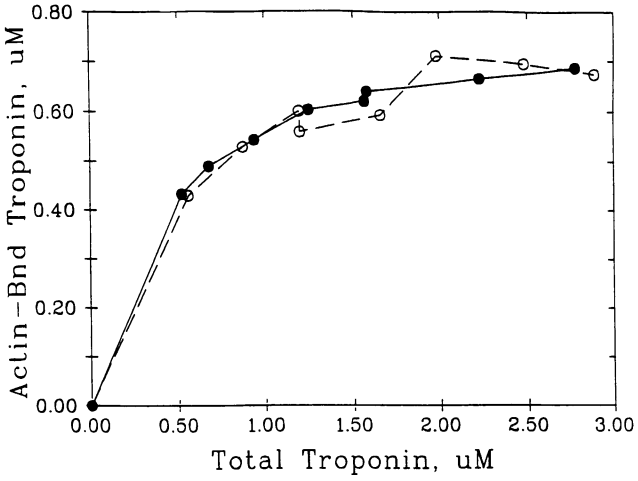

FIGURE 5 Binding of troponin to actin·tropomyosin in the presence of 150 mM KCl. Shown is an experiment indicating that conditions could be identified for analyzing thin filament assembly in the presence of an ionic strength closer to physiological conditions. Conditions: 150 mM KCl, 3 mM $MgCl_2$, 10 mM Imidazole HCl (pH 7.5), 0.5 mM 1,2-bis-(2-amino-5-bromophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (dibromo-BAPTA), 5 μM F-actin, and 1.4 μM tropomyosin. Increasing concentrations of radiolabeled troponin were added, and troponin cosedimentation with the thin filament was monitored. Both [^{3}H]troponin (λ) and [^{14}C]CBMII-troponin (μ) bound tightly to the thin filament. Note that the binding continues to slowly rise at the highest troponin concentrations. This is interpreted not to indicate incomplete saturation of the thin filament, but rather to correspond to the nonspecific association of actin and troponin that occurs at this ionic strength.

Fig. 5 shows the binding of ^{14}C-labeled CBMII-troponin or ^{3}H-labeled troponin to 5 μM actin plus 1 μM tropomyosin in the presence of more physiological ionic strength (150 mM KCl). Most of the added troponin cosedimented with the thin filament unless the total troponin concentration exceeded 0.6 μM. As still higher concentrations of troponin were added, a further, very gradual increase in troponin sedimentation was observed. This gradual increase had the same slope as the fractional binding of troponin to actin in the absence of tropomyosin (approximately 6%; data not shown) under these same ionic conditions. Therefore, the most likely explanation for the pattern in Fig. 5 is tight troponin binding to actin·tropomyosin, with superimposed weaker, nonspecific binding to additional sites on the actin filament. This experiment gave only a qualitative evaluation of these two processes, but did provide a guide for the concentration of troponin required to saturate the thin filament in the presence of 150 mM KCl, and suggested that nonspecific binding was a small proportion of total binding. Furthermore, unwanted formation of non-actin-bound troponin·tropomyosin complexes could be kept to a minimun in thin filament assembly experiments by using a tropomyosin concentration nearly equal to the available binding sites on F-actin. Parenthetically, this nonspecific binding may subtly complicate the interpretation of MgATPase rate experiments performed in the presence of excess troponin. For example, it is possible that the slightly altered regulation observed at a troponin/actin ratio of 2:7 in Fig. 2 is attributable to nonspecific troponin-actin binding.

Competitive binding of CBMII-troponin and troponin to the thin filament in the presence of 150 mM KCl is shown in Fig. 6. The results are similar to those obtained in the presence of higher ionic strength. In the absence of Ca^{2+} the two troponins behave indistinguishably from each other, resulting in values of 1.01 ± 0.05 for Y and 0.99 ± 0.02 for K_R. In the presence of Ca^{2+} the data indicate that removal of Ca^{2+} from site II causes troponin to bind to the thin filament 2.2-fold more tightly. Under these ionic conditions the observed cooperativity remains small, $Y = 1.6 \pm 0.2$, but is clearly present ($Y > 1$). This strongly suggests that thin filament-bound troponin molecules interact with each other, albeit weakly. Perhaps most significantly, these interactions depend upon the binding of Ca^{2+} to TnC site II. This implies that Ca^{2+} binding to a regulatory site on the thin filament causes a conformational change that is transmitted as far as an adjoining troponin molecule.

DISCUSSION

The striated muscle thin filament is a large, cooperatively activated (Brandt et al., 1987; Moss et al., 1986; Babu et al., 1987) system with multiple binding sites for Ca^{2+} and for myosin. Numerous interactions among proteins and among binding sites are involved in regulation. Despite the com-

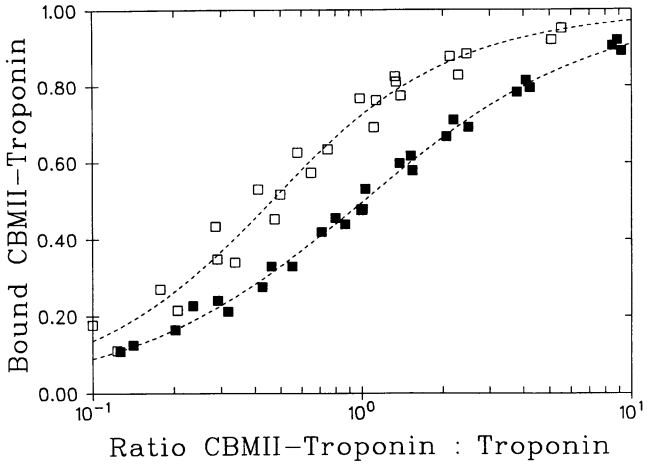

FIGURE 6 Competitive analysis of the effects of TnC site II Ca^{2+} on thin filament assembly and cooperativity. The competitition between CBMII-troponin and troponin for sites on the thin filament was assessed under the same conditions as in Fig. 4. ■, dibromo-BAPTA. □, 50 μM $CaCl_2$. Dashed lines are best fits to Eq. 1, with values for K_R and Y as listed in Table 1. The two forms of troponin bind equally and indistinguishably when there is Mg^{2+} present and no Ca^{2+} (■). The leftward shift in the presence of Ca^{2+} (θ) provides a precise measure of how much the affinity of troponin for the thin filament is strengthened by removal of Ca^{2+} from the TnC regulatory site (2.2-fold). The shape of the curve is determined by the value of the cooperativity parameter Y, which dictates the steepness of the transition. The transition is slightly steeper in the presence of Ca^{2+}, implying that Ca^{2+} binding to site II has an effect on thin filament conformation that is transmitted as far as a neighboring troponin molecule.

TABLE 1 Assembly of thin filaments containing both troponin and CBMII-troponin: effect of site II Ca^{2+} on thin filament assembly and cooperativity

	pCa = 4.3	pCa > 8
150 mM KCl		
K_R	2.18 ± 0.09	0.99 ± 0.02
Y	1.6 ± 0.2	1.01 ± 0.05
350 mM KCl		
K_R	2.19 ± 0.07	1.16 ± 0.05
Y	1.25 ± 0.11	0.89 ± 0.07

[^{3}H]Troponin and [^{14}C]CBMII-troponin were competitively bound to actin · tropomyosin in the presence of 3 mM $MgCl_2$, either 150 mM or 350 mM KCl, and either 50 μM $CaCl_2$ (pCa = 4.3) or 0.5 mM of a Ca^{2+} chelator (EGTA or dibromo-BAPTA) (pCa > 8). Shown are the results and standard error determinations for K_R and Y (see Fig. 1), as determined from the data in Figs. 4 and 6.

plexity of muscle activation, well-defined models for how site-site interactions within the thin filament mediate this process have been proposed, based upon known biochemical and structural data. For example, an elegant linear lattice model for the cooperative binding of myosin to the thin filament (Hill et al., 1980) successfully accounts for a great many experimental results (Williams et al., 1988; Greene, 1982, 1986; Ishii and Lehrer, 1990; Pan et al., 1989). A significant aspect of this model is that Ca^{2+} alone does not cause thin filament activation, but that Ca^{2+} serves to trigger myosin binding to the thin filament, which is the process that finally causes activation. Furthermore, the model implies no cooperative interactions between troponins in the absence of myosin. This latter feature is now shown to be a reasonable approximation, but a small amount of cooperativity is evident in our results.

There are substantial structural data indicating that, even in the absence of myosin, overall thin filament conformation is altered by Ca^{2+} alone (Kress et al., 1986; Lehman et al., 1994). Modeling of recent biochemical data suggests that, although cross-bridge binding may be crucial for thin filament activation, Ca^{2+} cooperatively induces a myosin-independent structural change in the thin filament (McKillop and Geeves, 1993; Head et al., 1995). This conclusion is based upon skeletal muscle results, so the observed cooperativity could reside within the N-domain of TnC, which has two Ca^{2+} binding sites. In contrast, the cooperativity observed in the present study indicates that Ca^{2+} binding to the single regulatory site of cardiac TnC has a conformational effect that is not purely local, but rather results in a measurable interaction (via tropomyosin and/or actin) with the regulatory site of a neighboring TnC on the thin filament. We had suggested such interactions previously, based on more indirect evidence (Tobacman, 1987; Tobacman and Sawyer, 1990). Furthermore, reports on the competitive binding of troponin and TnT·TnI to the thin filament directly indicated that one troponin influenced another (Wegner and Walsh, 1981; Mehegan and Tobacman, 1991). However, because these interactions between troponin and TnT·TnI were, quite unexpectedly, independent of the presence of Ca^{2+} (Mehegan and Tobacman, 1991), their relationship to regulation was problematical. The current study was designed to directly test whether such interactions existed when all three troponin subunits were present, and whether the interactions were dependent upon Ca^{2+} binding to site II. The pattern of competition between troponins that do and do not have Ca^{2+} bound at the regulatory site (i.e., the shape of the curve in Fig. 6) can only be explained by the existence of these very long-range interactions. However, there is nothing in the data to prove that these Ca^{2+}-sensitive interactions are within the troponin-tropomyosin strand. In a general sense, our data are consistent with any type of long-range conformational effect of Ca^{2+} on the thin filament, with the proposed movement of tropomyosin (Huxley, 1972; Lehman et al., 1994; Parry and Squire, 1973; Haselgrove, 1972) as a notable example.

Although the current results imply an interaction between TnC's, the strength of this TnC-TnC interaction, reported here for the first time, is quite small. In the absence of other sources of cooperativity, the values for Y in Table 1 would produce a Hill coefficient of only 1.2–1.3 (see Tobacman and Sawyer, 1990), much less than what is observed for pCa/tension data using both cardiac and skeletal muscle skinned fibers (Brandt et al., 1987; Babu et al., 1987; Fabiato and Fabiato, 1978; Kentish et al., 1986; Sweitzer and Moss, 1990; Metzger et al., 1993). This suggests that cooperative muscle activation by Ca^{2+} is primarily attributable to cooperative cross-bridge effects, described in many previous studies. Emerging but incomplete structural data from skeletal muscle are consistent

with this interpretation (Poole et al., 1995). It should be noted, however, that the competitive binding data in the present study may have underestimated the true value for the cooperativity parameter Y. This reservation is due to the impaired ATPase activation of thin filaments containing the radiolabeled, reconstituted troponin used in the competitive binding experiments. This impaired ATPase activation may have also resulted in altered Ca^{2+}-sensitive interactions between neighboring troponin and CBMII-troponin. Furthermore, any gaps between tropomyosins or incomplete saturation of the thin filament would have diminished the level of cooperativity detected in the competitive binding experiments. It seems reasonable to consider our measured values for Y as a lower boundary for the true value, which in any case is too low to be a sufficient explanation for cooperative muscle activation by Ca^{2+}.

Although K_R is a ratio fundamental to the long-standing proposal that Ca^{2+} causes a release of TnI from actin (Hitchcock et al., 1973; Potter and Gergely, 1974; Margossian and Cohen, 1973), CBMII has now provided the first direct means for measuring this important value. Ca^{2+} binding to the TnC regulatory site has a 2.2-fold effect on troponin binding to the thin filament. Remarkably good agreement was found between results in the presence of either 150 mM or 350 mM KCl, indicating that Ca^{2+} has an equally strong effect regardless of the ionic strength. It is possible that the incomplete ATPase activation, discussed above, may be a clue that this is an underestimate for K_R. However, our prior indirect determination of a similar ratio, the relative thin filament affinities of 3-Ca^{2+} troponin and 2-Mg^{2+} troponin, also suggested a low value for this parameter (Dahiya et al., 1994; Fisher et al., 1995). The present results are more definitive and pertain specifically to the effect of Ca^{2+} at the regulatory site. The small value for K_R and the small effect of Ca^{2+} on troponin binding to tropomyosin suggest two alternative interpretations. One possibility is that the azimuthal repositioning of tropomyosin (Lehman et al., 1994) involves Ca^{2+}-regulated, tight binding of troponin to two different locations on the actin filament, but with an affinity differing by only a factor of two. Alternatively, Ca^{2+} may be causing a more subtle conformational change in the thin filament that is more allosteric than steric (Chalovich and Eisenberg, 1982). Our results do not distinguish between these possibilities. However, they do establish that regulation by Ca^{2+} consists of a switch between two energetically similar states of troponin binding to the thin filament and does not involve the substantial change in binding energy one would expect from a release of TnI-actin binding alone. If there is a substantial release of troponin-thin filament binding on muscle activation, it must come (as suggested in a preliminary report; Cassell and Tobacman, 1995) from effects of cross-bridges on the thin filament, rather than from a direct effect of Ca^{2+}.

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