Familial hypertrophic cardiomyopathy (FHC) is caused by missense or premature truncation mutations in proteins of the cardiac contractile apparatus. Mutant proteins are incorporated into the thin filament or thick filament and eventually produce cardiomyopathy. However, it has been unclear how the several, genetically identified defects in protein structure translate into impaired protein and muscle function. We have studied the basis of FHC caused by premature truncation of the most frequently implicated thin filament target, tropomyosin. Electron microscope observations showed that the thin filament undergoes normal structural changes in response to Ca\(^{2+}\) binding. On the other hand, solution studies showed that the mutation alters and destabilizes troponin binding to the thin filament to different extents in different regulatory states, thereby affecting the transitions among states that regulate myosin binding and muscle contraction. Development of hypertrophic cardiomyopathy can thus be traced to a defect in the primary mechanism controlling cardiac contraction, switching between different conformations of the thin filament.

Familial hypertrophic cardiomyopathy (FHC) is caused by missense or premature truncation mutations in proteins of the cardiac contractile apparatus (1–4). Despite the varied functions of these many proteins, the clinical and histological manifestations of FHC define a common syndrome involving thickening of one or more parts of the left ventricular wall, myocyte disarray, fibrosis, and a variety of cardiac symptoms including sudden death (reviewed in Ref. 5). Cardiomyopathic mutations have been described for thick filament proteins, as well as for every thin filament component except troponin C (TnC), i.e. for α-tropomyosin and cardiac actin and troponin I and T (TnI, TnT). TnT appears to be the most frequent thin filament target, and mutations in TnT are associated with relatively high mortality despite only modest cardiac hypertrophy (2). Experimentally, TnT mutations produce physiological dysfunction in transgenic animals and in cultured cells and altered function of purified proteins assessed in vitro (reviewed in Ref. 6). However, the underlying mechanisms leading to these dysfunction(s) remain poorly understood.

Both the thin filament and the thick filament are dynamic interacting protein assemblies. Large structural transitions in myosin produce the cross-bridge stroke that results in muscle contraction (7, 8). Similarly, changes in thin filament structure are critical for Ca\(^{2+}\) regulation of contraction (9–11). FHC mutations in either filament presumably act by altering filament structure or dynamics, although no direct structural examination of FHC mutants has been reported. However, critical insights into the basis of FHC have come from mapping myosin mutations onto the atomic model of the myosin head (12, 13). Similarly for the thin filament, mutations can be mapped on to the atomic structures of the components where these are available. However, a full understanding of thin filament mutations has not been possible because of the lack of an atomic model of the thin filament as a whole and because no direct structural studies have been performed.

Our recent elucidation of thin filament molecular structure by three-dimensional reconstruction of electron micrographs approaches such a model and has provided essential structural insights into the thin filament regulatory mechanism (14, 15). These studies show that tropomyosin adopts three distinct positions on actin depending on Ca\(^{2+}\) binding to troponin and myosin binding to actin (10). In the absence of Ca\(^{2+}\), tropomyosin is localized on the periphery of the filament, where it sterically inhibits actin-myosin interaction, thereby causing relaxation (9, 16). Activation results from a two-step movement of tropomyosin away from the myosin binding site, the first induced by Ca\(^{2+}\), partially switching on the thin filament, and the second by myosin head binding leading to full activation (10). Our structural experiments have also enabled functional changes to be correlated with perturbations of regulatory transitions in thin filament structure. In this paper, we correlate the structural and functional effects of a FHC mutation in TnT to characterize the disease at the molecular level. We examine a 28-residue COOH-terminal truncation of cardiac TnT, similar to protein resulting from a FHC splice site mutation at the beginning of intron 15 (2). Heterozygotes for this mutation experience ~25% mortality by age 25, similar to the mortality associated with other TnT mutations (2). In transgenic animal models expressing the mutant protein, both systolic and diastolic function are compromised (17). Moreover, in a variety of in vitro experimental systems, thin filaments containing this mutation exhibit impaired regulation of actin-myosin interactions reflected in reduced inhibition of actomyosin ATPase activity in the absence of Ca\(^{2+}\), diminished activation of myosin cycling in the presence of Ca\(^{2+}\) (6, 18, 19), and diminished force (18, 20). Despite the obviously altered control mechanisms, the present report shows that tropomyosin adopts normal positions.
on the actin filament, both in the presence and in the absence of Ca\(^{2+}\). The origin of the thin filament functional abnormalities is instead shown to be due to weakened binding of troponin to the thin filament to different extents in the three regulatory states, thereby affecting the transitions among these states that control myosin binding and regulate contraction. Development of hypertrophic cardiomyopathy due to this mutation can thus be traced to a defect in the energetics of thin filament conformational switching.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Rabbit fast skeletal muscle actin and myosin subfragment 1 were purified to homogeneity as described previously (14). Cardiac troponin and troponin subunits were purified (14) from bovine heart obtained at a local slaughterhouse. Bovine cardiac TnT containing a 28-residue COOH-terminal truncation was expressed in DE3 cells using the pET3d-based expression vector, as described previously (6), as was wild type recombinant TnT. In humans, the FHC-inducing splice site mutation in cardiac TnT, intron 15 G>A, results in two truncated proteins, one missing 14 COOH-terminal residues, and the other in which the 28 COOH-terminal residues are replaced by seven novel residues. In some experiments (as indicated), TnT was carboxymethylated on Cys\(^{39}\) using \(N\)-Hydroxysuccinimide (Amersham Pharmacia Biotech). Labeled and unlabeled troponins were reconstituted by combining Tnl, TnC, and TnT under denaturing conditions in a 1:1:1 mixture, followed by sequential dialysis, and G100 chromatography monitored by SDS-polyacrylamide gel electrophoresis (6).

**Effect of the Mutation on Troponin’s Affinity for the Thin Filament—**

Troponin binds very tightly to the thin filament, making the affinity difficult to measure directly. Therefore, the effect of the mutation on this process was determined by competition (21). Unlabeled control or mutant troponin was used to displace radiolabeled control troponin from the thin filament. Increasing concentrations of unlabeled troponin were added to labeled thin filaments, and displacement was measured by determining the supernatant radioactivity after thin filament sedimentation in a TLA100 ultracentrifuge at 35,000 rpm for 30 min. Data were analyzed as in Hinkle et al. (21), to determine the value for \(K_D\), i.e. the ratio of the affinity of the competing troponin for the thin filament, relative to the thin filament affinity of control \(\text{[H]}\text{troponin. Conditions: 25 }^\circ\text{C, 7 }\mu\text{M actin, 7 }\mu\text{M myosin S1, 3 }\mu\text{M troponin, 1 }\mu\text{M }\text{[H]}\text{-labeled troponin, 10 mM Tris (pH 7.5), 300 mM KCl, 3 mM MgCl}_2, 0.2 mM dithiothreitol, 0.5 mM EGTA, 0.5 mM bovine serum albumin, 0.5 mM dithiothreitol, 0.5 mM CaCl}\(_2\). These high ionic strength conditions were used to impair troponin-troponin polymerization, which otherwise interferes with binding measurements (22). Competing unlabeled troponin was added to samples at concentrations ranging between 0 and 4 \(\mu\)M.

**Electron Microscopy and Three-dimensional Reconstruction of Thin Filaments Containing Mutant Troponin—** Thin filaments were reconstituted by mixing F-actin (24 \(\mu\)M) with cardiac troponycin (8 \(\mu\)M) and then troponin (8 \(\mu\)M, prepared as above from wild type troponin I and C and mutant TnT) in a solution of 250 mM KCl (used to prevent thin filament aggregation that tends to be induced by troponin), 3 mM MgCl\(_2\), 0.5 mM EGTA, 1 mM dithiothreitol, 10 mM sodium phosphate buffer (pH 7.1). Filaments were allowed to incubate at room temperature (\(-25^\circ\text{C}\)) for 5–10 min before making a 20-fold dilution with additional buffer lacking KCl such that the final KCl concentration was 12.5 mM. Samples of reconstituted filaments were also treated with Ca\(^{2+}\) by a comparable 20-fold dilution in the same buffer lacking both KCl and EGTA but containing 0.1 mM CaCl\(_2\). Diluted filaments were then applied to carbon-coated electron microscope grids and negatively stained using either Eikonix model 1412 or Imacon Flextight Precision II scanner. Regions of interest were selected and straightened as described previously (23). Electron micrograph images were recorded on a Philips CM120 electron microscope at \(\times 60,000\) magnification under low dose conditions (\(-12^\circ\text{e/Å}^2\)). Micrographs were digitized using either Eikonix model 1412 or Imacon Flextight Precision II scanners at a pixel size corresponding to 0.7 nm in the filaments. Regions of filaments were selected and straightened as described previously (24, 25). Helical reconstruction was carried out by standard methods (26–28) as described previously (10, 29). While actin and troponycin contributions are readily delineated in reconstructions, densities due to troponin are not apparent (see Ref. 30). Resolution (31) in all reconstructions was between 2.5 and 3.0 nm; comparison of reconstructions made from images digitized on the respective scanners showed no obvious differences at this resolution. Tropomyosin and actin densities displayed in reconstructions were significant (32, 33) at equal to or greater than 99.95% confidence levels.

**RESULTS**

**Effect of Cardiomyopathic TnT Truncation on Stability of Different Thin Filament Conformations—** Ca\(^{2+}\) controls muscle contraction by reversibly binding to the globular domain of troponin, which includes TnC, TnI, and a portion of TnT that contains the 28 residues removed by the FHC splice site mutation (reviewed in Ref. 36). The interaction of troponin’s globular domain with actin and troponin is Ca\(^{2+}\)-sensitive and is believed crucial for regulation. Previously, we showed that truncation of TnT’s 28 COOH-terminal residues weakens troponin binding to thin filaments (6). In the absence of Ca\(^{2+}\), the mutant troponin has only 22% the normal affinity for the thin filament, and in the presence of Ca\(^{2+}\), its affinity is 43% that of control troponin (Table I) (6). We show here that, in contrast, the formation of the myosin-binding block and the Ca\(^{2+}\) state of the thin filament, to degrees determined by curve-fitting of the myosin S1 binding data. All other parameters (35) were held constant.

| Condition | Troponin (\text{Aex 15,16}) |
|-----------|-----------------------------|
| pCa 4, myosin S1 | 0.95 ± 0.13\(^a\) 0.71 ± 0.05\(^b\) |
| pCa 4, no myosin | 1.03 ± 0.06 0.43 ± 0.02 |
| pCa > 8, no myosin | 1.1 ± 0.1 0.22 ± 0.03 |

\(^a\) Present study, average of four determinations using native troponin and one determination using reconstituted troponin.

\(^b\) Present study, average of four determinations.

\(^c\) From Ref. 6.

**Effect of Cardiomyopathic TnT Mutation on Troponin Binding to Thin Filaments**

The tabulated values are measurements of \(K_D\), the affinity of troponin for actin-tropomyosin thin filaments, relative to the affinity of wild type troponin labeled with \([\text{H}]\)iodoacetic acid on TnT Cys\(^{39}\). Results are based upon competitive binding experiments as shown in representative data in Fig. 1.

### Table I

| Condition | Control troponin | Troponin (Aex 15,16) |
|-----------|-----------------|---------------------|
| pCa 4, myosin S1 | 0.95 ± 0.13 | 0.71 ± 0.05 |
| pCa 4, no myosin | 1.03 ± 0.06 | 0.43 ± 0.02 |
| pCa > 8, no myosin | 1.1 ± 0.1 | 0.22 ± 0.03 |

\(^a\) Present study, average of four determinations using native troponin and one determination using reconstituted troponin.

\(^b\) Present study, average of four determinations.

\(^c\) From Ref. 6.
the mutant TnT, especially in the absence of both Ca\(^{2+}\) and myosin, when troponin binding to the thin filament is particularly weak. Electron microscopy was performed to determine the structural impact of the mutant TnT on thin filaments reconstituted with otherwise normal troponin subunits and tropomyosin. Thin filaments in electron micrographs of negatively stained samples containing normal and mutant troponin (Fig. 2) were well dispersed in both the presence and absence of Ca\(^{2+}\), so any effects were not due to possible nonspecific filament aggregation caused by the mutation. In three-dimensional reconstructions of thin filaments reconstituted using mutant TnT, the position of tropomyosin was readily identified in helical projection and cross-section (Fig. 3) and in surface view (Fig. 4), both in the presence and absence of Ca\(^{2+}\). In filaments examined in the absence of Ca\(^{2+}\), tropomyosin was positioned at the inner aspect of the outer domain of actin in close contact with actin subdomains 1 and 2. In contrast, in the absence of Ca\(^{2+}\), tropomyosin moved to the outer edge of the
outer domain of actin over subdomains 3 and 4, exposing most of the actin residues believed to interact with myosin. This regulatory movement of tropomyosin was indistinguishable from that observed in our previous work with cardiac muscle thin filaments containing wild type troponin examined under Ca²⁺ and Ca²⁺-free conditions (14, 37, 38). Since tropomyosin was found in the normal blocking and Ca²⁺-induced positions in these filaments, the effects of the TnT mutation on inhibition and activation of myosin S1-thin filament MgATPase rates and on tropomin-thin filament binding were not due to aberrant tropomyosin position.

Myosin S1-ADP Binding to Control and FHC Mutant Thin Filaments—The above structural results leave unanswered the question of how TnT truncation alters Ca²⁺-sensitive regulation of cardiac contraction. To address this, the effect of the mutation on myosin S1 binding to the thin filament was examined, since Ca²⁺-dependent control of this process is central to how tropinin and tropomyosin regulate contraction (10, 35, 36, 39). Our results show, as shown previously, that myosin binding to control thin filaments is very cooperative in the absence of Ca²⁺, resulting in a sigmoidal binding curve (Fig. 5, squares) (35, 40, 41). Virtually identical results were found for thin filaments containing the mutant TnT (triangles), with one important exception, namely, a much lower cooperativity in myosin binding to actin. As is evident when viewed with an expanded scale (see inset of Fig. 5) the binding was less sigmoidal when mutant TnT was present. This indicates a defect in inhibition of cross-bridge binding to the thin filament in the absence of calcium. However, only the initial portions of the binding curves differ in Fig. 5; once the filament was 30–40% saturated, the results were similar for mutant and control samples. Little or no cooperativity was evident in the presence of Ca²⁺, and therefore no significant effect of the TnT mutation on S1 binding was detected (data not shown).

The above results and interpretation qualitatively explain the impaired inhibition of myosin cycling induced by the mutation at low Ca²⁺, corresponding to incomplete diastolic relaxation in the intact heart: myosin binding is not suppressed, so cycling continues even at low Ca²⁺. The myosin binding data were further assessed by quantitative curve-fitting. Cooperative myosin S1 binding to the thin filament is a complex process involving the following features: (i) tropomyosin adopts a predominant position on the actin filament in the absence of Ca²⁺ that blocks the myosin binding site, a second position in the presence of Ca²⁺ that exposes much but not all of the myosin binding site on actin, and a fully active position in the presence of myosin in which the binding site is fully exposed (10); (ii) despite the above, tropomyosin and myosin reciprocally promote rather than weaken each other’s binding to actin (41–45), suggesting a conformational change on the thin filament binding surface. (14, 46); (iii) shifts in tropomyosin strand position tend to persist over contiguous sections of the actin filament (10, 35). Data displayed in Fig. 5 for the mutant and control filaments were fitted to a recent model incorporating these features (35), generating values for the equilibrium constant between the low Ca²⁺ (“blocked”) and fully active states of a thin filament. The presence of the mutant TnT caused a 3-fold enhancement of the transition from the blocked to the active state.
Both the present and previous work suggest that cardiac relaxation is altered by the TnT mutation examined here, but by a mechanism other than enhanced Ca\(^{2+}\) sensitivity. Although some assays showed that myosin cycling on actin was inhibited effectively by Ca\(^{2+}\) removal (6, 19), the predominance of the published data suggest that the TnT mutation impairs the ability of the regulatory proteins to shut off myosin cycling in the absence of Ca\(^{2+}\). This conclusion is supported directly by measurements of force production (18) and actomyosin and actomyosin S1 MgATPase rates (6, 18) and is indirectly supported by altered diastolic function observed in intact hearts (17). Defective interactions between the mutant TnT and the inhibitory TnI subunit (19) could be related to these instances of compromised regulation. Our studies here provide new mechanistic insights into these observations, showing that myosin binding to the thin filament is not as effectively inhibited by troponin containing the mutant TnT as by wild type tropo- nin and that the cooperative switching on and off of the filament is disrupted by the mutant.

Our reconstructions of thin filaments containing the mutant TnT show that tropomyosin occupies the same positions at high and low Ca\(^{2+}\) as it does in the absence of the mutation. Contrary to the expectation that tropomyosin, at low Ca\(^{2+}\), should sterically interfere with myosin cross-bridge attachment, steric blocking apparently is ineffective in mutant filaments judging from the incomplete suppression of actomyosin ATPase activity (6) and from direct measurement of myosin S1-actin binding (Fig. 5). This is readily explained by our observation that tropomyosin is less tightly held by troponin in its inhibitory position, presumably causing less steric hindrance of myosin binding to actin. Our data as a whole show that it is the energetics of thin filament conformational transitions that are altered by the mutant TnT. We suggest that, at low Ca\(^{2+}\), the mutant filament remains fully or almost fully in the blocked conformation with tropomyosin covering myosin binding sites on actin. Despite this, the mutation, in effect, lowers the energetic barrier for the cooperative thin filament transition to the active state leading to partial activation and cross-bridge cycling even in the absence of Ca\(^{2+}\). By destabilizing the blocked state more than the active state, the mutation diminishes the cooperativity of myosin binding and causes defective inhibition of myosin cycling in the absence of Ca\(^{2+}\). Defective inhibition would be of obvious importance in heterozygous patients, consistent with the dominant inheritance of this disorder. At the purified protein level, mixtures of wild type and mutant TnT produce intermediate functional behavior (51), except in the presence of troponin concentrations too low to saturate the thin filament. A more complex pattern could exist in vivo, where both direct and indirect effects of the mutation can occur. The present study addresses the direct effects of the mutation, which are ultimately responsible for the cascade of pathological events in the hearts of affected individuals.

The thin filament reconstructions show a normal position for tropomyosin in the presence of calcium, and our binding experiments show no effect of the mutation on the binding of myosin to thin filaments in the presence of Ca\(^{2+}\). Both observations seem consistent with normal activity once filaments are switched on, yet a number of studies indicate that not only relaxation but also myosin cycling is altered by the mutation (6, 18–20). This suggests that alteration of Ca\(^{2+}\)-induced activation involves perturbations of myosin cross-bridge kinetics, despite unaltered equilibria of myosin binding and tropomyosin position on actin in the presence of Ca\(^{2+}\). Interestingly, a mutually induced increase in binding of myosin and tropomyosin to thin filaments is thought to be accompanied by an actin or actin surface conformational change related to normal acti-

### Fig. 4. Surface views of reconstruction of mutant thin filaments showing the positions of tropomyosin strands on actin.

In EGTA (a), tropomyosin is associated with the inner edge of the outer actin domain of actin. Note the interaction of tropomyosin with actin subdomain-1 (single white arrowheads) and bridge of density over the neighboring subdomain-2, while subdomains-3 and -4 remain unobstructed (black cross). After Ca\(^{2+}\) treatment (b), tropomyosin is associated with the outer edge of the inner actin domain of actin. Note that tropomyosin now interacts with subdomain-3 (double white arrowheads) while bridging over subdomain-4 and that here subdomains-1 and -2 (black asterisk) are unobstructed.

**DISCUSSION**

The most commonly observed functional effect of cardiomyopathic mutations in the thin filament has been an increase in Ca\(^{2+}\) sensitivity, i.e. a decrease in the Ca\(^{2+}\) concentration needed for activation. In the heart, elevated Ca\(^{2+}\) sensitivity may both increase cardiac force in systole and impair relaxation-dependent cardiac filling during diastole. Both TnI and tropomyosin mutations increase the apparent Ca\(^{2+}\) affinity of the thin filament (48, 49), and there are several reports (Refs. 6, 18, and 50, for example) that TnT missense mutations can cause similar effects. Some TnT mutations also produce other functional abnormalities including decreased force (reviewed in Ref. 6). A variety of phenotypic effects is not unexpected considering the complexity of troponin-mediated regulation of contraction.
normal Ca\textsuperscript{2+} myopathy, myosin binding and cycling were inhibited despite tin (15) and tropomyosin (14) mutations unrelated to cardio-

tant TnT even though tropomyosin occupied the normal steric

blocking position and displayed normal Ca\textsuperscript{2+}. The difference in the initial portion of the curves shows that the mutant tropomyosin was poorly effective in suppression of myosin binding to the thin filament in the absence of Ca\textsuperscript{2+}. Dashed lines are best fit theoretical curves. These results (and data in the presence of Ca\textsuperscript{2+}, not shown) were fitted to a model of myosin S1-thin filament binding (35), attributing effects of the mutation to alteration in the stability of the myosin-blocking state of the thin filament (in the absence of Ca\textsuperscript{2+}) and in the stability of the Ca\textsuperscript{2+} state of the thin filament (in the presence of Ca\textsuperscript{2+}). This corresponds to effects of the mutation and of Ca\textsuperscript{2+} on the equilibrium constant (here defined as $K_T$) for the tropomyosin strand to shift to the fully active, inner domain position on actin. In the absence of Ca\textsuperscript{2+}, $K_T$ equaled 0.557 ± 0.009 for wild type filaments and 0.658 ± 0.019 for mutant filaments, demonstrating a greater tendency for myosin binding and switching on of mutant filaments. When expressed on a per regulatory unit basis (seven actins, one tropomyosin, one troponin), this equates to a 3.2-fold effect of the mutant troponin (see Table I, measuring relative troponin affinities (i.e. $K_T$ values), where a comparable 3.2-fold effect of the mutant was observed. In that case troponin binding in the active state (Ca\textsuperscript{2+} and myosin) is destabilized a factor of 0.71, in the low Ca\textsuperscript{2+} blocked state is destabilized by a factor of 0.22, and the ratio of these two factors equals 3.2. The myosin affinity for active state thin filaments was determined to be 2.72 ± 0.04 × 10\textsuperscript{6} M\textsuperscript{-1} (see “Experimental Procedures”), a value comparable with that previously obtained under slightly different conditions (35).

In addition to elucidating the mechanism of TnT-related FHC, our results provide key insights into muscle regulatory mechanisms in general. An interesting pattern is emerging from the current and previous studies in which tropomyosin shifts among its normal positions on actin, but thin filament-based regulation is profoundly abnormal (14, 15, 55). In the present study, the inhibited state was destabilized by the mutant TnT even though tropomyosin occupied the normal steric blocking position and displayed normal Ca\textsuperscript{2+}-induced movement. Moreover, in two previous investigations examining actin (15) and tropomyosin (14) mutations unrelated to cardiomypathy, myosin binding and cycling were inhibited despite normal Ca\textsuperscript{2+}-induced switching of tropomyosin away from the steric blocking position on actin. These results taken as a whole indicate that tropomyosin movement is not sufficient for relief of inhibition and that tropomyosin position on the outer domain is not sufficient to produce full inhibition. However, an excellent correlation has held in all these studies between cooperative inhibition of myosin binding and inhibition of myosin cycling. When cooperativity in binding was suppressed, regulation was defective (current study), and when cooperativity was abnormally increased, myosin cycling was impaired despite tropomyosin localization in the normal Ca\textsuperscript{2+}-induced position (14, 15).

The primary functions of the regulatory proteins are to inhibit myosin cycling in the absence of Ca\textsuperscript{2+} and to release this inhibition in its presence. Normal inhibition of actin-myosin interaction requires tight binding of the regulatory proteins to actin in the absence of Ca\textsuperscript{2+}, tight enough to hold tropomyosin in a position that sterically interferes with myosin binding. Similarly, normal activation requires tight troponin-tropomyosin binding to actin both in the presence of Ca\textsuperscript{2+} and, in a different position, in the presence of myosin. This complexity in activation arises because Ca\textsuperscript{2+} binding to troponin does not truly activate the system: Ca\textsuperscript{2+} does not cause tropomyosin to move far enough over the actin surface to permit the strong myosin-actin binding that is part of the myosin cross-bridge cycle. The stepwise regulatory effects of both Ca\textsuperscript{2+} and myosin binding depend on the dynamics and affinities of all components, including the correct stabilities of all three states of the thin filament. Disrupting any of these processes, as we have shown here in the case of a cardiomyopathic TnT mutation, can lead to defective conformational switching, defective regulation of contraction, and ultimately devastating clinical consequences.
Properties of Thin Filaments Containing Cardiomyopathic TnT

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