Structure of the mid-region of tropomyosin: Bending and binding sites for actin


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Tropomyosin is a two-chain α-helical coiled coil whose periodic interactions with the F-actin helix are critical for thin filament stabilization and the regulation of muscle contraction. Here we deduce the mechanical and chemical basis of these interactions from the 2.3-A-resolution crystal structure of the middle third of tropomyosin’s seven periods. Geometrically specific bends of the coiled coil, produced by clusters of core alanines, and variable bends about gaps in the core, produced by isolated alanines, occur along the molecule. The crystal packing is notable in signifying that the functionaly important fifth period includes an especially favorable protein-binding site, comprising an unusual apolar patch on the surface together with surrounding charged residues. Based on these and other results, we have constructed a specific model of the thin filament, with the N-terminal halves of each period (i.e., the so-called “α zones”) of tropomyosin axially aligned with subdomain 3 of each monomer in F-actin.

alanine | α-helix | cardiomyopathy | coiled coil | packing

Tropomyosin is a relatively “simple” molecule, whose partially periodic design controls its binding to F-actin and other related functional roles in muscle. The perceived simplicity of tropomyosin arises in part from its being a nearly uninterrupted coiled coil of two parallel and identical (or highly similar) α-helical chains. This class of proteins has a sequence with a short-range seven-residue (so-called “heptad”) repeat of the form (a-b-c-d-e-f-g) where the a and d residues are generally apolar. The winding of the α-helices around one another permits a systematic interlocking of the α-helices around the interhelical interface (or “core”), in a knobs-into-holes fashion to stabilize the molecule (1, 2). The basic conformation of a coiled coil was first confirmed at high resolution for the relatively short, stable leucine zipper (3). Tropomyosin also displays a long-range repeat in its sequence, which is especially regular for certain surface residues in its so-called “α zones” (4, 5). It was recognized early that the nearly 40-residue-long distance between these repeating units, or “periods,” along tropomyosin matches the distance between consecutive actin monomers along the long pitch of the F-actin helix (4–6).

Tropomyosin (which forms filaments of head-to-tail noncovalently bonded molecules) has been visualized in three different functional states by three-dimensional reconstructions of electron micrographs of the thin filament (for a recent review see ref. 7). The resolution has been too low, however, to observe tropomyosin’s detailed interactions with actin. Large fragments of tropomyosin (including residues 1–80 at the N terminus and 254–284 at the C terminus), however, have yielded relatively high-resolution structures of the molecule that show new features of its design. Near the C terminus of striated-muscle tropomyosin, for example, a group of bulky core residues causes the α-helices to splay apart and signals the location of a recognition site for troponin (ref. 8; see also ref. 9). The crystal structure of the N-terminal fragment also reveals how a cluster of core alanines (which are repeated elsewhere in tropomyosin) produces a local (1.2 Å) stagger of the helices and resulting (~6°) bends of the coiled coil, related to the winding of the molecule around the F-actin helix (ref. 10 and see below).

Here we describe newly recognized atomic features of tropomyosin’s surface as well as its core derived from a 2.3-A-resolution crystal structure of the middle three-sevenths of the molecule. New structural and dynamic consequences of isolated core alanines as well as another clear example of “alanine staggering” are shown. Moreover, analysis of this fragment’s crystal contacts gives insights into the binding properties of different regions of tropomyosin’s surface. These and other results permit the chemical features on the surfaces of tropomyosin and actin, in addition to their repeat distances, to be matched to each other. By this means, a model for the axial position of the tropomyosin filament on F-actin has been deduced.

Methods

Complete details and references for the expression, crystallization, and structure determination of the chimeric peptide here called MidTm may be found in Supporting Methods and Table 1, which are published as supporting information on the PNAS website. Briefly, MidTm was produced from cDNA encoding rat striated muscle α-tropomyosin residues 89–208 fused to GCN4 coiled-coil residues 8–33. Crystals of MidTm were obtained by combining 3 μl of protein solution (6.35 mg of MidTm per ml/20 mM Tris HCl, pH 7.0/100 mM NaCl/2 mM 2-mercaptoethanol/2 mM Na2P) with 2 μl of 14% polyethylene glycol 1000 monomethylene ether (PEG2K-MME) and equilibrating the resultant drop against 1 ml of reservoir solution (7.5% PEG2K-MME/120 mM NaCl/24 mM Tris HCl, pH 7.0) by vapor diffusion at 16°C. A 2.3-A-resolution native data set was collected at the Cornell High Energy Synchrotron Source at 100 K from a single crystal, cryoprotected in 22.5% PEG2K-MME, 25% glycerol, 120 mM NaCl, and 24 mM Tris HCl (pH 7.0). Phases were obtained from multiple anomalous dispersion data sets of a single crystal of selenomethionine-derivatized MidTm collected at 100 K with synchrotron radiation at beamline X29 at Brookhaven National Laboratory. The space group of the crystals is P212121 with unit cell dimensions a = b = 80.52 Å, c = 112.45 Å, 60% solvent, and one coiled-coil molecule per asymmetric unit. The model refined to an R/Rfree = 25.6/29.6. With a length of 211 Å and width of only 25 Å, MidTm is of one of the most elongated molecular species ever reported to high resolution.

Results

General Features. The crystal structure of MidTm displays many features anticipated from previous sequence and structural studies.

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Data deposition: Coordinates and diffraction data have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2B9C).

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Most of the fragment, from about residue 105 to residue 208 as well as the C-terminal leucine zipper, adopts the expected two-stranded \( \alpha \)-helical coiled-coil conformation (1, 3) (Fig. 1). The \( \alpha \)-helices in this fragment wind around each other with an average pitch of 146 Å [calculated by using TWISTER (11)], as seen previously in low-resolution crystal studies of native tropomyosin (12). In addition to the coiled-coil-defining knobs-into-holes interactions in the core (Fig. 2), the helices are held together by various other interactions, including eight observed salt bridges [five of the g-e’ type (13–15)] (see Table 2, which is published as supporting information on the PNAS web site), a pair of cation–π interactions at positions 161 and 162, and an apparent disulfide bond at position 190 (see Fig. 5, which is published as supporting information on the PNAS web site). (Here, a “pair” refers to the analogous features, residues, etc. found across from one another in the two chains in the homodimer.) The distance between the helical axes ranges from \( \approx 8 \) to 11 Å and, for the most part, is correlated with the size of the core residues (see Fig. 3 and exceptions below). The magnitude and extent of the axial stagger between the helices are greatest where there are three pairs of core alanines in a row (151–158) (blue in Fig. 3; see also Fig. 6, which is published as supporting information on the PNAS web site); the joining of this segment with neighboring in-register segments produces specific 6° bends about the broad face of the
coiled coil (right cartoon in Fig. 3; see also Fig. 6, figure 3 in ref. 10, and below).

B-Factor Profile. Additional analyses of the structure reveal other features of the molecule that have not been described before. One of the most obvious is that the temperature factors, although relatively low between residues 150 and 190, increase steadily toward the N terminus of the fragment (Fig. 1a). Moreover, residues 89–93 are totally disordered and not modeled, residues 94–97 are modeled only in the B chain, and residues 98–104 of the A chain do not appear to form an α-helical conformation.

Crystal Contacts. The region of MidTm with the lowest B factors (between 167 and 178) has the highest density of close crystal contacts (see Fig. 1b and c and legend). The apparent driving force of this interaction is the removal from solvent of the apolar surface of the coiled coil including b-position valine 170, c-position Ile-171, and f-position Ser-174, as well as the inner atoms of Arg-167 and Arg-178. This interaction is also strengthened by tight H-bonded salt bridges between the basic outer atoms of the arginines and the acidic side chains of residues 173, 175, and 177 (Fig. 1c). Note that only in this segment (167–178) do both helices of the coiled coil, despite being noncrystallographically related to one another, form the same crystal contacts. As suggested below, many of the residues involved in this crystal contact are also likely to form close interactions with F-actin.

Holes in Core Packing and Variable Bends. Whereas a specific pattern of outer surface residues appears to give rise to the low thermal disorder between residues 167 and 178 in the crystals, the pattern of residues in the core of the coiled coil (letters in Fig. 3) may account for the relatively high B factors in the N-terminal half of MidTm. This region shows little clustering of similarly sized core side chains. Such a pattern causes the helices at the locations of the small core side chains (e.g., at d-position Ala-102, Ala-109, and Ala-116 and a-position Ala-134) to be propped relatively far apart (9.5–10.7 Å) by nearby large apolar side chains directed into the core (especially Met-127 and Met-141). As a result, substantial holes are found here in the core (Fig. 2). In contrast, the core between residues 150 and 190 generally shows significant clustering of similarly sized side chains. Here, the coiled-coil radius is small at the locations of small core side chains, and the interface is well packed (see Figs. 2 and 3).

These large holes in the core of MidTm also coincide with the locations of an additional class of bends in the coiled-coil axis. A 4–8° bend occurs in the vicinity of residues 127–134 about the broad face of the coiled coil (middle cartoon of Fig. 3) so that one helix (the upper curved rectangle) follows a longer path than the other. An 11° bend is centered at residue 117, in this case about the narrow edge of the coiled coil (left cartoon in Fig. 3), so that both helices (the curved rectangle above the plane of the paper and the other one hidden below) locally follow similar path lengths (see also Fig. 6). The poor packing in these regions would appear to suggest a relatively flexible and nonspecific type of bending, and this concept is consistent with the two different directions of bending observed here.

Discussion

Early concepts of how tropomyosin interacts with actin, based on sequence studies and low-resolution images, stressed the remarkable “molecular matching” displayed by these two muscle filaments. X-ray analysis of tropomyosin crystals can, of course, yield a far more detailed picture of the molecule than can x-ray diffraction of whole muscle or three-dimensional reconstructions of electron micrographs of thin filaments. Thus far, however, we have no comparable high-resolution structures of tropomyosin bound to actin. Nevertheless, we have found that we can deduce key aspects of the interactions of these two proteins by analysis of crystals of certain tropomyosin fragments. In this report, we show that the high-resolution structure of the mid-region of tropomyosin yields information about both the core of the tropomyosin molecule and its surface, which gives new insights into its detailed interactions with actin.

Core Packing and How to Bend a Coiled Coil. α-Helical coiled coils, such as tropomyosin, are unique among protein classes in that many features of tertiary structure may be recognized directly from primary sequence information. This generalization is especially true for residues at the interface between helices. As previously noted (2), this is an important step toward determining protein structures from sequence data alone. These features include the helix repeat, signaling the coiled-coil conformation (1), the oligomeric state of the protein, and the orientation and pitch of the helices (for review see ref. 16). One role of the unusually large number of alanine residues in the core of tropomyosin was discovered by analysis of the crystal structure of an N-terminal fragment of tropomyosin, which includes the entire first period (10). Here it was shown that a cluster of core alanines, by producing an ~1.2-Å axial stagger between closely packed helices, gives rise to specific 6° bends along the broad face of the coiled coil. This same design is observed in the current structure, in the C-terminal half of the fourth period, where there is another segment containing three pairs of core alanines in a row (Figs. 3 and 6). There is also evidence that similar specific bends occur in other periods of tropomyosin (e.g., the second period) (see supporting information for ref. 10). Note that the clusters of core alanines promote the same geometrically specific bends even in different sequence contexts (i.e., whether preceded by an a-position lysine as in period 1 or by an a-position leucine as in period 4). These results strengthen the suggestion that this design promotes the bending of tropomyosin on the thin filament.

The current structure shows that other modes of bending also occur. Here, there are examples of local destabilization in the coiled coil that is produced by the presence of isolated alanine side chains in the core. In the third period and in the N-terminal half of the fourth period (in particular, at position 134), the helices at the core alanines are propped apart by neighboring large core apolar residues and hence display holes at the interhelical interface (Figs. 2 and 3). These structural observations of clustered and unclustered alanines are, in fact, consistent with calorimetric studies of designed coiled coils by Hodges and colleagues (17, 18). In their studies, a coiled coil whose core alanines and core leucines or isoleucines are each clustered in groups of three (e.g., L.L.L, A.A.A, L.L.L) is more stable than another coiled coil of identical composition but with a different arrangement that does not display such clustering (e.g., L.A.L, L.A.A, L.L.L). In addition to the high local temperature factors in the current tropomyosin crystals, the poor core packing at Ala-134 may also account for the instability of this region in solution (19) and the particularly susceptible tryptic site at Arg-133–Ala-134 (20). Taken together, the structural and biochemical results suggest that three or more consecutive pairs of core alanines (d- and a-positions) promote geometrically specific bends (semiflexibility) in the coiled-coil axis, as described above (10, 21), whereas an isolated pair or two consecutive pairs of core alanines located near large (and apolar) core side chains permit geometrically variable bends (see also refs. 22 and 23 and **). With new fragments that are now being crystallized, we expect to find an example of the specific bend at positions 235–242 and of the variable type at position 211. Both types of bends, in different regions of the molecule, are likely involved in tropomyosin’s interactions with F-actin.


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Crystal Packing and Surface Structure. Perhaps the most striking observation in the current crystal structure is the singularly high concentration of strong hydrophobic, as well as electrostatic, contact points in a 12-residue-long segment located in the N-terminal half of period 5. This period is distinguished by a stretch of four apolar residues in a row, including two surface apolar residues at 170 and 171, which form the center of the crystal contact. Not unexpectedly, this short region has the lowest temperature factors in the crystal lattice. Yet, in solution, this segment has been found to be part of the least stable (nonterminal) region of tropomyosin, which extends from $\sim130$ to 190 (20, 24). As previously noted (5), this loss of amphipathicity between residues 169 and 172, due to four consecutive apolar residues, may contribute to the flexibility of the molecule in this region. Taken together, these observations suggest that this segment of the tropomyosin coiled coil is an especially favorable target for binding another protein. It has long been recognized that highly apolar surface regions (with corresponding flexibility) are often found to be involved in protein interactions (25).

The high conservation of period 5, and especially its N-terminal half (26) (including the conservation of the apolarity of residues 170 and 171), points to its role in binding F-actin. This segment is also the most consistently important internal region for tropomyosin’s affinity to actin (and acto-S1), according to the deletion studies (26–28). The functional importance of period 5 in tropomyosin is also illustrated by the location of residues linked to hypertrophic cardiomyopathy. To date, mutations of 10 residues along tropomyosin [E62Q, A63V, K70A, I72T, D175N, E180G (E180V), L185R, E192K, and M281T] have been associated with this disease (refs. 29 and 30 and references therein). Mutations in different periods affect both the folding of tropomyosin and Ca$^{2+}$ sensitivity of muscle activation (31), but the majority of the mutations are concentrated in a 20-residue-long segment in the N-terminal half (or middle) of period 5. Inspection of the crystal structure suggests that most of these mutations would appear to affect the local stability of the coiled coil, as well as the electrostatic character of the coiled coil’s surface. In this connection, the best studied of these mutations, at positions 175 and 180, appear to diminish actin (but not tropinin) affinity (ref. 32; see also ref. 33).

Toward a Tropomyosin–Actin Model

These crystallographic and biochemical results, together with our current knowledge of thin filament structure (described below), allow us to deduce a relatively detailed structural description of certain interactions of period 5 and then, by extension, the rest of the tropomyosin molecule with the F-actin helix (Fig. 4).

Azimuthal Positions and Polarity of Tropomyosin on Actin. The interactions between tropomyosin and actin, inferred from small-angle x-ray studies of muscle (34, 35) and from sequence analyses of tropomyosin (4–6), has thus far been directly visualized only at low resolution in recent EM reconstructions. Despite the fact that the crystal structure of F-actin is not yet available, useful atomic models of F-actin (refs. 36 and 37; and see ref. 21) have been derived from x-ray fiber diffraction diagrams of F-actin (38, 39) and crystallographically determined G-actin structures (40, 41). It has therefore been possible to establish the azimuthal positions of tropomyosin on actin in three different functional states (42) (Fig. 4a). In striated muscles, these positions are controlled by troponin as well as by myosin and by tropomyosin itself (for review see ref. 21 and references therein). In summary, troponin positions tropomyosin primarily over actin’s outer domain (which consists of subdomains 1 and 2) in the Ca$^{2+}$-free “off” state. This position blocks the major myosin-binding sites of actin (on subdomain 1 and its junction with subdomain 3). The binding of Ca$^{2+}$ to troponin C weakens interactions of the troponin “head” region with actin–tropomyosin. As a result, tropomyosin moves azimuthally onto the outer edge of actin’s inner domain (consisting of subdomains 3 and 4). [This interaction is likely dominated by the binding of α zone residues of tropomyosin to recognition sites on actin (ref. 5 and see below)]. In this Ca$^{2+}$-activated state, most but not all of actin’s strong myosin-binding sites are exposed. Upon the strong binding of myosin to actin, tropomyosin moves azimuthally toward the inner edge of actin’s inner domain in the fully activated state. In the absence of both troponin and myosin, tropomyosin has been observed primarily in either the off or Ca$^{2+}$ positions on actin, depending on the specific isoforms examined (ref. 43; see also refs. 44 and 45).

In contrast to the globular actin structure, the different (axial) regions of the linear tropomyosin coiled coil cannot be distinguished at the resolution of the EM reconstructions, and its density appears uniform (but see below). It has therefore not been possible to observe directly either the axial position or the polarity of the tropomyosin filament on F-actin. Nevertheless, the polarity of the tropomyosin–actin complex, with the N terminus of tropomyosin oriented toward the “pointed” end of actin (subdomains 2 and 4 in Fig. 4a), has been inferred by immunoelectron microscopy of antibody-labeled TnT fragments on the thin filament (46); this polarity is consistent with the binding of tropomodulin to the pointed end of actin and to the N-terminal region of tropomyosin (47, 48).

Proposed Axial Position of Tropomyosin on Actin. Biochemical results and our current knowledge of the thin filament structure allow us to model the axial position of tropomyosin on the F-actin filament (Fig. 4b and c). The association between tropomyosin and actin is dynamic and is in large part electrostatic in nature. Nevertheless, because the removal of apolar residues from aqueous solvent is a critical driving force in the association between proteins, we have aligned tropomyosin on actin by first matching the most apolar patch of period 5 with a prominent apolar surface on actin. On tropomyosin, this apolar patch is on the helical turn containing residues 169–172 (including highly exposed b and c position residues 170 and 171 and partially exposed core position residues 169 and 172). This is the only highly conserved location on tropomyosin with four apolar residues in a row. They are located within one of the most important regions for actin binding according to mutational studies [i.e., the N-terminal half of period 5 (see above)], and they are at the center of the most stabilizing crystal contact of the current structure (see results). On actin, residues 329–333 on subdomain 3 appear to be the most prominent apolar surface for the binding of tropomyosin. Like the rest of actin, these residues are highly conserved. They are located at or just past the C-terminal half of an edge (surface) β-strand on a “ridge” on subdomain 3 (at its junction with subdomain 1) with Pro-333 highly exposed. According to EM studies, this is the primary apolar surface of actin that contacts tropomyosin in both the off and Ca$^{2+}$-activated azimuthal positions (42). [In the fully activated state, these residues appear to contact the so-called “cardiomyopathy loop” of myosin S1 (45, 50)].

The axial alignment that positions residues 169–172 of tropomyosin over 329–333 of actin is supported by strikingly favorable electrostatic interactions that result in the axially adjacent regions on either side (Fig. 4c). At the barbed end of subdomain 3 (and subdomain 1), a positively charged patch, consisting of Lys-326 and Lys-328 from the N-terminal half of subdomain 3 appear to be the most prominent apolar surface for the binding of tropomyosin. Like the rest of actin, these residues are highly conserved. They are located at or just past the C-terminal half of an edge (surface) β-strand on a “ridge” on subdomain 3 (at its junction with subdomain 1) with Pro-333 highly exposed. According to EM studies, this is the primary apolar surface of actin that contacts tropomyosin in both the off and Ca$^{2+}$-activated azimuthal positions (42). [In the fully activated state, these residues appear to contact the so-called “cardiomyopathy loop” of myosin S1 (45, 50)].
the pointed end of subdomain 3, Glu-334 of actin would here face Arg-167 of tropomyosin.

In this model, the azimuthal position of the tropomyosin coiled coil coincides with that of the Ca²⁺-activated state. This azimuthal position results from maximizing the electrostatic interactions, which, in turn, occurs at a specific rotational position of tropomyosin about its axis. Here, the second helix of tropomyosin is locally oriented toward the inner domain of actin. This overall position of tropomyosin on the actin subunit also results (for one helix) in the downward orientation of apolar residue 170 of tropomyosin, rather than apolar 171, to bury Pro-133 of actin. This model, in which residues 167, 171, and 178 (from both helices) would be solvent-exposed, is consistent with fluorescence quenching experiments of mutant tropomyosins that suggest that the analogous residues 90, 94, and 101 in period 3 are similarly exposed in the Ca²⁺-activated state (51). These same experiments also indicate that these residues
are less exposed in the off state, suggesting that burial of apolar 171 of tropomyosin may occur here.

Taken together, the segment of tropomyosin’s functionally critical fifth period that appears to best match the outer edge of actin’s protruding subdomain 3 spans residues 167–184. This stretch of tropomyosin also coincides perfectly with the fifth α zone identified in sequence analyses (4), consistent with the suggested dominant role of the α rather than β zones of tropomyosin for interactions with actin (5).

This kind of model would also apply to the rest of the tropomyosin molecule and filament, because most of the features described in the model for the α zone of period 5, including the basic residue at 167, the apolar residues at 170 and 171, and especially the acidic residues at 177, 181, and 184, are present in the other periods as well (4, 5) (Fig. 4d). On this view, tropomyosin filaments would traverse a repeating landscape on the surface of F-actin (Fig. 4b). The closest contacts would occur between the periodic F-actin ridges (subdo-

mains 3 and 1) and the semiperiodic contacts would occur between the periodic F-actin ridges (subdomains 3 and 1) and the semiperiodic α zones of tropomyosin. In contrast, the β zones, which alternate with the α zones (4) but are less periodic (ref. 5 and D. A. D. Parry, personal communication), would bridge over and make relatively few contacts with valley floors of subdomains 2 and 4 (Fig. 4b). (In this model, in the Ca2+-activated form, theseβ toβ interactions would be absent.)

Not that a singular region of the tropomyosin filament is the overlap between consecutive molecules, which involves approximately nine residues from the N and C termini (52), as well as a non-coiled-coil stretch observed to extend from the C terminus back as far as residue 263 in structures of fragments (8, 9). This region (residues 263–284) coincides precisely with the seventh β zone; its location over a valley rather than in close contact with a ridge of actin would support the picture of an uninterrupted interaction between F-actin and a tropomyosin filament across the head–tail overlap (52).

Further evidence for this specific model proposed here is provided by the observation of an increased affinity (~2-fold) for actin in certain short nonmuscle and chimeric smooth-muscle α-tropomyosins. The sequences of these tropomyosins differ from those of their lower-actin-affinity counterparts only at positions 189–213 (see refs. 53–55). In these tropomyosins, in addition to the four conserved apolar residues in period 5 described above (Fig. 4d), there is also another stretch of five apolar residues in a row at the analogous segment of period 6 (Fig. 7), which is published as supporting information on the PNAS web site). The most striking amino acid difference between the weaker and stronger actin-binding tropomyosins in this segment is the replacement of a charged residue, glutamate, with a large apolar residue, methionine, at position 208 (Fig. 7). A hydrophobic interaction of this methi-

onine with Pro-333 (and neighboring apolar residues) of actin, 

which is predicted by the specific axial and rotational position of tropomyosin on actin in the current model (Fig. 4 b and c), may account for the increased affinity of these tropomyosins for actin.

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