Three-Dimensional Organization of Troponin on Cardiac Muscle Thin Filaments in the Relaxed State

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ABSTRACT  Muscle contraction is regulated by troponin-tropomyosin, which blocks and unblocks myosin binding sites on actin. To elucidate this regulatory mechanism, the three-dimensional organization of troponin and tropomyosin on the thin filament must be determined. Although tropomyosin is well defined in electron microscopy helical reconstructions of thin filaments, troponin density is mostly lost. Here, we determined troponin organization on native relaxed cardiac muscle thin filaments by applying single particle reconstruction procedures to negatively stained specimens. Multiple reference models led to the same final structure, indicating absence of model bias in the procedure. The new reconstructions clearly showed F-actin, tropomyosin, and troponin densities. At the 25 Å resolution achieved, troponin was considerably better defined than in previous reconstructions. The troponin density closely resembled the shape of troponin crystallographic structures, facilitating detailed interpretation of the electron microscopy density map. The orientation of troponin-T and the troponin core domain established troponin polarity. Density attributable to the troponin-I mobile regulatory domain was positioned where it could hold tropomyosin in its blocking position on actin, thus suggesting the underlying structural basis of thin filament regulation. Our previous understanding of thin filament regulation had been limited to known movements of tropomyosin that sterically block and unblock myosin binding sites on actin. We now show how troponin, the Ca\(^{2+}\) sensor, may control these movements, ultimately determining whether muscle contracts or relaxes.

INTRODUCTION

Muscles contract by a mechanism in which myosin cross-bridges (heads) from the thick filament move by cyclic attachment and detachment along actin-based thin filaments. To function normally, muscles must possess on-off switching mechanisms to regulate cross-bridge / thin-filament interactions and hence contraction. The regulatory systems that control contraction vary in different muscles. In vertebrate skeletal and cardiac muscles, the thin filament binding proteins, troponin and tropomyosin, form the regulatory switch, which blocks cross-bridge attachment and cycling at low intracellular Ca\(^{2+}\)-concentration, leading to muscle relaxation. Upon excitation and the consequent rise in free Ca\(^{2+}\), this constraint is released and contraction follows (reviewed in (1)).

Current understanding of the structural mechanism of troponin-tropomyosin regulation comes largely from x-ray diffraction of intact muscle (2–4) and from electron microscopy (EM) and three-dimensional (3D) reconstructions of isolated and reconstituted thin filaments (5–7). These and other studies show that end-to-end bonded tropomyosin molecules form strands that run along the helically arranged actin subunits of the thin filaments (8). Each tropomyosin molecule contacts seven successive actin monomers every 385 Å and binds one troponin complex, giving a 1:1:7 stoichiometry to actin (1,7). Troponin functions to couple Ca\(^{2+}\)-concentration changes to azimuthal movement of tropomyosin on the thin filament. Tropomyosin’s location on actin controls the access of cross-bridges to the thin filaments and thus regulates the cross-bridge cycling that drives contraction. At low Ca\(^{2+}\), tropomyosin is held by troponin at a location that sterically blocks myosin binding sites on actin, thus producing relaxation; this is the blocked or B-state of the thin filament (5–9). Thin filaments are switched on when Ca\(^{2+}\) binds to troponin, which moves tropomyosin to the closed or C-state position, where the myosin binding sites are partly uncovered. Myosin binding to the thin filament also alters the position of tropomyosin, and full activation of the thin filament requires binding of both calcium and myosin (10–12).

Troponin is a complex of three subunits. Troponin-I (TnI) inhibits actomyosin ATPase; troponin-C (TnC) binds Ca\(^{2+}\); and troponin-T (TnT) links the complex to tropomyosin (1). The narrow 180 Å-long N-terminal tail of TnT (TnT1) runs along roughly half of the tropomyosin’s length (i.e., over three actin subunits), whereas its C-terminal region (TnT2) is incorporated into the more globular end of troponin, where TnI and TnC are located (1, 7, 8; Fig. 1). Crystal structures (13,14) of this core-domain of troponin show a W-like structure (the TnIT arm) where each of the four segments of the W represents a different TnI or TnT helix, with the central two segments forming a TnT-TnI coiled-coil (the IT-helix). TnC is a bilobed structure with
There were several reasons for these difficulties (1). The symmetry of troponin organization on thin filaments differs from the helical symmetry of actin and tropomyosin. Thus, standard helical reconstruction protocols used for actin filaments (20,21) are inappropriate for revealing troponin, as they are based on actin helical symmetry. The helical symmetrization used in these procedures treats all actin monomers and any associated densities as equivalent. Because troponin is not equally distributed on each actin subunit along the thin filament, it is averaged out and not reconstructed (2). In principle, single particle reconstruction of thin filament segments comprising a full repeating unit of the thin filament (14 actin monomers and two tropomyosin-troponin complexes) overcomes these problems. However, poor visualization of the relatively weak troponin density (molecular mass ~80 kDa) in thin filament electron micrographs has made accurate identification of its position impossible. Thus, the selection of identical segments from thin filaments that is required for single particle reconstruction was compromised. Moreover, the conformational dynamics of troponin and plasticity of its interactions with tropomyosin and actin also probably hamper a high resolution reconstruction of its density on the thin filament. In previous work, we approached the problem by creating different models and positions of troponin on thin filaments and scoring the models by cross correlating to thin filament EM data. This method located the general position and polarity of the troponin core domain on actin-tropomyosin. However, when the best model was used as an initial reference for thin filament single-particle reconstruction, the troponin signal degraded during iteration and then disappeared, suggesting that either the modeling or the EM data was not optimal (18).

In the current extension and refinement of our previous work, we have developed methods to overcome the obstacles to single-particle EM reconstruction of thin filaments, enabling us to reveal key features of troponin organization. We first developed improved methods to isolate and negatively stain native cardiac thin filaments that display much more robust and regular troponin densities than seen previously. These native cardiac filaments did not suffer from artifacts sometimes encountered with filaments reconstituted from engineered or tissue purified proteins. We also established new, to our knowledge, protocols to preprocess EM images to determine the axial position of troponin and allow us to acquire the near identical filament segments necessary for reconstruction. Following these preliminary steps, we have applied single particle methods (without imposing actin helical symmetry) enabling us to reconstruct thin filaments showing clear and recognizable troponin density.

Single particle methods normally require a starting reference model for initial image alignment and reconstruction, and the final result can suffer from bias toward this reference. We have avoided this problem by comparing reconstructions obtained using multiple different starting models. In one, we used a featureless sphere to represent troponin. In others, we used variously oriented and positioned core domain structures. All of the models produced the same final structure,
demonstrating that the reconstructions had little or no model bias. Each of the independently generated reconstructions, as well as their average, showed strong actin and tropomyosin densities, together with consistent troponin density and polarity relative to actin. These features made possible a low resolution docking of troponin crystal structures to the reconstruction, enabling an evaluation of the molecular orientation of troponin and assessment of previous conclusions based on in vitro regulatory protein interactions (22, 23) and fluorescence polarization studies on muscle fibers (24). Of importance, our reconstructions also showed regions of troponin that are not present in crystal structures and that were not a feature in the initial reference models. These include densities attributable to the TnT1 tail running adjacent to tropomyosin, and the C-terminal mobile domain of TnI, which is considered responsible for pinning tropomyosin in the blocked state. By defining the geometry of troponin on thin filaments, our results suggest the first, to our knowledge, detailed structural mechanism for understanding muscle relaxation and activation.

**MATERIALS AND METHODS**

Detailed methods are provided in the Supporting Material text. In brief, native thin filaments were isolated from porcine hearts (25, 26), negatively stained, and EM performed as previously ((27–29), see Fig. S1 in the Supporting Material). Image processing was carried out on 755 filaments as outlined in Fig. 2. Following filament unbending using ImageJ (30, 31), power spectra were computed as a first screen to select troponin-decorated filaments for use in the reconstruction and to eliminate filaments deficient in troponin. 72 filaments lacking a 385 Å meridional reflection were assumed to have lost their troponin and were not considered further (Fig. 3, d–f). Cross correlation against a model structure was used to determine up-down filament polarity (32), and filaments were oriented with pointed ends facing up in subsequent work. Estimation of troponin axial positions on filaments, essential for successful single-particle reconstruction, was carried out by autocorrelation and cross correlation steps (Fig. 4), and filaments then computationally divided into equal length segments with troponin centered in each segment. One-dimensional density profiles of troponin-containing segments showed extra density due to the presence of troponin (Fig. 4, i and j), and the absence of this indicator was used as a second screen to eliminate troponin-deficient filaments; 138 more filaments lacking troponin were eliminated by this step.

The relative rotation of filament segments about their long axis was determined by matching against two-dimensional projections of a reference structure (21, 33, 34), built from models of actin, tropomyosin, and troponin (14, 35–39) (Fig. 5). Back projection using the rotation angles thus determined produced an initial 3D reconstruction. The reconstruction was used as a new reference and the process iterated 80 times. To increase signal/noise ratio in the reconstruction, the two troponin densities on the
the peaks show that troponin axial positions are correctly determined. The asymmetry of these profiles (a and right) confirms that troponin is present, and the central positions of the peaks show that troponin axial positions are correctly determined.

two sides of the filament were aligned and averaged in each cycle. Reconstructions were made beginning with each of 10 different reference models (Fig. 5) and these reconstructions averaged. Fig. S2 shows that all angles were represented. Alignment of atomic structures to the reconstruction was carried out using Chimera (38).

RESULTS AND DISCUSSION

EM and 3D reconstruction of thin filaments

Porcine cardiac muscle thin filaments in low Ca$^{2+}$ relaxing solution were well separated and showed minimal background material when observed by negative staining (Fig. S1). They displayed helically arranged actin-subunits and, in many cases, prominent densities (Fig. 3 a), projecting at 380 ± 8 Å intervals (Fig. 3 d), which are the core domain of the troponin complex.

To determine troponin organization, images of filaments were divided computationally into equal length segments and 3D reconstructions carried out by single particle analysis (cf. 21,32–34). To ensure preservation of troponin density in the reconstruction, helical averaging was avoided, and the segments treated as true, asymmetric single particles. Single particle analysis is normally performed on individual, identical macromolecular complexes. When it is carried out on thin filaments, the segments used must mimic true single particles, and ideally will meet the following requirements: 1), be completely or near completely decorated with troponin; 2), show multiple views of troponin; 3), have known polarity; 4), have troponin positioned identically in each segment. Our protocol (Figs. 2–4) allowed particles containing troponin to be selected, accurately aligned, and properly averaged (see Materials and Methods and Detailed Methods in the Supporting Material).

Single-particle reconstructions can suffer from bias toward the reference model used to initiate the reconstruction. To circumvent model bias, we used a variety of starting models to determine the angle of rotation of each troponin-containing segment about its own axis and to refine its axial position. These reference structures were built from an atomic model of F-actin-tropomyosin (37) together with various models of troponin (13,14) positioned equivalently on two azimuthally adjacent actin subunits on opposite sides of the actin double helix (Fig. 5, a–j). Each model was rotated about its long axis and projections made every 4°. The rotational angle of each experimental filament segment was then determined by finding the best match to the 90 reference projections, and these angles were used to calculate a backprojection. The reconstruction thus produced was used as a new reference, and the process iterated for 80 cycles. The position and appearance of actin, tropomyosin, and troponin in the reconstructions generally stabilized by the tenth round and remained stable through the 80 cycles, generating an ~25 Å resolution map according to the 0.5 Fourier Shell Correlation threshold criterion (Fig. S3). Ten separate reconstructions, each initiated from a different starting model, were produced in this way (Fig. 5, k–t).

In the first model, troponin was represented by a sphere comparable in mass to the core domain (Fig. 5 b). This produced a reconstruction showing actin, tropomyosin, and extra densities on each side of the actin helix that are attributable to troponin (Fig. 5 l). The helically arranged actin subunits were well defined, with clear subdomain structure, and were associated with continuous tropomyosin strands following the two actin helices (located in the blocking position, over actin subdomains 1 and 2). Troponin features included a mass slanting at ~50°, and extending over parts of two adjacent actin subunits, together with a well-separated smaller globular density. Although weak, these densities nevertheless resembled features expected of troponin, viz., the TnIT arm and the N-lobe of TnC (see Fitting crystal structures of the troponin core domain (below) and Figs. 6 and 7). These densities (and their refinements noted below) differed significantly (by ~15 Å axially and ~30° rotationally) from their location in our earlier work (18), the model shown in Fig. 5 a).
Because troponin features in the first reconstruction were weak, we decided to test their validity by carrying out additional reconstructions using filaments with variously oriented and positioned core domains as reference models. Nine models were built with the low Ca\textsuperscript{2+} core domain structure (14) (filtered to 20 Å resolution) substituted for the sphere and located at different axial and azimuthal positions on actin, or with different rotations and tilts, or with part of the core domain missing (Fig. 5, a–j). The first used the arrangement of troponin we had determined in earlier work using reconstituted filaments and a different structural approach ((18), Fig. 5 a). The others differed from this model by movement of troponin by ±20 Å and 20° to 30° from its initial position. No attempt was made to include TnT1 or the C-terminal mobile regulatory domain of TnI in any of the reference models, as crystal structures for these domains are not available (13,14,40). Despite the substantial differences in the starting models (Fig. 5, a, c–j), troponin density in the final reconstructions converged to a new consistent shape, site, and orientation on actin (Fig. 5, k, m–s). This displayed clear and recognizable features of the core domain similar to those obtained with the sphere model (Fig. 5, b and l; note the evolution of the final structure from one of the starting models is shown at different iterations in Fig. S4). The consensus position of troponin in these reconstructions was up to ~45 Å away from its position in the starting models. Rotation and tilt of the core domain relative to the filament axis also differed dramatically from that in the different models by up to 100° (e.g., Fig. 5, c and m, Fig. S4). The key conclusion from this common organization of troponin in the different reconstructions is that the initial model did not significantly bias the final structure. In one initial model (Fig. 5 j), where troponin was positioned furthest (~70 Å) from this consensus position, troponin, actin, and tropomyosin densities were degraded after three cycles of iteration (Fig. 5 t), and thus did not even converge on a well-defined F-actin-tropomyosin structure. Similarly, when the sphere
domain of TnI (from (15)). In this helical reconstruction, the fragment binds equivalently to every actin subunit; arrows point to the TnI density on one actin subunit. (b) The troponin difference densities in (f) superposed on the map in (g) showing that the putative mobile domain in native filaments overlaps with the TnI peptide in the reconstituted filament. Note: TnI and TnIT difference densities appear larger than actual relative to the TnIT arm density due to the low surface contour used to depict the differences, as well as possible mobility of these regions. The resolution of the full reconstruction in (b) was 25 Å, although those of the troponin and of the actin-tropomyosin parts of the map were 32 Å and 21 Å, respectively (Fig. S3). The latter compared well to previous 18 Å reconstructions of actin-tropomyosin (42).

model was centered at a similar distance from the consensus site, the reconstruction rapidly degraded (data not shown). Failure of the latter reconstructions to converge (when the troponin model is far from its consensus position) implies that troponin contributes significantly to the alignment of segments, and further supports the validity of the consensus

FIGURE 6 Surface views of thin filament reconstructions (pointed end facing up). (a) Control F-actin with subdomains marked on one subunit. (b and c) native thin filaments. In (b), the reconstruction was made using the average of individual reconstructions (Fig. 5, k–s), as a reference. Densities resembling the TnIT arm and N- and C-lobes of TnC are labeled. Tropomyosin strands are marked with black arrows and the putative C-terminal TnI extension with a double-sided arrow. (c) Higher threshold version of (b) showing that the tropomyosin strand is wider at the barbed end of the filament than at the pointed end, an indication of the additional presence of TnTI at the barbed end of the core domain; white arrows indicate the relatively narrow tropomyosin strand at the pointed end clearly bridging over actin subdomain 2, leaving a gap which, in the lower actins (yellow arrows), is filled with extra density. (d and e) cross sections through the top (d) and bottom (e) of the reconstruction in (c), again indicating the increased width of the strand in the region below the core domain (yellow arrows) compared with above (white arrows); actin subdomains numbered. (f) Difference map (gold), formed by subtracting actin-tropomyosin densities from the map in (b) and then superposing it on a map (purple) generated by imposing helical symmetry on (b), highlights densities attributable to troponin; in addition to the core domain, densities attributable to TnT (open arrow), the C-terminal mobile domain of TnI (double-sided arrow), and possibly the cardiac muscle-specific N-terminal TnI chain (bracket) are visible. (g) Reconstruction of F-actin-tropomyosin decorated with an 80 residue long fragment representing the C-terminal mobile domain of TnI (from (15)). In this helical reconstruction, the fragment binds equivalently to every actin subunit; arrows point to the TnI density on one actin subunit. (h) The troponin difference densities in (f) superposed on the map in (g) showing that the putative mobile domain in native filaments overlaps with the TnI peptide in the reconstituted filament. Note: TnI and TnIT difference densities appear larger than actual relative to the TnIT arm density due to the low surface contour used to depict the differences, as well as possible mobility of these regions. The resolution of the full reconstruction in (b) was 25 Å, although those of the troponin and of the actin-tropomyosin parts of the map were 32 Å and 21 Å, respectively (Fig. S3). The latter compared well to previous 18 Å reconstructions of actin-tropomyosin (42).

FIGURE 7 Fitting troponin core-domain crystal structures to the 3D reconstruction. (a and b) Two orthogonal views of the thin filament with the low Ca<sup>2+</sup> core domain (14) fitted into corresponding densities, showing back and front views of the fitting. The core domain is shown in ribbon view with TnI, cyan, TnT, yellow, and TnC red (cf. Fig. 1). Note the ~50° orientation of the TnIT helix of the troponin core domain relative to the filament long axis. (c) In addition to the core domain, the atomic model of actin-tropomyosin (37) was fitted to the reconstruction and substituted for corresponding densities shown in (b). Troponin difference densities made translucent are superposed. The tropomyosin structure is shown in ribbon view and actin subunits in space-filling view; actin residues 222, 226, and 311 are highlighted in red on one actin subunit lying under the putative TnI mobile domain.
organization arrived at from the other nine models. The non-convergent reconstructions were discarded.

**Analysis of troponin features in the thin filaments**

To enhance shared features and decrease noise, reconstructions in Fig. 5, k–s, were averaged and the average used as a new reference to build a final reconstruction. The troponin mass in this reconstruction again had an outline resembling the troponin IT arm, the TnC C-lobe protrusion on the upper part of the arm, and the N-lobe of TnC (Fig. 6b, cf. Fig. 1, Movie S1). The two troponin densities lay on the outer aspect of the central pair of azimuthally neighboring actin subunits, on subdomains 1 and 2, and were quite distinct from actin and tropomyosin densities at lower radius. The putative TnIT arm was the strongest feature, consistent with its mass in core-domain crystal structures, and was oriented obliquely and tilted away from the filament axis (cf. (24)). This density appeared to be attached to the tropomyosin strand and was centered over, but not obviously in contact with, actin subdomain 2 (Fig. 6b, Fig. 7, a and b). A density most simply attributable to the N-lobe of TnC (see section on alignment below) was seen on the upper surface of actin subdomain 1, to the side of the TnIT arm. Density putatively coming from the TnT tail (TnT1), thought to be a single α-helix over much of its length (41), was suggested by a greater width of the tropomyosin strand over ~3 actin subunits from the base of the core-domain in the direction of the filament’s barbed end (Fig. 6c, yellow arrows), compared with the pointed end (white arrows). The widening can also be seen in filament cross sections (Fig. 6, d and e). This orientation of TnT1 from the base of the core domain toward the filament’s barbed end was supported by analysis of one-dimensional projection profiles of averaged filament segments (Fig. 4, i and j). In addition, extra mass is noted adjacent to the TnC N-lobe on actin subdomain 1, possibly showing the C-terminal region of TnI (Fig. 6b, discussed below).

**Difference density analysis**

To illustrate more clearly the features of troponin suggested by the reconstruction, these features were analyzed further by computing a difference map between the reconstruction and a reconstruction lacking troponin (Fig. 6f). In the difference map, the troponin core domain is positioned on the outer side of the upper part of the elongated TnT1-like density. This is seen to run alongside tropomyosin in the direction of the barbed end of the filament, confirming the position of the TnT1-like density and the orientation of the troponin complex deduced in the original reconstruction. Based on the difference map, the entire complex is ~200 Å long. This is ~60 to 70 Å shorter than the length estimated in other studies (41,43), possibly because the N-terminal end of TnT1 is not resolved from actin and tropomyosin in the reconstruction. This part of TnT varies greatly between TnT isoforms (41) and may not form a discernible mass on the thin filament. In contrast, a short extra density runs above the core domain, and may be the C-terminus of TnI, which is absent from troponin crystal structures (13,14). We speculate that this end of the TnT2 might also interact with tropomyosin and/or TnI (cf. 44–46).

A second region of density, separate from actin and tropomyosin, appears to emerge from behind the putative N-lobe of TnC, on the extreme outer edge of actin subdomain 1, to form an obliquely oriented tangent to the neighboring actin monomer on the opposite F-actin helical strand (Fig. 6f, double-sided arrow, cf. the original reconstruction in Fig. 6b, where this density is seen to fill the cleft between two azimuthally adjacent actin subunits observed in Fig. 6a). This density is present only on the actin subunits at the level of the troponin core domain and is absent from neighboring actins above and below (Fig. 6b, Movie S1). It has the same location, dimensions, and orientation as density found in a previous reconstruction of F-actin-tropomyosin decorated with an 80 amino acid C-terminal construct of TnI, representing the TnI mobile regulatory domain (15) (Fig. 6g). This construct inhibits actin-tropomyosin stimulated myosin ATPase by binding to troponin-free F-actin-tropomyosin in a 1:1 molar ratio to actin, constraining tropomyosin in the blocking position (15,47). Although the density attributed here to the C-terminal domain of TnI is weak, its coincidence with that in the C-terminal TnI-decorated filaments (Fig. 6h) suggests that we are observing the native organization of the C-terminal TnI regulatory peptide in the current maps.

**Fitting crystal structures of the troponin core domain**

Additional insights into the reconstruction were gained by fitting atomic structures of subunits into corresponding features of the EM density map (Fig. 7). An actin-tropomyosin atomic structure (37) was first fitted to corresponding densities in the reconstruction, as previously (37). An approximate fitting of the low Ca²⁺ troponin core-domain crystal structure (14) was then carried out, using the asymmetric geometry of the TnIT arm, which is invariant in crystal structures (13,14), as the key guide to orientation and position. Although the volume of troponin in the reconstruction was smaller than the atomic structure, its major feature was similar in appearance to the TnIT arm, which is in turn the main component of the crystal structure. Similar asymmetric features seen in the crystal structure and reconstruction include the tapering of the TnIT arm density at its lower end and a characteristic bulge due to confluence with the C-lobe of TnC at its upper end (Figs. 7, a and b, Fig. S5). This asymmetry made it possible to fit the TnIT arm in only one plausible orientation; the only other reasonable way of alignment (by reversing its orientation) led to a poor match. A closely related modification of the crystallographic model, based on fluorescence polarization (24) fit
equally well. There are several possible reasons why the volume of the TnIT arm in the average reconstruction did not fully envelop the corresponding atomic structure. These include inherent or stain-induced variability or disorder of troponin on native filaments, partial troponin dissociation, penetration of stain into troponin, or imperfect alignment in the reconstruction procedure. Although this ambiguity precludes high resolution atomic docking, we are nevertheless able to characterize the location and orientation of the major domains of troponin, and thus describe structure/function relationships.

In the average and all nine individual convergent reconstructions, a small globular feature was consistently observed to the right of the TnIT arm, on actin subdomain 1 (Figs. 5, 6 b, and 7 a). When the above mentioned atomic models (14) were aligned to the reconstructions based solely on the TnIT arm, the TnC N-lobe in the models was found to coincide with this density, strongly suggesting that it shows this domain of TnC. The reliability of this feature is strongly supported by its appearance in reconstructions even when absent from starting models (Fig. 5, b, l, d, and n). The C- and N-lobes of TnC in the fitted structure are separated by ~22 Å, and the line joining them is oriented roughly perpendicular to the F-actin axis, consistent with fluorescence polarization studies (24). Because the N-lobe of TnC is attached to the rest of the core domain by a single helix that may be disordered at low Ca²⁺ (14), the position of the N-lobe on the thin filament may be variable. Such local disorder of the TnC N-lobe may account for its relatively small mass in reconstructions compared to that in atomic models and to that of the C-lobe density in reconstructions, as the C-lobe is firmly linked to the TnIT arm (Fig. 7).

The location of the core domain next to tropomyosin in the fitted structure positions the N-terminal end of TnT2 in the atomic model (Fig. 1, NTerm. TnT2) close to the start of the putative TnT1 density (Fig. 7, b and c), with which it is continuous (41), which further supports the proposed alignment. Because the ends of tropomyosin and the troponin complex are not resolved in the reconstruction, alignment of the troponin core domain to any particular tropomyosin pseudorepeat on actin is ambiguous. However, the likely arrangement can be inferred from published cross-linking data, binding studies, and antibody competition analysis (22,23). Accordingly, the troponin core domain was placed next to tropomyosin pseudorepeats 3 and 4 on the actin-tropomyosin model (37) so that TnT residues in the low Ca²⁺ crystal structure (14) would be closest to their natural target (near residue 174 on tropomyosin (22,23)). This alignment orients the tip of TnT2 toward subdomain 1 of actin (Fig. 7 c, see models of the troponin core domain position in Fig. S6). This arrangement is in agreement with the model of Jin and Chong (22), in which troponin binding to tropomyosin occurs at two major sites, one via interactions with TnT1, as mentioned above, and the other by contacts with the N-terminal end of TnT2, as indicated here.

In the low Ca²⁺ crystal structure, the bulk of TnI ends behind the C-lobe of TnC, but the C-terminal TnI regulatory domain emerging from this point is mobile and not resolved (14). It is at this position in the reconstruction that the putative TnI extension discussed earlier is expected to begin. Mapping the extension onto the atomic model of actin-tropomyosin shows that it bridges the cleft between azimuthally adjacent actin subunits on opposite strands of the double helix, extending from subdomain 1 of the actin from which it originates to subdomain 4 of the neighboring actin in the direction of the filament barbed end (15) (Fig. 7 c). The extension, known to contain an excess of basic residues, then passes over the region of actin containing helix 222–230 (including acidic surface residues Asp-222 and Glu-226, shown as red spheres on actin subdomain 4 in Figs. 7 c, Fig. S6), and ends on actin subdomain 3 near Asp-311. Here, the tip of the extension abuts tropomyosin and/or TnT1 on the inner edge of actin subdomain 1, where it could constrain tropomyosin on the opposite side of the filament in the myosin blocking position (Fig. 7 c). We note that TnT1 lying alongside may augment the rigidity of tropomyosin in addition to strengthening its end-to-end bonding (48,49).

Tissue- and species-specific peptide extensions frequently distinguish troponin subunits in different muscles. For example, mammalian cardiac muscle TnI contains an N-terminal extension absent in the skeletal isoform (50,51). Phosphorylation of this extension by protein kinase A modifies Ca²⁺ affinity of the N-lobe of TnC, presumably by its interactions with this domain (51). Narrow density emerging from the top of the core domain can be seen to approach the TnC N-lobe and may be this cardiac-specific TnI extension (bracket in Fig. 6 d).

Summary: Functional implications of troponin organization

Our single-particle reconstruction of native cardiac thin filaments clearly reveals troponin and shows greatly improved detail over previous models, including features not present in crystal structures. Multiple distinct reference models, with different troponin definition, starting positions, orientations, and structures all led to very similar final locations and orientations of troponin in the reconstructions, supporting the reliability of our model. Although alignment of crystal structures to the reconstruction (Fig. 7) is approximate due to the moderate resolution (±5° to 10° error in any direction), fitting of major domains is unique; when the core domain is reoriented, for example by inverting or rotating it to switch the positions of the TnIT arm and TnC, or by turning it back to front to reverse the location of the lobes of TnC, none of these variants fits sensibly into the EM volume. The appearance of troponin is qualitatively the same as that displayed by rotary shadowed molecules, which first demonstrated the characteristic structure of a globular head
(the core domain) at the end of a narrow stalk (TnT) (43). Our reconstruction yields additional detail not resolved by rotary shadowing, or in any previous thin filament reconstruction, and is in good agreement with crystal structures of the core domain. The orientations of TnT1-like and core-domain densities provide clear evidence for the polarity of troponin on the thin filament consistent with that proposed originally (43,52), and in contrast to reports suggesting the opposite (17,19). In addition, the −50° angle of the TnIT arm (Fig. 7) relative to the filament axis supports the conclusion of Knowles et al. (24), based on spectroscopic data from intact muscle, relating orientations of specific probes on troponin to the axis of the filament. Although the spectroscopic methods do not reveal troponin polarity or azimuthal or axial positions on thin filaments, our reconstruction is most consistent with the orientation of the TnIT arm in Model 2 rather than Model 1 of Knowles et al. (24). Our reconstructions do not have sufficient resolution to distinguish differences in rotation of the N-lobe of TnC within the core domain, which is what discriminates the Vinogradova and Knowles models (14,24), nor to pin-point exact amino acid to amino acid contacts between troponin and actin or tropomyosin.

3D reconstruction from electron micrographs has yielded abundant evidence of steric control of actin-myosin interactions by tropomyosin (5−7). In contrast, 3D EM relating troponin structure to tropomyosin regulatory function has been ambiguous, and the molecular mechanism of troponin-tropomyosin regulation has remained unresolved. Here, we have visualized the troponin complex at −25 Å resolution, and, through this, have been able to infer its influence on tropomyosin position in the relaxed state, providing new, to our knowledge, insights into the structural basis of troponin function. Our results show that the troponin core domain is mounted on top of the TnT1 tail where it forms a scaffold for regulatory domains of TnC and TnI. Our results on native filaments support previous suggestions based on synthetic filament constructs (15) that, at low Ca2+, TnI bridges azimuthally adjacent actin subunits across the filament, interacting with tropomyosin or TnT1 on the opposite actin helix from its origin in the core domain. The end of the C-terminal mobile domain of TnI appears to target actin subdomain 3, where it is likely to constrain TnT1 and tropomyosin to positions on actin that sterically interfere with cross-bridge binding and cycling, thus leading to muscle relaxation. In this scheme, blocked-state tropomyosin would be wedged between two tropomyosin complexes, on one side by the C-terminal TnI domain (and TnT) and on the other by the troponin core domain, suggesting a functional interaction between the two sides of the thin filament (Fig. 1). We propose that this structural inhibition is reversed during muscle activation, following Ca2+-binding to the N-lobe of TnC. The TnI mobile domain would then be attracted to the TnC N-lobe and less so to actin, favoring release of tropomyosin from its blocking position, leading to myosin cross-bridge cycling on actin (1,39,50,51). To our knowledge, these new insights into troponin organization and its relation to actin and tropomyosin provide a framework for a deeper understanding of the structural mechanics of the thin filament and its role in contractile regulation.

SUPPORTING MATERIAL

Eight figures, one movie, and detailed methodology are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00008-X.

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REFERENCES


Three-Dimensional Organization of Troponin on Cardiac Muscle Thin Filaments in the Relaxed State

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Supporting Material

Detailed Methodology

Filament Isolation and Electron Microscopy: Native thin filaments were prepared from porcine hearts according to the method of Spiess et al. (25) as modified by Matsumoto et al. (26). Samples were dialyzed against 100 mM KCl, 2 mM MgCl₂, 10 mM MOPS and 0.2 mM EGTA (pH 7.1), then centrifuged in a Beckman TLA-100 Ultracentrifuge at 30,000 RPM for 30 minutes to remove any particulate material and filament aggregates. Aliquots of the supernatant, containing the thin filaments, were diluted to 1 μM using the same buffer immediately before staining. Carbon-coated grids were irradiated with a UV-lamp (Type R51 (ozone-producing), UV Products, Pasadena, CA) for ~ 40 minutes to make carbon surfaces hydrophilic, and used for staining within 4 hours (27-29). 5 μl samples of freshly diluted filaments were applied to the grids and negatively stained with 1% uranyl acetate. Samples were examined in a JEOL 1200EX electron microscope with a LaB₆ source operating at 80 kV. Electron microscope images were recorded at a nominal magnification of 40,000x on SO-163 film (Kodak, Rochester, NY) (27-29) using conventional electron doses (~50-100 e⁻/Å²). 200 micrographs were collected and digitized on an Imacon Flextight 848 scanner at a pixel size of 5.2 Å, calibrated by measuring the 1/59 Å⁻¹ F-actin layerline.

Filament Unbending and Boxing: 755 filaments were first unbent using ImageJ (see Fig. 3 in main text) (30,31). Further image processing used the SPIDER software package (34). Filaments were windowed in a 51 pixel-wide box, which fully included troponin density, but excluded as much noise as possible.
Filament Selection: Native thin filaments can sometimes lose their troponin during sample preparation, or the troponin present may not be readily visualized (if the filament is rotated such that troponin is above and below the filament rather than at the sides). Successive troponins, binding to the same strand of F-actin, are separated by approximately 385 Å, which generates a corresponding meridional reflection in the power spectrum. Filaments where troponin was present were distinguished from those where it was not by computing their power spectra following straightening. When troponin lies on the sides of the filament, it is clearly visualized (Fig. 3a in the main text) and its presence is confirmed by a strong 385 Å meridional reflection in the power spectrum (Fig. 3d). Many filaments lacking such projections (Fig. 3b) still show a clear meridional reflection (Fig. 3e), suggesting that troponin is present, but in these cases lies above and below the filament. Similar looking filaments (Fig. 3c) show no meridional reflection (Fig. 3f), suggestive of troponin loss. Inclusion of filaments that displayed a strong 385 Å meridional reflection (whether troponin was directly visible or not), and rejection of filaments that did not, removed filaments that lacked troponin from further processing.

683 of the filaments initially selected had a 385 Å meridional reflection (Fig. 3d, e), suggesting that these filaments retained a large proportion of their troponin. The remaining 72 filaments lacked this reflection (Fig. 3f), suggesting that their troponin complement was low; these filaments were discarded. While this first screen removes poorly decorated or inadequately stained filaments, it is possible that some filaments showing a 385 Å meridional reflection may still lack troponin (this can happen because the stain surrounding a filament adsorbed to the substrate may not always have true helical symmetry). Such filaments were removed at a later stage of processing, when their 1D profile was computed (see “Selection of particles for reconstruction” below).

Filament polarity determination: Single particle reconstruction depends on matching individual particle images to reference projections of a model structure. This determines three Euler angles of view and two translational shifts (x and y, in the plane of the micrograph) for each particle, which are then used in the SPA back-projection procedure (21,29,34). The accuracy of these parameters strongly impacts the quality of the reconstruction. One of the Euler angles is related
to the orientation of the filament (pointing up or down). However, reference projections of a model thin filament segment resemble those of the same model with the opposite polarity (this is because actin monomers do not have a very pronounced asymmetry). Thus polarity determination of individual segments is not very reliable. To eliminate this problem, the polarity of filament segments was determined using the method of Narita and Maeda (32), in which cross-correlation coefficients are determined for full length filaments, pointed up or down, matched against F-actin or thin filament models. Both models gave the same result, suggesting that the F-actin backbone dominates the alignment. The polarity assignments determined were applied to all segments contained in any given filament.

**Determination of troponin axial position:** To use filament segments as single particles, the filament must be cut so that troponin is identically located in each segment. One way to do this is to find the axial position of the segment that gives the maximum cross-correlation with a thin filament model containing troponin. However, when this method was used, the reconstructions showed strong model bias. A different method for localizing troponin was therefore devised which avoided using a model structure. In this approach, troponin positions along filaments were determined by a series of filtering, auto-correlation and cross-correlation steps (see Fig. 4 in the main text). 830 Å-long segments were cut based on these positions, so that a troponin-pair density was precisely at the center of the segment, and another was at one end.

Filaments (Fig. 4a) were filtered to include frequency components from 1/450 Å⁻¹ to 1/300 Å⁻¹. The filtered image (Fig. 4b) derives mainly from the density of troponin, which contains noisy peaks with a ~385 Å periodicity. To extract the troponin positions from this noisy filtered image, the auto-correlation function of the filtered image was first calculated (Fig. 4c) as a reference, and the cross-correlation function (Fig. 4d) between this reference (Fig. 4c) and the filtered image (Fig. 4b) was calculated. It can be seen that the peaks in Figure 4d correctly point to troponins when these are directly visualized in side-view in the original filament (up arrows in Fig. 4a-d), demonstrating the accuracy of this method. To obtain a wide range of views of troponin, the same procedure was used for filaments in which troponin was bound to the top and bottom of the filament and therefore not directly visible (Fig. 4e-h). The cross-correlation map (Fig. 4h) has a similar pattern to that in Figure 4d. This procedure was essential in such
filaments, in which troponin could not be visualized directly by eye. 1D density profiles of averaged segments selected from filaments with troponin centered in this way showed clear peaks representing troponin, demonstrating the accuracy of this method (Fig. 4i, j).

Selection of particles for reconstruction: Using each troponin axial position in a filament as the center, 51 x 160 pixel particles were cut containing ~2.1 actin crossovers. An average image of the particles in each filament in which troponin is seen at the sides (see Fig. 4a in the main text) was calculated, enhancing the signal-to-noise ratio, especially the troponin signal. Each particle contains two pairs of troponins. The members of one pair are at the center, on opposite sides of the filament and axially separated by one actin subunit rise (27.5 Å), while those of the other pair are 74 pixels (~385 Å) away, near the end of the particle. A one-dimensional (1D) projection of the average image perpendicular to the filament axis has two density peaks corresponding to these troponin pairs, one at the center, and the other at the right (down arrows, Fig. 4i). A similar 1D projection (Fig. 4j) is found for filaments with troponin binding to the top and bottom (Fig. 4e), and is distinct from 1D projections of F-actin filaments, which lack such density peaks. 512 filaments showing the central and right peaks in the 1D projection were selected, including 4813 particles that were boxed from these filaments and padded to 160 x 160 pixels for subsequent image processing. The other 138 filaments (about 20% of the total) did not show this pattern, indicating that troponin had partially or completely dissociated from F-actin or was otherwise disordered (Fig. S7); these filaments were discarded. This second screen eliminated from further consideration additional troponin-deficient filaments that had passed the first screen based on the 385 Å meridional reflection. Even without troponin, these filaments had shown this reflection, possibly due to non-helical staining (see “Filament selection” above).

The 1D projection contains important information on the polarity of troponin. The left and right sides of the central peak (see Fig. 4i, j in the main text) have different density distributions. The greater, extended density on the right side (the direction of the barbed end of the filament) is most simply attributed to TnT1, implying that TnT1 is on the barbed side of the core domain, as also concluded from the high threshold reconstruction (see Fig. 6c in the main text) and confirmed by the difference map (Fig. 6f). The polarity of 98% of the filaments assessed in this way matched that from the earlier determination.
Classification analysis: To better visualize different views of the thin filaments, the 4813 particles were classified using multivariate statistical analysis. Particles were first low-pass filtered to remove frequencies larger than 1/20 Å\(^{-1}\), and reference-free alignment was performed to align particles using the SPIDER operation AP SR (34). Correspondence analysis and hierarchical clustering were used for classification. Following classification, images in each class were averaged together, creating 250 class averages. 36 representative views are shown in Figure S8. Troponin density is clearly visualized in some class averages, when on the sides of the filaments (black arrow, Fig. S8), its varying appearance being due to different rotations of the segments. In the extreme case where troponin is on the top of the filament, discrete troponin density disappears (white arrow in Fig. S8). In this position, superposition of troponin on actin contributes to observed modulation of actin subunit densities compared with F-actin-troponin-tropomyosin. It is important to note that visualization of troponin in the class averages depended on the use of a high number of factors (200) in the SPIDER CA S operation (34).

Single particle reconstruction and difference mapping: Reconstruction was carried out on the 4813 selected particles by back-projection using the SPIDER operation BP 3F (34). For each of the reference models used (see Fig. 5 in the main text), reconstruction was carried out for 80 iterations. During each iteration, ~3500 particles were included in the back-projection, the others being removed in each cycle based on their low cross-correlation coefficient in the alignment, or on the large shift in x or y directions, the large in-plane rotational angle, or the wrong polarity after alignment. All particles were available again for the next cycle. Particle matching statistics showed good sampling of filament segment orientations around the full 360° rotation (Fig. S2).

Following computation of the back-projection during each iteration, the two strands of actin, together with their respective tropomyosin-troponin complexes, were averaged together to enhance the signal and reduce noise. The two strands are related geometrically to each other on average by a rise of ~27.5 Å and a rotation of ~167.1°. To determine the precise values in each iteration, helical symmetry parameters were searched using IHRSR (21), with a small search radius of 45 Å to ensure that only actin density was used in the search. A FORTRAN program (avg2strand.f) was written to average the two strands, based on principles developed for the IHRSR method (21). The reconstruction is converted to a polar coordinate system, and a density
in one strand is searched for its corresponding density pair in the other strand using the determined helical symmetry parameters. Corresponding densities in every hollow cylinder at different radii are then averaged together. The actin subunit at the top of one strand and at the bottom of the other lacks a subunit in its partner strand. The original densities in these special positions were maintained without averaging, and do not interfere with the averaging of the troponin density at the center.

To exclude model bias, ten different reference models were tested, built using CHIMERA (38) and SITUS (39). The atomic structures of 42 actin subunits (35), 6 tropomyosin dimers (36) and 6 troponin complexes (14) were assembled together using the appropriate helical symmetries for actin, tropomyosin and troponin, with tropomyosin placed in the blocking position (37). These atomic models were converted to SPIDER format at a pixel size of 5.2 Å and filtered to 20 Å. The central 160 x 160 x 160 pixel volume was windowed to generate a reference projection. In all models, actin and tropomyosin were invariant, while troponin position and orientation were altered. In seven models (Fig. 5c, e-j in the main text), troponin was translocated, tilted or rotated relative to the starting model (Fig. 5a in the main text, Pirani et al. (18)). In one (Fig. 5d), the N-lobe of TnC was removed. In another, a sphere was used as a model for troponin (Fig. 5b). The sphere was positioned approximately at the center of troponin in the reconstruction, and had a diameter of ~ 70 Å, large enough to embrace the entire troponin core domain.

To better understand the structure of troponin in the reconstruction, troponin density was isolated by calculating a difference map between the reconstruction (Fig. 6b in the main text) and a reconstruction lacking troponin (Fig. 6c). The latter was produced by searching the helical symmetry parameters of F-actin in the reconstruction using IHRSR (21). These parameters were then imposed on the reconstruction in Figure 6b within a radius of 52 Å, to generate a helically symmetric structure. The majority of the troponin density is outside this radius and is removed during this procedure, leaving a reconstruction that consists mainly of actin and tropomyosin. The difference map (Fig. 6d) was generated by subtracting this symmetric structure from that in Figure 6b.

SUPPORTING CITATIONS: References (13,14,18,21-23,25-32,34-37) appear in the main text reference list.
Figure S1. Field of negatively stained thin filaments showing two filaments with periodically distributed troponin densities crossing another with no obvious troponin decoration. Scale bar represents 500 Å.
Figure S2. Angular distribution of segments used in the reconstruction determined by projection matching. The histogram demonstrates that all angles are represented in the reconstruction.
Figure S3. Fourier Shell Correlation (FSC) curves calculated for three different reconstructions. In each case, two reconstructions were made, each from randomly selected particles comprising one or the other half of the data set. (a) The resolution for the reconstruction as a whole was ~ 25 Å based on the 0.5 FSC cutoff criterion. (b) It appeared likely that the resolution of the troponin component alone might be lower owing to its flexibility, partial dissociation, and/or imperfect alignment in the segments (see main text). We tested this by first calculating reconstructions from two randomly selected data sets as in (a). F-actin and tropomyosin density was then subtracted from the two EM maps (cf. Fig. 6f), and the FSC calculated between the two difference maps. The resolution thus obtained for troponin was 32 Å. (c) We also tested the resolution of a reconstruction obtained by the IHRSR method, in which F-actin helical symmetry was imposed on the reconstruction and troponin density was thus averaged out. Helical averaging produced a resolution of 21 Å, comparable to our previous work in which helical reconstruction methods were used.
Figure S4. Illustration of the progression of the reconstruction through different iterations, showing how the initial reference model for troponin evolves into the final consensus structure. In this example, the core domain in the reference model (a) is rotated 100° counter-clockwise from that of the final position (considerably more than the rotation in Fig. 5 of the main text). During 20 cycles of refinement, the successive reconstructions diverge from the starting model then converge on the consensus structure. (b-f) show the first, second, third, fourth and twentieth iterations. Troponin density (red) is highlighted on actin-tropomyosin (green). The structure resembles the starting model after the first iteration, but changes dramatically in the second iteration, converging on the final consensus structure between iterations 4 and 20. A similar progression was seen with the models in Fig. 5 in the main text. Additional reference models, also very different from the final structure, were also tested. In one, the core domain was tilted 40° towards the filament; this also generated the converged consensus structure even though the troponin in the starting model clashed with F-actin (data not shown). Two other models, generated by shifting the core domain azimuthally an additional 40° and 80° from that shown in Fig. 5j in the main text, generated corrupted reconstructions as in Figure 5t in the main text.
Figure S5. Asymmetry of core domain in reconstruction. A key feature used to align the atomic model of the TnIT arm into the reconstruction was the similar asymmetry observed in both. The tapered shape of the TnIT density in the thin filament reconstructions, used to align the respective core domain structures, is very evident when viewed as a semitransparent solid (rather than as an isosurface volume, as in text Figures 6 and 7) and selecting for low contour densities. (a) thin filament reconstruction (C- and N-lobes of TnC labeled); note the wide aspect at the top end of the TnIT arm and the tapering toward the narrow base, (b) fitting of the troponin core domain atomic model, which shows similar broad-to-narrow asymmetry in the TnIT arm, constraining the fit to the orientation shown.
Figure S6. Orienting the troponin core-domain crystal structures on F-actin-tropomyosin. Actin, tropomyosin and Vinogradova and Takeda troponin core domain models (13,14) were aligned to the thin filament reconstruction as in Figure 7 in the main text and then EM densities removed to illustrate the fitting. Actin subunits are shown in space-filling view, while the troponin core domain and tropomyosin are shown in ribbon view with TnI, cyan, TnT, yellow, TnC, red, and tropomyosin, lavender/magenta (cf. Fig. 1). Actin residues 222, 226 and 311, highlighted in red, would be expected to lie under the putative TnI mobile domain, a key feature of troponin which is not resolved in crystallographic models (cf. Figs. 6,7). Also highlighted in yellow is tropomyosin residue 174 that is thought to interact with the N-terminal end of TnT2 (22,23), i.e. the end of the TnT chain seen close to this site. Putative differences in the orientation of the N-terminal helix of TnT2 about a possible flexible loop at the base of the TnIT helix (arrow) in the Vinogradova (a) and the Takeda (b) structures of the core domain are seen. Note that in the Takeda model (b) the N-terminal end of TnT2 is closer to residue 174 on tropomyosin (double arrow).
Figure S7. Screening of filaments for troponin binding using the 1D density profile. Most filaments that passed the initial screening for the presence of troponin (Fig. 3) showed two density peaks separated by 385 Å (and an asymmetric tail of density associated with each peak) in a one dimensional projection of the average filament segment. This is consistent with the presence of troponin (Figs. 4i, j). However, 138 filaments did not show these features, suggesting that troponin was lost or was not well ordered or preserved. These filaments were not processed further. (a) The majority of the rejected filaments had an almost random density distribution. Although there are small peaks near ~ 385 Å and ~ 770 Å (arrows) (possibly arising from buildup of stain at actin crossovers), the asymmetric density profile seen in Fig. 4i,j is missing, consistent with troponin absence or disorder. (b) ~ 10 filaments had a distribution with shorter and variable distances between two maximum peaks (arrows), suggesting anomalous filament staining. (c) Other filaments showed a broad peak near the center, with a similar density distribution on the two sides. This broad peak might result from disordered troponin or uneven
staining around the troponin core domain. (d) ~20 filaments had a symmetric density distribution around the central peak. Here it is possible that the TnT1 has dissociated from the filaments. Again these filaments were not processed further.

To further validate our protocol, power spectra of 11 F-actin-tropomyosin (no troponin) and 10 F-actin (no tropomyosin or troponin) “control” filaments were examined. All showed clear 385 and 59 Å layer lines in their power spectra, but only 3 showed a meridional reflection at 385 Å. Filtered images (equivalent to Fig. 4b,f) showed no regular repeat, and there were no regular peaks in the cross-correlation function (equivalent to Fig. 4g,h). 1D projections (equivalent to Fig. 4i,j) showed variable and noisy profiles and no sign of the two peaks (separated by 385 Å) or asymmetric density distribution that we saw in our “selected” troponin-tropomyosin decorated thin filaments and which we attribute to troponin. All of the troponin-free filaments examined would have been rejected using our selection criteria, a further indication that our image analysis procedures are reliable.
Figure S8. Class averages from multivariate statistical analysis. To better visualize different views of the thin filaments, the 4813 particles were classified using multivariate statistical analysis. Particles were first low-pass filtered to remove frequencies larger than 1/20 Å⁻¹, and reference-free alignment was performed to align particles using the SPIDER operation AP SR (34). Correspondence analysis and hierarchical clustering were used for classification. Following classification, images in each class were averaged together, creating 250 class averages. 36 representative views are shown. Troponin density is clearly visualized in some class averages, when on the sides of the filaments (black arrow), its varying appearance being due to different rotations of the segments. In the extreme case where troponin is on the top of the filament,
discrete troponin density disappears (white arrow). In this position, superposition of troponin on actin contributes to observed modulation of actin subunit densities compared with F-actin-tropomyosin. It is important to note that visualization of troponin in the class averages depended on the use of a high number of factors (200) in the SPIDER CA S operation (34). Scale bar represents 200 Å.
Movie S1. Rotation of thin filament reconstruction shown in text Figure 4b about its longitudinal axis.

Snapshot of first frame of movie.