Cardiomyopathic Tropomyosin Mutations That Increase Thin Filament Ca\textsuperscript{2+} Sensitivity and Tropomyosin N-domain Flexibility*

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The relationship between tropomyosin thermal stability and thin filament activation was explored using two N-domain mutants of α-striated muscle tropomyosin, A63V and K70T, each previously implicated in familial hypertrophic cardiomyopathy. Both mutations had prominent effects on tropomyosin thermal stability as monitored by circular dichroism. Wild type tropomyosin unfolded in two transitions, separated by 10 °C. The A63V and K70T mutations decreased the melting temperature of the more stable of these transitions by 4 and 10 °C, respectively, indicating destabilization of the N-domain in both cases. Global analysis of all three proteins indicated that the tropomyosin N-domain and C-domain fold with a cooperative free energy of 1.0–1.5 kcal/mol. The two mutations increased the apparent affinity of the regulatory Ca\textsuperscript{2+} binding sites of thin filament in two settings: Ca\textsuperscript{2+}-dependent sliding speed of unloaded thin filaments in vitro (at both pH 7.4 and 6.3), and Ca\textsuperscript{2+} activation of the thin filament-myosin S1 ATPase rate. Neither mutation had more than small effects on the maximal ATPase rate in the presence of saturating Ca\textsuperscript{2+} or on the maximal sliding speed. Despite the increased tropomyosin flexibility implied by destabilization of the N-domain, neither the cooperativity of thin filament activation by Ca\textsuperscript{2+} nor the cooperative binding of myosin S1-ADP to the thin filament was altered by the mutations. The combined results suggest that a more dynamic tropomyosin N-domain influences interactions with actin and/or tropomyosin that modulate Ca\textsuperscript{2+} sensitivity, but has an unexpectedly small effect on cooperative changes in tropomyosin position on actin.

Contracting muscle is controlled by the reversible binding of calcium to the N-domain of TnC, which is the regulatory subunit of troponin (for reviews, see Refs. 1 and 2). Tropomyosin and tropomyosin bestow Ca\textsuperscript{2+} dependence on the productive interactions of actin and myosin: rapid ATPase activity, generation of force, and generation of movement. Three-dimensional, helical reconstructions of thin filament electron micrographs imply three positions of tropomyosin on the actin filament. These data, supported by x-ray diffraction of thin filaments (4), indicate that a major component of regulation consists of tropomyosin sterically interfering with myosin binding to actin. In the absence of Ca\textsuperscript{2+}, much of the myosin-binding site on actin is obscured by tropomyosin. Calcium binding to troponin causes tropomyosin to shift position, exposing much of the myosin-binding site. Strong actin-myosin binding requires a further repositioning of tropomyosin. These findings do not imply that steric interference fully explains regulation. For example, addition of troponin and tropomyosin to bare actin filaments increases acto-myosin affinity, force production, and sliding speed in the presence of Ca\textsuperscript{2+} (5–7). Nevertheless, as was first proposed 30 years ago (8), most recent reports (albeit not all (e.g. Refs. 9 and 10)) point to the shifting position of tropomyosin on actin as a critical aspect of regulation (3–5, 11–17).

Each muscle tropomyosin binds to one troponin and spans seven actin monomers on the thin filament. This stoichiometry implies that regulation is extended in space, with calcium ions directly affecting a regulatory unit that is seven actins long. Also, tropomyosin polymerizes in solution, and successive troponin-tropomyosin complexes form a continuous strand along the actin filament. Therefore, the thin filament not only undergoes kinetic transitions, but also can have spatial transitions. Any local shift in tropomyosin position on the actin surface, for example caused by a lone, strongly bound cross-bridge, implies spatial transition(s) between actins with tropomyosin in one position, and actins with tropomyosin in another. The highly cooperative behavior of muscle fibers and of isolated thin filaments suggests that these spatial transitions have considerable physiological importance. Thus, the flexibility of tropomyosin on the actin filament is significant for full appreciation of its regulatory function.

Recently, a new approach to understanding tropomyosin has been provided by the discovery that tropomyosin missense mutations can cause the autosomal dominant disorder familial hypertrophic cardiomyopathy (HCM). Several studies have appeared describing functional effects of these mutations in solution (18–20), in fibers or cells (21–23), and in whole animals (22, 24, 25). Most of this work (with the exception of Ref. 23) concerns the first two mutations detected, E180G and D175N, located near where tropomyosin interacts (weakly) with the globular, Ca\textsuperscript{2+} binding domain of troponin. In examining cardiomyopathic mutations A63V and K70T (26, 27), located instead in the N-terminal domain of tropomyosin, the current report combines with the earlier evidence to imply that a characteristic result of the cardiomyopathic tropomyosin mutations is to decrease thermal stability, which presumably reports an increased tropomyosin flexibility. Despite these findings, the cooperativity of thin filament activation, either by myosin or by Ca\textsuperscript{2+}, was not altered by the mutations. The data suggest that the flexibility of the tropomyosin N-domain influences Ca\textsuperscript{2+} sensitivity.

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¶ The abbreviations used are: Tn, troponin; HCM, hypertrophic cardiomyopathy; MOPS, 4-morpholinepropanesulfonic acid.
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**EXPERIMENTAL PROCEDURES**

**Protein Purification and Construct Design**—Bovine cardiac whole troponin was purified as previously described from a heart muscle ether powder (28, 29). Actin (30) and myosin S1 (31) were obtained from rabbit fast skeletal muscle. Recombinant control and mutant bovine tropomyosins were expressed in DE3 cells using vector pET3d, and purified to homogeneity as described (32). A63V or K70T mutations were introduced into cDNA encoding rat striated muscle α-tropomyosin by the same PCR-based approach used previously to create other missense mutations (33). Both wild type and mutant constructs were designed to include an N-terminal Ala-Ser dipeptide, added to functionally compensate for lack of acetylation of bacterially expressed tropomyosin (34, 35). The coding sequences of the expression plasmids were confirmed by automated DNA sequencing at the University of Iowa DNA Facility.

**Protein Folding and Circular Dichroism**—The circular dichroism of tropomyosin (θ222) was monitored using an Aviv DS65 circular dichroism spectrometer, recording as a function of increasing temperature, beginning at 5 °C. To correct for minor differences in protein concentration, data were normalized so that the average ellipticity between 5 and 9 °C was the same for all curves. Conditions were: 50 mM NaH2PO4 (pH 6.5), and either 50 or 300 mM KCl. Data were fit to a model in which N-terminal and C-terminal regions of tropomyosin fold independently, except for a stabilizing cooperative interaction between the N- and C-regions of tropomyosin, plainly evident by shifts in midpoint of the higher temperature, N-domain transition. However, note that the full thermal denaturation curves were affected by mutational N-domain destabilization, not just the higher temperature transitions, implying cooperative interactions between the N- and C-regions of tropomyosin. Solid lines are best-fit curves corresponding to parameters in Table I (50 mM KCl data).

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**Fig. 1. Thermal denaturation of control and mutant tropomyosins.** Increasing temperature caused a biphasic change in the circular dichroism signal produced by control tropomyosin (filled circles), consistent with two unfolding transitions. Mutations A63V (squares) and K70T (open circles) destabilized the N-terminal portion of tropomyosin, plainly evident by shifts in midpoint of the higher temperature, N-domain transition. However, note that the full thermal denaturation curves were affected by mutational N-domain destabilization, not just the higher temperature transitions, implying cooperative interactions between the N- and C-regions of tropomyosin. Solid lines are best-fit curves corresponding to parameters in Table I (50 mM KCl data).
RESULTS

Effects of Tropomyosin Mutations on Thermal Stability—Tropomyosin folding stability is weakened by HCM mutation E180G, and by mutation D175N in pyrene-labeled tropomyosin. Both mutations are located in a tropomyosin region that interacts with troponin, albeit weakly (1). Similar assessments of folding stability have not been reported for mutations in the N-terminal half of tropomyosin, far from residues directly binding to troponin. However, structural reports suggest that such mutations might alter protein folding. Mutation V95A, located in the N-terminal half of tropomyosin, far from residues directly binding to troponin, also affects protein folding and induces tropomyosin bending (43). Lysine 70, eliminated in patients with mutation K70T, forms a stabilizing intrahelical salt bridge with Asp-66 in the x-ray structure of tropomyosin fragment 1–81 (43). Of the four identical strands in the unit cell (on two molecules), the one (helix B) with this salt bridge is the most ordered locally, i.e. its C-terminal portion has the lowest refined temperature factors.2

Against this background, circular dichroism was employed to monitor protein folding as a function of temperature for wild type tropomyosin and for HCM mutants K70T and A63V (Fig. 1). At intermediate temperatures, each mutation significantly altered \( \theta_{222} \), a measure of \( \alpha \)-helical content. Wild type tropomyosin unfolded in two distinct transitions, separated by \( \sim 10^\circ C \). The lower temperature transition can be assigned to the more C-terminal portion of tropomyosin, both previous work indicates that tropomyosin is more stable toward its N terminus (44, 45), and because these N-terminal mutations primarily alter the higher temperature transition. The N-terminal transition is smaller than the C-terminal transition: \( \sim 98 \) versus \( 185 \) peptide bonds, respectively, based on fitting to the model described below. A63V decreases the N-terminal transition midpoint (\( T_m^N \)) by \( 3.7^\circ C \) (Table I). For K70T tropomyosin, the shift in \( T_m^N \) is more pronounced \( (10.3^\circ C) \), and the C-terminal transition also is plainly affected.

Insight into tropomyosin folding thermodynamics per se, plus further analysis of the mutations, was obtained by global fitting of an equilibrium model to \( \theta_{222} \) data from all three molecules. Because two transitions are evident for wild type tropomyosin, the model adopts the approximation that tropomyosin has two folding domains under the examined conditions. The A63V and K70T mutations affect only the more N-terminal domain in the model, i.e. the unfolding enthalpy of the domain \( (\Delta H_m^N) \), and/or melting temperature \( (T_m^N) \). The CD results indicate major changes in these parameters (Table I), implying that the mutations affect the structure and dynamics of a large region of tropomyosin. Furthermore, both mutations also affect (indirectly) the behavior of the more C-terminal portion of tropomyosin, as is particularly evident for K70T. The entire circular dichroism curve is shifted toward lower temperatures, not just the portion of the curve above \( 40^\circ C \). This shows that cooperative interactions within tropomyosin span virtually the full length of the coiled-coil, and that a tropomyosin point mutation can affect the entire molecule in this sense.

Fig. 1 was more precisely analyzed by assuming there is a cooperative interaction free energy (36) between two folding domains \( (\Delta G_i) \), which is lost when either of them unfolds. This is equivalent to stating that the N-and C-terminal regions can fold separately, but that the results imply that folding together is accompanied by a facilitating interaction. In this model, N-domain mutations should have little effect on \( \Delta G_i \) itself, on C-domain folding thermodynamics \( (T_m^C, \Delta H_m^C) \), or on the relative sizes of C- versus N-domain transitions. With these assumptions, global analysis of all three tropomyosins revealed \( \Delta G_i = 1.5 \pm 0.1 \) kcal/mol in the presence of \( 50 \) mM KCl and \( 1.0 \pm 0.3 \) in the presence of \( 300 \) mM KCl (Table I). These calculations should be interpreted cautiously, because other experiments (46–48) imply that tropomyosin folding is more complex than accounted for by this two-domain model. Additionally, the present data do not permit separate assessment of dimerization, which is omitted from the model. However, the measurement of a significant value for \( \Delta G_i \) implies long range

\[ \text{Table I} \]

|            | \( T_m^N \) | \( \Delta H_m^N \) | \( T_m^C \) | \( \Delta H_m^C \) | \( \Delta G_i \) |
|------------|------------|------------------|------------|------------------|----------------|
|            | °C         | kcal/mol         | °C         | kcal/mol         | kcal/mol       |
| 50 mM KCl  |            |                  |            |                  |                |
| Wild type  | 47.3 ± 0.1 | 98 ± 3           | 77 ± 4     |                  |                |
| A63V       | 43.6 ± 0.1 | 77 ± 4           | 45 ± 4     |                  |                |
| K70T       | 36.9 ± 0.1 | 45 ± 4           | 35 ± 0.3   | 109 ± 2          | 1.5 ± 0.1      |
| All forms  | 38 ± 1     | 88 ± 2           | 73 ± 6     |                  |                |
| 300 mM KCl |            |                  |            |                  |                |
| KCl        |            |                  |            |                  |                |
| Wild type  | 49.6 ± 0.2 | 103 ± 5          | 66 ± 5     | 4                  |                |
| A63V       | 45.8 ± 0.1 | 66 ± 5           | 73 ± 6     |                  |                |
| K70T       | 42.1 ± 0.1 | 73 ± 6           | 38 ± 1     | 88 ± 2            | 1.0 ± 0.3      |

2 J. H. Brown and C. Cohen, personal communication.
cooperativity within tropomyosin. This is consistent with the folded tropomyosin structure having a continuous α-helix, as is likely from its amino acid sequence.

**Effects on In Vitro Motility—**Actin filament sliding over a heavy meromyosin-coated surface is in several respects a suitable, purified protein correlate of the unloaded shortening velocity of muscles fibers (41, 42). This correlation is now shown to apply to the complex effects of pH on troponin-regulated thin filaments. Consistent with muscle fiber results in the absence of Ca²⁺, acidic pH decreases maximal sliding speed, decreased the apparent Ca²⁺ sensitivities were increased. The apparent Ca²⁺ affinities when binding is to adjacent as opposed to isolated sites (1). For ligand binding to Ca²⁺, the -fold increase in ligand sensitivity only slightly, 1.2-fold for K70T and 1.5-fold for A63V (see “Results”). Notably, the cooperativity of ATPase activation was not significantly affected by the mutations.

![Figure 3](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**Fig. 3.** Thin filament-myosin S1 ATPase rate regulation. Ca²⁺-sensitive ATPase regulation was similar for thin filaments containing control (triangles), K70T (circles), or A63V (diamonds) tropomyosin. The mutations increased the apparent Ca²⁺ sensitivity only slightly, 1.2-fold for K70T and 1.5-fold for A63V (see “Results”). Notably, the cooperativity of ATPase activation was not significantly affected by the mutations.

**Activity.** Similar to findings with many other HCM-causing thin filament mutations (33, 54–60), regulation was preserved. Addition of Ca²⁺ increased the ATPase rate more than 10-fold, regardless of the presence of either mutation (Fig. 3). However, Ca²⁺ sensitivities were increased. The apparent Ca²⁺ affinities were 3.10 ± 0.11 × 10⁵ M⁻¹ (pCa₅₀ 5.49 ± 0.01) for control ASTm, 3.73 ± 0.11 × 10⁵ M⁻¹ (pCa₅₀ 5.57 ± 0.01) for K70T tropomyosin, and 4.63 ± 0.15 × 10⁵ M⁻¹ (pCa₅₀ 5.66 ± 0.01) for A63V tropomyosin. The changes in affinity are small, 20% for K70T and 50% for A63V, corresponding to 0.08 and 0.17 on a log scale.

ATPase activation by Ca²⁺ was highly cooperative for all three tropomyosins, despite the fact that cardiac troponin has only one regulatory Ca²⁺ binding site (1). For ligand binding to a series of sites (e.g. many troponins) along a linear lattice such as the thin filament, Y ≈ nH is the -fold increase in ligand affinity when binding is to adjacent as opposed to isolated sites (39, 61). The cooperative ATPase activation of thin filament-myosin S1 ATPase regulation was similar for thin filaments containing control (triangles), K70T (circles), or A63V (diamonds) tropomyosin. The mutations increased the apparent Ca²⁺ sensitivity only slightly, 1.2-fold for K70T and 1.5-fold for A63V (see “Results”). Notably, the cooperativity of ATPase activation was not significantly affected by the mutations.

**Effects on Cooperative Binding of Myosin S1 to the Thin Filament—**Myosin S1 binds very cooperatively to the thin filament in the absence of both Ca²⁺ and ATP. The full structural
basis for this is not clear, but an important component may be a rigidity in tropomyosin position on actin. Tropomyosin sterically interferes with the myosin-binding site on actin in the thin filament B-state, observed in the absence of Ca\(^2+\) or myosin. For filaments saturated with myosin (regardless of Ca\(^2+\)), the position of tropomyosin is shifted, and in this M-state position there is no steric interference between myosin and tropomyosin (3). According to this mechanism of cooperativity, an energetic cost results from shifts in multiple short sections of the tropomyosin polymer, resulting in multiple B to M transition points along a thin filament, relative to the free energy with fewer transition points and fewer but longer sections shifting position (5). A more weakly folded tropomyosin, such as the A63V and K70T mutants, might be more flexible, diminishing this contribution to the cooperativity of myosin binding. However, neither mutation affected cooperative binding of S1-ADP to regulated thin filaments in the absence of Ca\(^2+\) at 25 °C (Fig. 4A). (Additionally, they had minimal effect on less cooperative S1-thin filament binding in the presence of Ca\(^2+\) (Fig. 4B).) For both control and mutant thin filaments, the binding curves in the absence of Ca\(^2+\) were S-shaped, indicating high and indistinguishable cooperativity. Furthermore, this was not the result of the temperature of the experiment, 25 °C. At 37 °C there is significant unfolding of the N-domain of K70T tropomyosin in the absence of actin or troponin (see Fig. 1). Nevertheless, the K70T mutation had no effect on cooperative S1-thin filament binding at this higher temperature (Fig. 4C). Similar results were obtained for A63V tropomyosin (data not shown).

**DISCUSSION**

Of the tropomyosin mutations implicated in cardiomyopathy, E180G and D175N were the first identified (62) and at present are the best characterized, both at the protein level (18–20) and in transgenic mice (22, 24, 25). These mutations increase muscle Ca\(^2+\) sensitivity (21, 22, 24, 25, 63), perhaps related to their location within a putative troponin-binding region of tropomyosin (see review in Ref. 1). More surprisingly, Michele et al. (23) have shown that muscle Ca\(^2+\) sensitivity is also increased by mutations A63V and K70T, and we have shown a similar effect in solution studies of these molecules (present study) and of mutant V95A (55). Many thin filament HCM mutations increase Ca\(^2+\) sensitivity, regardless whether in TnT, TnI, or tropomyosin (reviewed in Ref. 64). However, the mechanism(s) for these effects are poorly understood, and are particularly unapparent in the case of the tropomyosin N-domain mutations.

HCM missense mutations have not, as a general rule, been detected in residues that directly participate in binding interactions or, in the case of myosin, in catalysis. Exceptions may be found as structural information advances, but current data are consistent with malfunction arising from indirect effects on the dynamics, structure, and/or interactions of protein regions in which the mutations occur. The present manuscript is concordant with this pattern, establishing that the A63V and K70T mutations diminish tropomyosin N-domain folding stability. Because E180G and D175N mutations also have destabilizing effects, this appears to be a characteristic property of HCM tropomyosins. Increased thin filament Ca\(^2+\) sensitivity is equally characteristic, making it interesting to consider whether these two properties are causatively linked.

The dynamic aspects of tropomyosin important for thin filament function almost certainly are distinct from the folding-unfolding reactions explored in the present study. Bound to actin, the tropomyosin N-domain does not unfold. Nevertheless, a more dynamic tropomyosin is likely in the setting of decreased folding stability. Furthermore, this may be functionally important, because tropomyosin flexibility, or rather, inflexibility, has been suggested as critical for cooperative regulation of myosin binding to the thin filament (see above). Surprisingly, the present data suggest that the mutant tropomyosins are more flexible, but this does not produce the ex-
spected effect on cooperative aspects of regulation. With one exception (the effect of A63V on sliding speed), the mutations failed to decrease the cooperativity of thin filament activation in several different assays. Instead, it appears that the tendency of the tropomyosin strand to shift position on many rather than few adjacent acts (3) is preserved in the presence of the mutations. The explanation for this apparent discrepancy may be that the cooperative shifting of the tropomyosin strand depends only in part on tropomyosin (or troponin-tropomyo- 

s) stiffness; it also depends on the shape of the free energy profile for tropomyosin contacting all possible positions across the actin filament surface (32, 65), and this energy profile depends on actin-tropomyosin interactions (5). In the M-state position, these interactions are very tight, and tropomyosin binds with affinity \( \geq 10^9 \text{M}^{-1} \) (66). Therefore, the data support the possibility that tropomyosin-actin interactions are more important for the cooperativity of the transition to the M-state, than is the intrinsic flexibility of the tropomyosin N-domain.

Tropomyosin N-domain dynamics could affect Ca\(^{2+}\) sensitivity by a related mechanism. The rate of the Ca\(^{2+}\)-regulated weak to strong binding transition of myosin (67, 68) may depend on tropomyosin shifting from its C-state to M-state position on actin. A more dynamic tropomyosin might lower the activation energy for this crossbridge step, increasing the apparent Ca\(^{2+}\) affinity. This would not be detected in equilibrium binding measurements, consistent with Fig. 4. It would instead affect activation in the presence of cycling myosin (Figs. 2 and 3), and cause the largest change in \( pC_{250} \) in experiments involving major cross-bridge effects on activation. In agreement with this, A63V and K70T have larger effects on \( pC_{250} \) in force versus \( pCa \) experiments (23) than they do in the present ATPase and motility studies, in which fewer myosin actin patches to actin.

Alternatively, the increased Ca\(^{2+}\) sensitivity could be a result of alteration of direct troponin-tropomyosin interactions, via effects on conformation that are propagated from the mutation sites to the troponin-binding site. We do not favor this possibility, because it would require that the mutations alter the Ca\(^{2+}\) dependence of cardiac troponin-tropomyosin binding, and there is no such dependence for skeletal muscle tropony- 

osin-tropomyosin affinity (69, 70). However, the structure of the troponin-tropomyosin complex is not known, and long range conformational propagation within tropomyosin is shown both by Fig. 1 of the current study and by previous results (44, 46, 48, 71).

In summary, thermal denaturation studies of control and mutant tropomyosins show a cooperative interaction between folding of the N-domain and C-regions of tropomyosin, of ~1.5 kcal/mol. HCM mutations A63V and K70T prominently destabilized the tropomyosin N-domain, which indirectly affected cooperative interactions between N- and C-terminal regions of tropomyosin. Both A63V and K70T increased the apparent affinity of the regulatory Ca\(^{2+}\) binding sites of thin filament in \textit{in vitro} motility and ATPase experiments. Neither mutation had more than small effects on the maximal ATPase rate in the presence of saturating Ca\(^{2+}\) or on the maximal sliding speed. Despite the increased tropomyosin dynamics implied by destabilization of the N-domain, neither the cooperativity of thin filament activation by Ca\(^{2+}\) nor the cooperative binding of myosin S1-ADP to the thin filament was altered by the mutations. The current and previous results together suggest that HCM mutations locally destabilize tropomyosin, leading to an increased thin filament Ca\(^{2+}\) sensitivity. A more dynamic tropomyosin N-domain has an unexpectedly small effect on cooperative changes in tropomyosin position on actin.
Cardiomyopathic Tropomyosin Mutations

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