Dual Regulatory Functions of the Thin Filament Revealed by Replacement of the Troponin I Inhibitory Peptide with a Linker*

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Striated muscles are relaxed under low Ca2+ concentration conditions due to actions of the thin filament protein troponin. To investigate this regulatory mechanism, an 11-residue segment of cardiac troponin I previously termed the inhibitory peptide region was studied by mutagenesis. Several mutant troponin complexes were characterized in which specific effects of the inhibitory peptide region were abrogated by replacements of 4–10 residues with Gly-Ala linkers. The mutations greatly impaired two of troponin’s actions under low Ca2+ concentration conditions: inhibition of myosin subfragment 1 (S1)-thin filament MgATPase activity and cooperative suppression of myosin S1-ADP binding to thin filaments with low myosin saturation. Inhibitory peptide replacement diminished but did not abolish the Ca2+-dependence of the ATPase rate; ATPase rates were at least 2-fold greater when Ca2+ rather than EGTA was present. This residual regulation was highly cooperative as a function of Ca2+ concentration, similar to the degree of cooperativity observed with WT troponin present. Other effects of the mutations included 2-fold or less increases in the apparent affinity of the thin filament regulatory Ca2+ sites, similar decreases in the affinity of troponin for actin-tropomyosin regardless of Ca2+, and increases in myosin S1-thin filament ATPase rates in the presence of saturating Ca2+. The overall results indicate that cooperative myosin binding to Ca2+-free thin filaments depends upon the inhibitory peptide region but that a cooperatively activating effect of Ca2+ binding does not. The findings suggest that these two processes are separable and involve different conformational changes in the thin filament.

Ca2+ binding to the troponin-tropomyosin complex switches striated muscle contraction on and off by altering myosin’s mechanical and enzymatic interactions with thin filaments (1, 2). Despite many years of investigation, the inactivation mechanism that occurs when Ca2+ is dissociated is not well understood. Muscle inhibition depends upon Ca2+ dissociation from troponin subunit TnC,2 which triggers the release from TnC of an amphiphilic “switch” helix of the troponin subunit TnI. It is unclear how this causes muscle relaxation, in large part because the structure of the thin filament is not known at atomic resolution.

One aspect of regulation is that tropomyosin’s azimuthal position on actin filaments is troponin-modulated in a Ca2+-controlled manner. Intermediate resolution studies of purified thin filaments and muscle fibers show that Ca2+ binding to TnC shifts tropomyosin’s position on actin, reducing tropomyosin’s steric interference with strong myosin-actin binding (3–5). Such steric interference with myosin must be relieved for contraction to occur. Electron microscopy indicates that the effect of Ca2+-free troponin on tropomyosin’s azimuthal position (inhibitory to contraction) is attributable to the TnI C-terminal ~75 residues (6). Correspondingly, extensive biochemical work indicates that the inhibitory functions of TnI are due to this region of the subunit, a region in which the TnI switch helix is a small internal part (7–16). These findings have established some aspects of the overall regulatory mechanism and of troponin’s role in mediating inactivation at low Ca2+ concentrations.

One of several unknown aspects (and the instigation for this study) is to explain how troponin’s inhibitory effects can be mimicked by a 12-residue TnI peptide termed the inhibitory peptide. The isolated inhibitory peptide suppresses actomyosin ATPase activity, an effect that can be reversed by Ca2+-dependent binding of the peptide to TnC. The peptide gains inhibitory potency in the presence of tropomyosin. Ca2+-triggered changes in this region have been demonstrated in several experimental contexts (17–20), and point mutations in it can derange troponin’s inhibitory capacity (21–25). The binding of the isolated inhibitory peptide to actin, its inhibitory actions in a variety of preparations, and its Ca2+-dependent interactions with TnC all suggest that this small segment of troponin is important. More specifically, the segment may have a central and direct role in troponin’s effecting muscle relaxation in the presence of low Ca2+ concentrations.

Bounded on one end by the TnI switch helix, the inhibitory peptide region is bounded at its other end by the TnI strand of a TnI-TnT coiled coil (see Fig. 1). In Ca2+-saturated cardiac troponin, the inhibitory peptide region is a disordered loop (26, 27). In skeletal muscle troponin crystals, the region flips back and forth between alternative positions and conformations, driven by the Ca2+-dependent binding of the adjacent TnI switch helix to TnC (28). Thus, the inhibitory peptide region is a distinct, dynamic, structural element with properties consistent with a role in Ca2+-dependent regulation. Mutagenesis of

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‡ The abbreviations used are: Tn, troponin; S1, subfragment 1.
this segment can be used to investigate how the inhibitory peptide region alters the thin filament to affect or effect regulation. Accordingly, we describe the functional properties of troponins containing mutations within the inhibitory peptide sequence. Smaller or larger portions were replaced with Gly-Ala linkers. If specific binding of the inhibitory peptide region to actin and/or tropomyosin in the absence of Ca\(^{2+}\) is an essential aspect of inhibition, then replacement would abolish Ca\(^{2+}\)-dependent regulation. The results show that the mutations impaired Ca\(^{2+}\)-mediated regulation of both ATPase rates and myosin binding to the thin filament. The regulatory impairment was considerable, supporting an important role for the inhibitory peptide region. On the other hand, even for thin filaments with complete replacement of the inhibitory peptide region, Ca\(^{2+}\) removal caused a cooperative decrease in myosin-thin filament ATPase activity. This points to the co-importance of other parts of troponin as mediators of inhibition generally and of cooperative regulation specifically. The relationship of these findings to the overall regulatory mechanism is discussed.

**EXPERIMENTAL PROCEDURES**

*Protein Purification*—Striated muscle \(\alpha\)-tropomyosin, rabbit fast skeletal muscle F-actin, and myosin subfragment 1 were prepared as described previously (29–32). Human cardiac TnC and TnT were expressed in BL21(DE3) cells using pET vectors and purified using established methods (33–35), with the addition of a final purification step on an AKTA column (Resource Q for TnC and Resource S for TnT and TnI). Purified protein concentrations were determined by absorbance using extinction coefficients calculated as described by Gill and Von Hippel (36).

*Tnl Mutagenesis*—PCR was used to alter the cDNA for human cardiac TnI so that it would encode several different inhibitory peptide region mutations. The mutations were designed to replace normal residues with alanines, except in several positions, glycines were inserted instead to ensure the creation of a flexible replacement linker. The glycine positions were arbitrary, except for cloning convenience, they were inserted at positions 142 and 144. In brief, the cDNA was inserted into an altered form of the expression vector pET3d in which the ApoI site at vector bp 1 was eliminated. Next, the inserted WT sequence was altered by creation of mutationally silent restriction sites flanking the inhibitory region: for ApoI, at TnI bp 391; and for SacII, at TnI bp 449. The DNA between these sites was excised and replaced by ligation with an insert encoding changes in either 8 (mut-a) or 10 (mut-b) amino acids of the intervening region (see Fig. 1). The mut-a DNA sequence contained AgeI and KpnI sites including the codon for unchanged Thr\(^{143}\), Thr\(^{143}\) is not conserved between cardiac and skeletal muscle TnI. These two restriction sites were used to restore part of the WT sequence by insertion of WT PCR fragments either 5’ to the Thr\(^{143}\) codon (to create mut-c) or 3’ to the Thr\(^{143}\) codon (to create mut-d). All coding regions were confirmed by automated DNA sequencing at the University of Illinois Biotechnology Center.

*Troponin Complex Formation*—Complexes of TnT, TnC, and each of the TnI mutants were made by mixing equimolar amounts of each subunit under denaturing conditions and per-forming serial dialysis as described previously (37). The resultant ternary complexes were isolated using a 6-ml AKTA Resource Q column; each 1:1:1 complex eluted as a single peak regardless of the presence of inhibitory peptide region replacements. Troponin complexes were stored in the freezer at \(-80^\circ C\) in buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 0.01% Na\(_2\).

**ATPase Assays**—Steady-state MgATPase rates in the presence of myosin subfragment 1 (S1) were determined as described previously (38). The NADH-coupled assays were performed under the following conditions: 25 °C, 0.3 \(\mu\)M myosin S1, 10 mM MOPS (pH 7.1), 4 mM MgCl\(_2\), 5 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 5 \(\mu\)M F-actin, 0.8 \(\mu\)M tropomyosin, 0.8 \(\mu\)M troponin, 0.1 mg/ml lactate dehydrogenase, 1.5 mg/ml pyruvate kinase, 0.5 mM phosphoenolpyruvate, 200 \(\mu\)M NADH, 0.5 mM EGTA, and between 0 and 520 \(\mu\)M Ca\(^{2+}\). Control experiments established that the troponin and tropomyosin concentrations were saturating for each form of troponin. The ATPase reactions were started by the addition of ATP, and absorbance at 340 nm was monitored continuously for 5 min in a Cary 100 spectrophotometer. The absorbance decreased linearly throughout this period unless the NADH was consumed before 5 min.

**Competitive Measurement of the Affinity of Troponin for the Thin Filament**—Thin filament samples containing an affinity reference \(^3\)H-labeled bovine cardiac troponin (TnT Cys\(^{39}\)) (39) in saturating amounts were prepared for centrifugation. Unlabeled experimental troponin (WT, mut-a, or mut-b) was added in varying amounts to these samples, causing displacement of some of the \(^3\)H-troponin from the thin filament. Because the filaments were saturated, this displacement did not depend upon absolute troponin-thin filament affinity, which is too tight to readily measure. Instead, displacement depended upon a ratio of affinities: the experimental troponin/\(^3\)H-labeled reference troponin affinity ratio. Non-actin-bound \(^3\)H-troponin was measured as supernatant radioactivity after ultracentrifugation (35). The conditions were 25 °C, 10 mM Tris-HCl (pH 7.5), 300 mM KCl, 3 mM MgCl\(_2\), 0.2 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, 2.5–4.0 \(\mu\)M F-actin, 1 \(\mu\)M bovine cardiac troponomyosin, 0.6–0.75 \(\mu\)M \(^3\)H-troponin, and unlabeled troponin concentrations as shown in the figures.

**Binding of Myosin S1 to the Thin Filament**—Myosin S1-ADP binding to the thin filament was measured as described previously (40, 41). Briefly, a FluoroMax spectrofluorometer (HORIBA Jobin Yvon) was used to monitor the decrease in fluorescence intensity of Cys\(^{374}\) pyrene-labeled actin as increasing myosin S1 was added. Fluorescence data were analyzed as described by Rosol et al. (42). Intensities were corrected for dilution as myosin S1 was added (8% or less). The conditions were 25 °C, 10 mM Tris-HCl (pH 7.5), 170 mM KCl, 5 mM MgCl\(_2\), 2 mM ADP, 1 mM glucose, 10 units of hexokinase, 20 \(\mu\)M \(P\), \(P\)_5-di(adenosine 5’)-pentaphosphate, 0.3 mg/ml bovine serum albumin, 1 \(\mu\)M F-actin, 0.4 \(\mu\)M tropomyosin, 0.8 \(\mu\)M troponin, 0.5 mM EGTA, and either 0 or 600 \(\mu\)M Ca\(^{2+}\).

**Curve Fitting**—Nonlinear least-squares analysis was used for parameter and parameter error estimation with the program SCIENTIST (Micromath). Thin filament-activated myosin S1 ATPase rates as a function of free Ca\(^{2+}\) concentration were
assessed using a linear lattice cooperative binding isotherm (Equation 12 in Ref. 29). Data from two or more independent calcium titrations for a given troponin (WT or mutant) were fit separately, normalized, and then merged into one data set that was again subjected to curve fitting. This procedure yielded values for apparent Ca$^{2+}$ affinity and for the cooperativity parameter, $Y$. Alternatively, but less valuably because there is no specific physical interpretation, a Hill coefficient ($n_H$) could be measured from the data, providing an alternative metric of cooperativity. Fitting using both methods invariably yields a Hill coefficient equaling $Y^{45}$ (29).

Data for myosin S1 binding to the thin filament were averaged from three or four experiments and then analyzed by non-linear least-squares fitting to Equation 3 in Ref. 43. Contemporaneously obtained binding data for thin filaments with WT or mutant troponins were globally fit using the assumption that the mutations altered the stability of either the off-state (thereby changing $K_T$ and $L$ equally and oppositely) or the on-state (thereby changing $K_{T'}$ only). The troponin mutations were assumed in this procedure to have no effect on the affinity of myosin for the thin filament active state ($K_T$) or on the tendency of tropomyosin to move to the M-state position on actin cooperatively ($Y$). The magnitude of the effect of the mutations on a regulatory unit equals (WT $K_{T'/mutant} K_T$)$^3$, where $K_{T'}$ represents a myosin-independent equilibrium constant from an off-state to on-state of the thin filament. $K_T$ is expressed per actin, as was done in the original derivation (43). In the model of McKillop and Geeves (44), the term $K_{T'}$ has a somewhat similar meaning and is expressed per tropomyosin.

Competitive binding of troponin to the thin filament was assessed according to Equation 1 in Ref. 35 using least-squares fitting. This yielded values for $K_{T'}$, the ratio of the affinities of the competing troponin and [$^{3}$H]troponin for actin-tropomyosin. The experiment was repeated until suitably precise values for $K_{T'}$ or four similar experiments for each condition, analyzed globally to obtain $K_{T'}$. $K_{T'}$ values for the mutant troponins were then normalized to the identically measured $K_{T'}$ values for human control WT cardiac troponin in the presence of either EGTA or Ca$^{2+}$. After this normalization, the resultant troponin affinities for the thin filament, $K_{T'}$, indicate the -fold effects of the mutations on this process.

**RESULTS**

**Troponins Containing Mutations across the TnI Inhibitory Peptide Region**—The inhibitory peptide region was first identified as a functional 21-residue cyanogen bromide fragment of rabbit fast skeletal muscle TnI (15, 45). Work over the following decade indicated that this fragment’s inhibitory function resides in a smaller region, corresponding to residues 137–148 of human cardiac TnI (16, 46). When the Ca$^{2+}$-saturated troponin core domain structure was first solved (26), a notable feature was that cardiac TnI residues 137–147 composed a disordered or poorly ordered segment very nearly coincident with the boundaries of the inhibitory peptide residues that had been identified ~20 years previously. In this study, mutagenesis was performed across residues 137–147. Specifically, between 4 and 10 residues in the disordered region were replaced with an arbitrary Gly-Ala linker (Fig. 1). Non-conserved Thr$^{143}$ was not altered. Functional analyses were performed after reconstituting each of the mutants into a ternary TnT-TnI-TnC troponin complex.

**Effect of Mutations on Myosin S1-ADP Binding to the Thin Filaments**—Tropomyosin induces modest cooperativity in the strong actin binding of either nucleotide-free or ADP-saturated myosin S1 (47). In the absence but not in the presence of Ca$^{2+}$, this cooperativity is considerably exaggerated if troponin is also present (44, 47–49). More specifically, the initial portion of binding isotherms is suppressed by troponin-tropomyosin when the Ca$^{2+}$ concentration is low. The current panel of TnI mutants was used to determine whether this inhibitory effect of normal troponin on strong myosin binding to thin filaments is dependent upon specific effects of the inhibitory peptide region. Strong myosin binding to thin filaments was assessed using pyrene-labeled actin fluorescence.

The results (Fig. 2, upper panel) show that the inhibitory peptide region is indeed critical for this inhibitory function of troponin. Myosin S1-ADP bound with high cooperativity to Ca$^{2+}$-free thin filaments containing WT troponin (closed circles), but with much less cooperativity to thin filaments containing mutant troponins (open symbols). Thus, troponin-dependent suppression of the initial portion of the binding curve was dependent upon an intact inhibitory peptide region. Replacement of four residues abolished this troponin action.

To determine the magnitude of the inhibitory effect eliminated by mutagenesis, such data can be analyzed according to alternative models of the conformational changes producing the cooperativity (44, 50–53) based upon alternative conceptions of the properties of the thin filament. The solid lines in Fig. 2 are the best fit curves using one such model (43, 54). The

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**FIGURE 1. Gly-Ala linker replacements of the inhibitory peptide region.**

*Upper panel,* cardiac troponin core domain structure, with TnI in black ribbon format, TnT in white ribbon format, and TnC in grey sphere format. Cardiac TnI residues 137–147 compose the TnI minimal inhibitory peptide region identified previously. In the high resolution structure of the troponin core domain, these residues are not ordered and are shown here as a dotted line. Note that they lie immediately N-terminal to the TnI switch helix, which binds to the Ca$^{2+}$-saturated regulatory domain of TnC (26, 28, 63). *Lower panel,* underlined residues indicate mutations examined in this study. Whereas earlier work perturbed this region more narrowly using single-residue changes, here, the region was progressively replaced with an arbitrary linker.
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The model assumes a continuous tropomyosin strand that can shift its position on actin cooperatively; steric blocking of myosin depending upon the position of tropomyosin; cooperative effects of myosin and tropomyosin on each other’s attachment to actin; and most pertinently to the present experiment, Ca\(^{2+}\)-sensitive effects of troponin on the equilibrium between more active and more inactive states of the thin filament. A change in this equilibrium constant by a factor of 4.7 ± 0.8 per seven actins, without alteration in any other parameters, results in the two fitted curves in the panel, one curve matching the WT data and the other curve fitting the grouped mutant troponin data. (Mutants were assessed as a group because the data among them were not distinguishable.) Either destabilization of the inactive state or stabilization of the active state gave indistinguishable pairs of curves. Intermediate state alteration could not be determined from these results in part because intermediate state calculations are highly model-dependent. Finally, in the presence of Ca\(^{2+}\), the mutations had no discernible effect on myosin S1-ADP binding to thin filaments (Fig. 2, lower panel). This is not surprising because myosin binding is very similar in the presence of tropomyosin alone compared with tropomyosin-troponin-Ca\(^{2+}\).

Effect of TnI Mutations on Myosin S1-Thin Filament MgATPase Rates in the Presence of Either EGTA or Ca\(^{2+}\) —The effects of the four mutational sequences on the inhibition and activation of the thin filament by troponin-tropomyosin were also examined by measuring MgATPase rates in the presence of either EGTA or saturating amounts of Ca\(^{2+}\) (Table 1, first two columns). As tabulated, the assay behaved in the manner required for such an analysis, i.e. control thin filaments containing troponomyosin and WT troponin were Ca\(^{2+}\)-regulated: steady-state ATPase rates were an order of magnitude higher in the presence of Ca\(^{2+}\) than in the presence of EGTA. Also, Ca\(^{2+}\) had a negligible effect on actin-activated myosin S1 ATPase activity in the absence of the troponin-tropomyosin complex.

Substituting mutant for WT troponin produced defective inhibition, as assessed by comparing ATPase rates measured in the presence of EGTA but different troponins. Each of the various Gly-Ala substitution mutants increased the ATPase rate measured under EGTA conditions. These mutational effects were 2.1–5.8-fold, depending upon the particular mutant, compared with the findings with WT troponin, i.e. the normalized ATPase rate with WT troponin was 0.11, and the mutations increased this value, with a range of 0.23–0.63 depending upon the specific mutant troponins (Table 1). Gly-Ala substitution of residues 139–142 in the inhibitory region (mut-d) had the smallest effect on ATPase rates of any of the mutants. Larger functional effects resulted from alteration of residues 144–147.

![Graph of Fractional Actin Saturation](image)

**FIGURE 2. Effect of TnI mutations on binding of myosin S1-ADP to thin filaments.** Under low Ca\(^{2+}\) concentration conditions, myosin S1 bound to thin filaments with exaggerated cooperativity, as shown by the differing pattern of the WT troponin data in the upper versus lower panel (●). This inhibitory effect of troponin was eliminated by each of the TnI inhibitory peptide region mutations examined, shown in these representative data. Replacement of 4 residues toward either the N-terminal (mut-d) or C-terminal (mut-c) end of the inhibitory region was sufficient to eliminate the normal high cooperativity observed in the presence of EGTA. ○, mut-a; □, mut-c; △, mut-d; ●, WT. mut-d is omitted in the lower panel for clarity. The solid lines are the best fit curves to previously derived Equation 3 (43). The fitted yields measurements of the magnitude of the effect of an intact inhibitory peptide region on the equilibrium process responsible for the normal cooperativity. Upper panel, per actin $k_r = 0.601 ± 0.036$, -fold effect of mutations on $k_r$ per tropomyosin = 4.7 ± 0.8, $Y = 200$, $K_{s} = 2.3 \times 10^6$ M\(^{-1}\), and $L = 5.6 ± 0.3$; lower panel, per actin $k_r = 0.867 ± 0.165$, $Y = 200$, $K_{s} = 1.8 \times 10^6$ M\(^{-1}\), and $L = 10$.

**TABLE 1**

| Regulatory protein | Myosin S1-actin ATPase rate (ATPase rate, $I/\mu$) | Thin filament assembly-troponin affinity | ATPase rate (ATPase rate, $I/\mu$) |
|--------------------|-----------------------------------------------|---------------------------------------|-----------------------------------|
|                    | EGTA | Calcium | $K_{s}^{+}$ ($K_{s}^{−}$) | Cooperativity (T) | EGTA | Calcium |
| None               | 0.86 ± 0.01 | 0.80 ± 0.02 | 0.69 ± 0.04 | 6.2 ± 1.2 | 1.00 ± 0.11 | 1.00 ± 0.08 |
| WT Tn + Tm         | 0.11 ± 0.01 | 1.00 ± 0.02 | 1.32 ± 0.10 | 6.9 ± 2.7 | 0.64 ± 0.06 | 0.86 ± 0.12 |
| mut-a Tn + Tm      | 0.61 ± 0.02 | 1.34 ± 0.01 | 1.40 ± 0.09 | 6.5 ± 2.1 | 0.47 ± 0.07 | 0.39 ± 0.04 |
| mut-b Tn + Tm      | 0.63 ± 0.02 | 1.60 ± 0.03 | 1.80 ± 0.06 | 6.0 ± 1.4 | ND | ND |
| mut-c Tn + Tm      | 0.51 ± 0.03 | 1.08 ± 0.02 | 1.11 ± 0.18 | 4.0 ± 2.5 | ND | ND |
either alone (mut-c) or combined with mutation of residues 139–142 (mut-a) or 137–142 (mut-b).

Mutational replacement of 4 residues (mut-c and mut-d) had a minimal effect on ATPase rates in the presence of Ca\textsuperscript{2+}/H\textsubscript{11001} (Table 1, second column). This was unexpected because single-residue Ala substitutions at these same sites tend to decrease the ATPase rate (55). Furthermore, replacements of 8 (mut-a) and 10 (mut-b) residues actually increased ATPase activity compared with WT results by 34 and 60%, respectively. This finding suggests that the normal inhibitory peptide region suppresses the level of thin filament activation in the presence of not only EGTA but also Ca\textsuperscript{2+}/H\textsubscript{11001}. Interestingly, similar findings were reported when the C terminus was truncated by 18 residues (56). Removing these residues results in tropomyosin shifting to an “enhanced” C-state position in the presence of Ca\textsuperscript{2+}/H\textsubscript{11001} and more than normal activation of myosin S1 ATPase activity (57).

Effect of Tnl Mutations on Thin Filament Activation as a Function of Ca\textsuperscript{2+}/H\textsubscript{11001} Concentration—Interestingly, none of the mutations abolished Ca\textsuperscript{2+}/H\textsubscript{11001}-dependent regulation. Rather, regardless of the mutation, Ca\textsuperscript{2+} increased the myosin S1 ATPase rate, with a range of 2.1–4.6-fold activation by Ca\textsuperscript{2+}/H\textsubscript{11001} in the presence of the mutations (Table 1, first column versus second column). This residual regulation was unexpected because of the high importance generally ascribed to the inhibitory peptide region in effecting inhibition. It strongly suggests that some other part of troponin has a Ca\textsuperscript{2+}/H\textsubscript{11001}-dependent interaction with actin and or tropomyosin. In this regard, the C terminus of Tnl is one notable possibility (9–12).

To explore this further, activation was studied as a function of Ca\textsuperscript{2+} concentration for thin filaments containing each of the four mutant troponins (Fig. 3). Progressive activation of the myosin S1-thin filament ATPase rate closely parallels fractional Ca\textsuperscript{2+}/H\textsubscript{11001} binding to the cardiac TnC regulatory sites (29). Results are shown in a normalized format, indicating the fractional increase produced at each Ca\textsuperscript{2+}/H\textsubscript{11001} concentration. If the only effects of the mutations were to remove stabilizing interactions of an inhibited thin filament state, regulatory site Ca\textsuperscript{2+}/H\textsubscript{11001} affinity would increase. Consistent with this, the mutations modestly increased the apparent Ca\textsuperscript{2+}/H\textsubscript{11001} affinity by 1.6–2.6-fold relative to WT troponin (Table 1, comparisons within the third column). These effects are in the same direction and are similar in magnitude to those observed by alanine scanning single-residue changes in the inhibitory region (55). Thus, there is no additive effect from replacing the entire region with a flexible linker, relative to the effects of single-residue changes, on the Ca\textsuperscript{2+}/H\textsubscript{11001} affinity of the thin filament regulatory sites.

Significantly, the mutations hardly diminished the cooperativity of activation by Ca\textsuperscript{2+}/H\textsubscript{11001}. The shapes of the curves in each panel of Fig. 2 indicate that regardless of the mutation and regardless of whether inhibition in the presence of EGTA was defective, residual regulation by Ca\textsuperscript{2+}/H\textsubscript{11001} was distinctly cooperative. The solid lines are the best fit curves using a linear lattice model to measure the cooperativity (Table 1). In this model, the Ca\textsuperscript{2+}/H\textsubscript{11001} affinity of a given troponin is increased Y-fold if Ca\textsuperscript{2+} is already bound to a linearly adjacent troponin along the length of the thin filament (29). The data indicated Y ≈ 6 regardless of...
which troponin was present, implying a 6-fold effect of one troponin on the adjacent troponin. mut-d may have had somewhat less cooperativity, but the data in Fig. 3D are not sufficiently consistent to be certain. Regardless of this uncertainty, overall, the results show that this cooperative interaction did not depend upon the presence of the normal inhibitory peptide region of TnI.

Effect of Mutations on Troponin Binding to the Thin Filament—
To further characterize the mutational alterations in function, the effects of the TnI mutations on the affinity of troponin for actin-tropomyosin were measured. Normally, troponin binds tightly to actin-tropomyosin, with an affinity of $10^8$ M$^{-1}$ or greater (58). To assess the effects on this tight attachment, a competitive assay was used (35), focusing on the troponins with the greatest mutational changes, mut-a and mut-b. A radioactively tagged troponin was added to actin tropomyosin in saturating amounts. As increasing amounts of a competitor troponin (WT or mutant) were added, the displacement of this troponin from the thin filament was measured via supernatant radioactivity after thin filament centrifugation. Fig. 4 shows the results, which depended upon the concentration of troponin-binding sites, the amount of labeled troponin, and the ratio of relative affinities of unlabeled and labeled troponins. Each panel shows three separate experiments of the same competition, all fit globally to a single affinity ratio.

Both mutants were able to displace labeled troponin from thin filaments under both high and low Ca$^{2+}$ concentration conditions. Thus, even the most altered of the troponins examined in this study bound tightly to thin filaments, displacing a strongly attached control troponin. Normalizing WT $K_{Tn}$ = 1.00 ± 0.11 to the findings with WT troponin in last two panels in Fig. 4, the data indicate that $K_{Tn}$ = 0.64 ± 0.06 and 0.47 ± 0.07 in the presence of EGTA for mut-a and mut-b, respectively (Table 1). This modest decrease in affinity caused by the TnI mutants could mean that this region of TnI is directly involved in binding to actin-tropomyosin. However, the effect is small. Furthermore, it is notable that the effects of the mutations on $K_{Tn}$ in the presence of Ca$^{2+}$ were quantitatively similar to the effects of the mutations in the presence of EGTA (Table 1, compare last two columns).

DISCUSSION

This study supports an important role for the inhibitory peptide region in muscle regulation. The inhibitory properties of troponin were considerably impaired by replacement of the region with a linker in which any specific interactions of the WT residues with actin and tropomyosin were eliminated. Myosin S1-thin filament ATPase activity was poorly suppressed in the presence of EGTA, relative to the normal effects of Ca$^{2+}$ removal. Similarly, mutation of the inhibitory region
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eliminated the highly cooperative interference of troponin-tropomyosin with myosin S1-ADP binding to thin filaments under low Ca\(^{2+}\) concentration conditions. Quantitatively, this effect can be rationalized by elimination of the 5-fold contribution of the inhibitory peptide region to the equilibrium away from a thin filament state(s) that is permissive for strong myosin binding. These results confirm previous data derived from other types of experiments that this region of TnI helps to effect muscle relaxation.

An interesting implication of this study is that the inhibitory peptide region is not the sine qua non of the relaxation mechanism for striated muscle. Previous work showed that single-residue changes in the inhibitory region could degrade troponin function significantly (14, 55, 59). Our work shows that this functional degradation is not amplified by replacing the entire inhibitory region. Residual 2-fold Ca\(^{2+}\)-dependent regulation can occur without the inhibitory region sequence present. How might this limited regulation be effected? A simple or partial answer may be that the inhibitory region is but one part of a larger C-terminal region of TnI that holds tropomyosin in a position that blocks strong myosin binding to actin (6) unless this blocking is relieved by Ca\(^{2+}\)-dependent association of the TnI switch helix with the TnC N-terminal domain. In other words, the mutational replacement of the inhibitory region may impair the stability or the fractional occupancy of such a blocked state, without eliminating it.

However, one cannot fully rationalize our findings by proposing that the inhibitory peptide region and TnI residues more C-terminal act together to stabilize a blocked position for tropomyosin. This mechanism readily explains the effects of the mutations on myosin S1-ADP binding to the thin filament and on ATPase rates in the presence of EGTA. It leaves unexplained, however, how Ca\(^{2+}\) cooperatively increases the ATPase rate in the presence of the mutant troponins. A separate mechanism, different from that described above, must underlie the preserved Ca\(^{2+}\) cooperativity (Fig. 3) in the setting of thin filaments lacking troponin’s cooperative effects on myosin binding (Fig. 2). One possibility is that the two troponins in longitudinal register, on opposite long pitch actin strands, jointly affect myosin activity, as suggested by the location of the TnI C terminus, which spans the two actin strands (6). The inhibitory peptide region is within or immediately proximate to the core domain and therefore does not comprise this strand-to-strand bridge. It is difficult to state the likelihood of any such proposal, however, because structural features that have not yet been identified may be essential to the regulatory mechanism.

The high resolution structure of the troponin core domain has been docked onto thin filaments only provisionally, and some parts of troponin have not been solved at all at atomic resolution. Also, there are no structural findings to explain how the TnI C-terminal 18 residues alter tropomyosin position on actin in the presence of high Ca\(^{2+}\) concentrations (57). Perhaps troponin movement accompanies tropomyosin’s movement to the latter’s active M-state position (5), and this does not require the inhibitory peptide.

Regardless of the above uncertainties, these results imply several aspects of the cooperative ATPase mechanism: it does not greatly depend upon the inhibitory peptide region; it is an effect transmitted between troponins; and it can exist in the absence of troponin’s inhibitory effect on strong myosin binding to the thin filament. If the latter is due to changes in tropomyosin position on actin, then the ATPase cooperativity involves some other Ca\(^{2+}\)-dependent change in the thin filament.

Significantly for the above interpretation, independent evidence suggests that the control of tropomyosin position on actin is not the entire explanation for regulation. Transient kinetic results show that Ca\(^{2+}\) regulation of myosin S1-thin filament ATPase activity is rather profound, several hundredfold (60, 61), as would be expected for an on-off switch of muscle contraction. Contrarily, Ca\(^{2+}\) appears to have a much smaller effect on the distributions of tropomyosin among its three azimuthal positions on actin (62). Similarly, the calculated effects of Ca\(^{2+}\) on thin filament states, based on analyses of strong myosin binding to actin (44), are not as great as are the effects of Ca\(^{2+}\) on thin filament activation inferred from actomyosin ATPase kinetics. Thus, changes in tropomyosin position on actin provide a compelling explanation for how Ca\(^{2+}\) controls strong myosin binding to actin, but are a doubtfully complete explanation for the regulation of the myosin-actin kinetic mechanism essential for force production. This study suggests that the more complete explanation, i.e. the missing parts, whatever their structural basis, involves regulatory site Ca\(^{2+}\) cooperativity and interactions between troponins and does not depend upon the inhibitory peptide region of TnI.

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