Ca\(^{2+}\)-induced Rolling of Tropomyosin in Muscle Thin Filaments

THE \(\alpha\)- AND \(\beta\)-BAND HYPOTHESIS REVISITED\(^*\)

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Tropomyosin is a filamentous coiled-coil protein directly involved in the regulation of the actomyosin interaction responsible for muscle contraction: it transmits the local calcium-induced conformational change in troponin to the helical array of myosin-binding sites on the surface of the actin filament. McLachlan and Stewart (McLachlan, A. D., and Stewart, M. (1976) J. Mol. Biol. 103, 271–298) proposed that the tropomyosin coiled-coil structure can be divided into 14 alternating 19- to 20-residue “\(\alpha\)- and \(\beta\)-bands,” which could act as alternate 7-fold sets of sites for specific binding to actin in the different conformational states of the regulated thin filament. Here we present the first direct experimental evidence in support of the \(\alpha\)- and \(\beta\)-band hypothesis: we analyze the acrylamide quenching of the fluorescent probes placed in the \(\alpha\)-bands, which we show that fluororescent probes placed in the \(\alpha\)-bands become less solvent-exposed in the absence of calcium, whereas those in the \(\beta\)-bands become less solvent-exposed in the presence of calcium. A model in which the tropomyosin coiled-coil rolls across the actin surface in response to Ca\(^{2+}\)-binding to troponin most easily explains these observations.

Skeletal muscle tropomyosin (Tm)\(^1\) is a dimer of 284-residue \(\alpha\)-helical chains that associate as an in-register parallel coiled-coil (1–4). The coiled-coil tertiary structure of Tm is derived from a heptad repeat in its primary structure (abcdefg) in which positions \(a\) and \(d\) are preferentially occupied by hydrophobic residues buried at the interface between the two \(\alpha\)-helices. The other positions in the heptad repeat are occupied predominantly by hydrophilic residues, with charged residues at positions \(e\) and \(g\) often being involved in interhelical salt bridges (5).

Tm molecules polymerize in a head-to-tail fashion along the long pitch strands of F-actin filaments. One trimeric tropomyosin (Tn) complex associates with the C-terminal half of Tm, which in turn spans seven actin monomers on each F-actin strand.

Calcium binding to Tn triggers a series of conformational changes (6–9) that reposition the Tm molecule in a manner such that myosin binding sites on the actin surface are exposed, leading to strong stereospecific actomyosin binding and the generation of force (7, 10–22). The majority of models for Tm-mediated regulation of actomyosin activity assume that regulated thin filaments may exist in two or three biochemically distinguishable states, which are distinguished in part by the two or three distinct positions of Tm fibers along the actin filament: the “blocked” or “off” state, where Tm sterically blocks specific myosin binding to actin, the “off” or “closed” or “Ca\(^{2+}\)^{-induced}” state, which permits weak specific binding, and the “on” or “open” or “myosin-induced” state, in which strong rigor-like actomyosin cross-bridges may form (2, 10–14, 23, 24). The binding of Ca\(^{2+}\) and/or myosin to regulated thin filaments can influence the equilibrium controlling the population of the thin filament conformational states (25, 26), and electron microscopy studies of regulated thin filaments have provided evidence of Tm movement across the actin surface (13–15, 19). Narita and co-workers (27) have suggested that Tm may kink at specific sites in response to Ca\(^{2+}\) such that different regions of a single Tm molecule may adopt different azimuthal positions on the actin surface. Recently (21, 22) a model was presented where Tm fibers associated with actin are treated as a weakly confined continuous flexible chain. Here, thermal fluctuations within a weak electrostatic potential result in kinks with dimensions that may be significantly smaller or larger than that of a full-length Tm molecule, thereby producing a range of orientations (permitted sinusoidal paths) of the Tm fiber along the actin filament.

The degree of resolution of the above models, however, have so far been insufficient to address a fundamental and as yet unsolved question central to the understanding of thin filament-based regulation of muscle contraction: which Tm residues are involved in the binding to actin in the presence and absence of Ca\(^{2+}\) ions and/or myosin? The elucidation of these sites could reveal details of the conformational changes involving Tm in the thin filament. For example, the means by which Tm is translated between positions on the thin filament is still not clear: Tm could “roll” or “slide” across the actin surface. Tm rolling would involve a rotation of the coiled-coil superhelix with respect to actin and implies the use of different residues to bind actin in each state, as previously suggested (2, 23). On the other hand, Tm sliding would imply the use of the same Tm surface to bind different sites on actin.

Based on an analysis of the chemical nature of solvent-exposed residues in the Tm sequence, McLachlan and Stewart (23) identified 14 pseudo-repeats of 19–20 residues, which they divided into 7 pairs of \(\alpha\) and \(\beta\) bands. They hypothesized that the \(\alpha\)- and \(\beta\)-bands may in fact represent alternative 7-fold sets of sites that bind to complementary sites on the actin surface in

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\(1\) The abbreviations used are: Tm, tropomyosin; Tn, troponin; 5-OHW, 5-hydroxytryptophan; ASTm, Ala-Ser N-terminal fusion; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol.

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the absence and presence of Ca\(^{2+}\). Because the -20-residue spacing between α- and β-bands corresponds to an -90° rotation in the coiled-coil superhelix, this α- and β-band hypothesis implies a corresponding rolling of Tm triggered by the binding of Ca\(^{2+}\) to the thin filament. Bacchioni and Lehrer (28) showed evidence for the rolling of Tm on the actin surface, based on frequency-domain fluorescence energy transfer data between 5-(2-iodoacetylaminonaphtalene)naphtalene-1-sulfonic acid at Cys-190 of Tm and phallloidintetramethylrhodamine B isothiocyanate bound to F-actin. However, the preferential binding of specific Tm residues or surfaces to actin in a Ca\(^{2+}\)-dependent manner, although a highly attractive model, remains as yet in need of direct experimental support.

One efficient and direct method to localize specific contacts between two proteins is to place fluorescent probes at unique positions and measure the attenuation of fluorescence as a function of neutral quencher concentration (29–31). Because protein–protein interfaces usually involve the exclusion of solvent and small solutes, the fluorescence of probes at binding sites is quenched with less efficiency than for those at solvent-exposed positions. Here we present the results of an extensive set of fluorescence quenching experiments using regulated muscle thin filaments reconstituted with recombinant Tms containing 5-hydroxytryptophan (5-OH-W) probes at nine different positions. Our results are consistent with the model originally proposed by McLaughlin and Stewart (23) in which Tm α-band residues interact with the actin surface in the absence of Ca\(^{2+}\), whereas β-band residues move toward actin in the presence of calcium.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Tryptophan codons were created at positions 90, 94, 101, 107, 111, 122, 261, and 278 of the chicken fast skeletal Tm expressed and purified separately and the Tn complex was reconstituted with recombinant Tms containing 5-hydroxytryptophan (5-OH-W) probes at nine different positions. Our results are consistent with the model originally proposed by McLaughlin and Stewart (23) in which Tm α-band residues interact with the actin surface in the absence of Ca\(^{2+}\), whereas β-band residues move toward actin in the presence of calcium.

**Actin Binding Assay**—Actin binding assays were performed using an AVIV Instruments ATF 105 spectrofluorometer. Excitation and emission bandwidths were 1.0 and 5.0 nm, respectively, and excitation and emission wavelengths were 312 and 337 nm, respectively. Tm (1 μM), actin (7 μM), and the Tn complex (2 μM) were combined in 25 mM MOPS, pH 7.0, 50 mM NaCl, 5 mM MgCl\(_2\) and 1 mM EDTA plus either 1 μM EGTA or 1 mM CaCl\(_2\). The solution was titrated with acrylamide up to 0.5 M. Data was analyzed using the modified Stern-Volmer relation for collisional and static quenching,

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F/F_o = 1 + K_{SV} [Q]
\]

where \(F\) is the fluorescence of the sample in the absence of the quencher, \(F_o\) is the fluorescence as a function of acrylamide concentration \([Q]\), and \(K_{SV}\) is the Stern-Volmer or dynamic quenching constant, and \(V\) is the static quenching constant (29–31). Curvature fitting was performed using the SigmaPlot 2000 program (SPSS Inc.). The inner filter effect was insignificant under all conditions tested.

**RESULTS**

**Mutant Tms: Rationale of Design, Conformational Stability, Actin Binding, and Regulation of the Actomyosin Mg\(^{2+}\)-ATPase**—We have produced mutant ASTms (32) containing 5-OH-W residues at one of nine positions along the molecule: positions 90, 94, 101, 107, 111, 122, 261, 276, and 278. The mutants were named 5OH90W, 5OH94W, and so on (34). The original residues in the chicken skeletal Tm that were mutated are as follows: Arg-90, Leu-94, Arg-101, Ala-107, Glu-111, Glu-122, Tyr-261, His-276, and Leu-278. Expression and purification vector (36). The original residues in the chicken skeletal Tm cDNA—band residues move toward actin in the presence of calcium.

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**Fig. 1.** The primary sequence of chicken skeletal α-Tm (58). The Tm sequence is arranged to highlight the 7- and 14-fold wih\(\hat{\zeta}\)l-repeat pattern (23). The heptad repeat position is indicated in italics below each line of the primary sequence. Residues that were mutated to Trp are bold and underlined.

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**Fig. 2** presents molecular models of the segments of the Tm coiled-coil corresponding to the third and seventh pairs of α-
and β-bands corresponding to residues 86–125 and 244–283, respectively. In these models, we have indicated the residues that were mutated to 5-OHW in the recombinant proteins. In Fig. 2 (A, D, B, and E) we assume that both Tm segments adopt a coiled-coil polypeptide backbone structure based on a Tm model (44). However, recent crystal (45) and solution NMR (46) studies of C-terminal fragments indicate that the C terminus of Tm may in fact not adopt a canonical coiled-coil structure. In both structures, the two α-helices splay apart over the last twenty residues. For this reason, in Fig. 2 (C and F) we present a model of the seventh α/β repeat in which residues 254–283 appear as found in the crystal structure (45).

We analyzed the effect of the mutations on the thermal stability of Tm. Thermal denaturation was accompanied by circular dichroism. All mutants except for 5OH90W was presented a similar denaturation temperature (T_m ~ 47 °C) to that observed for Astm (Fig. 3). This indicates that the probes at these positions do not affect the global stability of the Tm molecule to a great deal. That these mutations may in fact be causing local changes in Tm structure and stability is witnessed by the observation that the 5OH90W mutant repeatedly aggregated at temperatures above 40 °C under the conditions tested. We do note, however, that the denaturation profile up to this temperature was similar to the other proteins tested (data not shown). These results indicate that, although the mutation promotes aggregation of the molecule at higher temperatures, its stability at lower temperatures seems to be similar to that of the other tropomyosin molecules tested.

To determine the effect of the 5-OHW mutations on the regulatory properties of the mutant Tms, we first analyzed their capabilities to bind actin (Fig. 4). Actin binding assays were performed under four different conditions (Fig. 4): (i) Tm plus actin; (ii) Tm, actin, and Tn in the absence of free calcium ions (1 mM EDTA); (iii) Tm, actin, and Tn in the presence of calcium ions (1 mM CaCl_2); and (iv) Tm and actin in the presence of 0.5 μM acrylamide. All mutant Tms were able to bind actin on their own at levels comparable to that observed for Astm in the absence of acrylamide (Fig. 4). All Tms also bound to actin in the presence of Tn complex in both the absence and presence of Ca^{2+}. Because the fluorescence quenching assays (described below) were carried out in the presence of up to 0.5 μM acrylamide, actin binding experiments were also carried out under these conditions (Fig. 4A, column 4). Only the mutant with 5-OHW at position 90 showed a significant reduction in its binding to actin in the presence of 0.5 μM acrylamide (Fig. 4). However, in the presence of the Tn complex, 5OH90W was found to bind actin even in the presence of acrylamide (Fig. 4A).

We tested all mutants for their ability to mediate Ca^{2+}-dependent regulation of the actomyosin and acto-S1 myosin Mg^{2+}-ATPase in the presence of tropinin (Fig. 5). What we focus on here is the nature of the Ca^{2+}-induced changes in the observed Mg^{2+}-ATPase activities. The presence of acrylamide (500 mM) did not affect the regulatory properties of Astm to a great degree. Several of the mutants displayed regulatory properties different from that of Astm. All mutants except for 5OH90W inhibited the acto-S1 myosin ATPase to varying degrees (10–50%) in the absence of tropinin (Fig. 5B). On its own, mutant 5OH90W activated the acto-S1 myosin Mg^{2+}-ATPase to a level ~20% greater than that observed in the absence of Tm (Fig. 5B). Activation of the acto-S1 myosin ATPase by 5OH90W has been observed before (34). In the case of full-length myosin, ATPase activity was activated by 5OH90W, 5OH261W, and 5OH278W, whereas the other mutants either inhibited or had no effect (Fig. 5A). For both actomyosin and acto-S1 myosin, the addition of Tn in the absence of Ca^{2+} (+EGTA) maintained or increased inhibition, and the addition of Ca^{2+} relieved this inhibition to varying extents (Fig. 5). The variation in the degrees of inhibition and disinhibition of ATPase activity does not seem to correlate with the localizations of 5-OHW residues in specific positions within the αβ or heptad repeats. We note that the degree of Ca^{2+}-dependent regulation observed in these assays is not as complete as that observed previously for Astm (Monteiro et al. (32)). We do not know the cause for this discrepancy at the moment and can only speculate that it may be due to differences in the assay conditions or in the preparation of one of the protein components.

Acrylamide Quenching—Acrylamide quenching studies were
FIG. 2. Molecular models of αβ repeats III and VII of Tm. A and B, axial views from the C-terminals of αβ repeats III (residues 86–125) and VII (244–283) showing side chains (extended all-trans conformation) of residues Arg-90 (light green), Leu-94 (blue), Arg-101 (light blue), Ala-107 (gray), Gln-111 (red), Glu-122 (orange), Tyr-261 (dark blue), His-276 (yellow), and Leu-278 (salmon). The backbone conformations of both models are based on the low resolution model for repeat III (44). α-Bands (blue main chain) and β-bands (red) are related by 90° rotations of the coiled-coil super-helix. The horizontal lines represent a plane separating the two α-helices of the coiled-coil. Because Tyr-261 is only two residues away from the beginning of the β-band, a small azimuthal overlap is observed with His-276 when viewed down the coiled-coil axis. D and E, longitudinal views of αβ repeats III and VII. All side chains are shown except those in the internal positions a and d of the heptad repeat. Coloring is the same as in A and B. C and F, a model of αβ repeat VII based on the crystal structure of the Tm C terminus resolved by Li et al. (45) (in this figure, Tm residues 244–253 were modeled by mutating GCN4-derived residues of the crystal structure).
Fig. 3. Thermal denaturation of the tropomyosin mutants monitored by CD. The curves show fraction unfolded versus temperature for AS-Tm and the tropomyosin mutants. Fraction unfolded was calculated based on the ellipticity at 222 nm (see “Experimental Procedures”). The midpoints of the denaturation transitions \( (T_m) \) are shown in parenthesis. Conditions: 10 mM protein, 5.73 mM NaH\(_2\)PO\(_4\) (pH 7.0), 50 mM NaCl, 5 mM MgCl\(_2\), and 1 mM DTT.

Fig. 4. Actin binding assay. Actin (7 \( \mu \)M), Tm (1 \( \mu \)M), and Tn complex (1 \( \mu \)M) were dissolved in 25 mM MOPS (pH 7.0), 50 mM NaCl, 5 mM MgCl\(_2\), and 1 mM DTT containing either 1 mM CaCl\(_2\) or 1 mM EDTA. Each sample was ultracentrifuged. The initial mixture (left lane), the supernatant (center), and the pellet (right) were then analyzed by SDS-PAGE. A displays results for all the mutants tested. B displays the full gels for mutants 5OH90W, 5OH122W, and 5OH276W, demonstrating that they all mediate troponin binding to actin. Equivalent results were observed for all other mutants (data not shown). Tm.Ac, actin plus Tm; Tm.Ac.Tn, actin, Tm, and Tn in the absence of calcium ions; Tm.Ac.Tn.Ca, actin, Tm, and Tn in the presence of calcium; Tm.Ac (or Tm.Ac.Tn.Ca) + \( y \), actin and Tn (or actin, Tm, and Tn) in the presence of calcium in the presence of 0.5 M acrylamide. *, actin; **, tropomyosin; T, TnT; I, Tnf; C, TnC.

carried out on the nine Tm mutants under four conditions: Tm alone, Tm plus actin, Tm plus actin plus Tn minus Ca\(^{2+}\), and Tm plus actin plus Tn plus Ca\(^{2+}\). Fig. 6 presents representative Stern-Volmer \( (F_0/F \text{ versus } [\text{acylamide}]) \) curves of the quenching data obtained for Tms with 5-OHW at positions 101 and 122. The values of the dynamic \( (K_{SV}) \) and static \( (V) \) quenching
constants (29–31) obtained for all nine mutants under each condition are presented in Table I. The $K_{SV}$ values for each mutant were normalized with respect to the value obtained in the absence of other proteins ($K_{SV}/K_{SV0}$) and presented in Fig. 7 (B and E).

In the following paragraphs we will consider the quenching results under each condition separately to facilitate their interpretation in terms of Tm and thin filament structure. Before doing so, however, we should consider to what extent changes in $K_{SV}$ values caused by actin, Tn, or Ca$^{2+}$ binding to each mutant do in fact reflect increases in fluorescence lifetime and in only a few specific cases do we observe fluorescence intensity changes induced by actin, Tn, or Ca$^{2+}$ binding (Table I). These specific cases are the following: (i) Ca$^{2+}$ binding does not result in significant changes in the fluorescence intensity changes in any of the mutants except for 5OH122W (34) (Table I). In the case of 5OH122W, Ca$^{2+}$ binding to regulated thin filaments causes a 15% increase in fluorescence intensity (34), whereas the $K_{SV}$ value decreases from 5.4 M$^{-1}$ to 4.9 M$^{-1}$ (Table I). Thus, for 5OH122W, the Ca$^{2+}$-induced reduction in solvent.

**Fig. 5.** Regulation of myosin (A) or myosin S1 fragment (B) Mg$^{2+}$-ATPase activity by reconstituted thin filaments containing Tm mutants. The graphs display the relative ATPase activity for each mutant containing 5-hydroxytryptophan residues at the indicated positions. All values are reported relative to that of actomyosin or acto-S1 (1.0, horizontal line). Error bars show the standard error of at least four determinations. Conditions: actin (7 μM), Tm (1 μM), Tn (2 μM), and myosin or myosin S1 fragment (0.2 μM) were combined in 25 mM MOPS (pH 7.0), 50 mM or 75 mM NaCl (for S1 or myosin respectively), 5 mM MgCl$_2$, 0.5 mM EGTA, 1 mM DTT, and 1 mM CaCl$_2$ where indicated at 25 °C. The unmutated recombinant ASTm protein was also tested in the presence of 500 mM acrylamide (+acryl.).
exposure of the probe is greater than at first suggested by the reduction in the $K_{SV}$ value alone. (ii) The only mutant whose fluorescence intensity is reduced significantly by the binding of actin is 5OH261W (Table I). For this mutant, the actin-induced reduction in fluorescence intensity is $-23\%$ (47), a value only slightly less than the reduction in the $K_{SV}$ value (28%). This indicates that the actual variation in the $K_{SV}$ value due to the addition of actin to this mutant is less than the value reported ($-5\%$ in the degree of exposure of the probe). We should note, however, that neither the introduction of troponin nor of calcium ions to this system causes any further variation on the intensity of the fluorescence of this mutant (Table I). The only other mutant whose fluorescence intensity is sensitive to actin binding is 5OH122W. The fluorescence intensity of 5OH122W increases $-40\%$ upon actin binding (34); that is, in the direction opposite to that observed for the change in $K_{SV}$ (Table I).

Thus, again, the change in $K_{SV}$ value correctly indicates the nature of the change in $k_q$, but underestimates the extent of the change. The fluorescence intensities of all the other mutants do not change upon binding to actin (34) (Table I). (iii) The only probes with fluorescence intensities sensitive to the binding of troponin ($-\text{Ca}^{2+}$), in the presence of actin, are those situated at positions 276 and 278. These residues are most likely directly involved in an interaction with troponin. The increases in the intensity of their fluorescence spectra are 24 and 14\%, respectively. These values are similar to the variations observed in their $K_{SV}$ values (Table I). Nonetheless, the introduction of calcium ions does not cause any further variation in the intensity of the fluorescence spectra for these mutants.

The above considerations indicate that to more directly reflect changes in probe exposure (changes in $k_q$) we may divide the $K_{SV}$ values by the -fold change in $F_o$ (which is directly proportional to the -fold change in $\tau_o$). These “corrected” values for $K_{SV}$, after normalization with respect to the value obtained in the absence of other proteins ($K_{SV}/K_{SVo}$), are shown in Fig. 7 (C and F). Comparison of Figs. 7B, C, E, and F shows that the $\alpha/\beta$-band dependence of the $K_{SV}$ value is maintained even when taking intensity changes in the fluorescence spectra into account. Finally, we note that, as each mutant probably has a different fluorescence lifetime, comparisons of the magnitudes of $K_{SV}$ between mutants are not as important as the changes in $K_{SV}$ in a single mutant that result from the conformational changes induced by the binding of other proteins (actin and Tn) and $\text{Ca}^{2+}$.

In the absence of other proteins, the $K_{SV}$ values of the 5-OHW probes at different positions in Tm ranged from 4.7 $\text{m}^{-1}$ to 10 $\text{m}^{-1}$ (Table I). These values are comparable to the acrylamide quenching $K_{SV}$ constants observed for tryptophan residues in proteins in partially to fully solvent-exposed environments (48). This is as expected, because all nine Tm mutants have 5-OHW residues in solvent-exposed positions $b$, c, e, or f of the heptad repeat (Fig. 1 and Table I). In comparison, the $K_{SV}$ values for the acrylamide quenching of indole, acetyltryptophanamide and Trp in water are 30.5, 17.5, and 14.8 $\text{m}^{-1}$, respectively (49). It is worth noting that the $K_{SV}$ value obtained for the 5OH90W mutant, which aggregates irreversibly above 40 $^\circ\text{C}$, is greater than that obtained for any of the other mutants. This may indicate that the probe in this position may be more exposed than in the other positions tested due to some specific structural feature of this region of the Tm molecule or due to a mutation-derived structural abnormality.

Acrylamide quenching experiments performed in the presence of actin resulted in decreased $K_{SV}$ values (Table I and Fig. 7C and F), signifying reduced accessibility of at least one of the probes to the quencher for all mutants (except perhaps for 5OH261W, see above). Probes at positions 276 and 278 had great proportional decreases in accessibility (Fig. 7). This may be due to the localization of these C-terminal residues in the head-to-tail overlap region, which includes the first and last 9–11 residues of Tm (50). Under the conditions employed, less than half of the Tm C termini are expected to be involved in head-to-tail interactions in the absence of actin (33). Because this fraction probably increases upon binding to actin, the relatively large burial of the probes at positions 276 and 278 could be due to a combination of interactions with both actin and the N-terminal of a neighboring Tm molecule.

The addition of Tn ($-\text{Ca}^{2+}$) affects the degree of accessibility of the fluorescent probes in a position-dependent manner. The accessibility of the probes in $\alpha$-bands was either unchanged (positions 90 and 101) or significantly reduced (94 and 261) by increasing $-40\%$ upon actin binding (34); that is, in the direction opposite to that observed for the change in $K_{SV}$ (Table I). Thus, again, the change in $K_{SV}$ value correctly indicates the nature of the change in $k_q$, but underestimates the extent of the change. The fluorescence intensities of all the other mutants do not change upon binding to actin (34) (Table I). (iii) The only probes with fluorescence intensities sensitive to the binding of troponin ($-\text{Ca}^{2+}$), in the presence of actin, are those situated at positions 276 and 278. These residues are most likely directly involved in an interaction with troponin T. The increases in the intensity of their fluorescence spectra are 24 and 14\%, respectively. These values are similar to the variations observed in their $K_{SV}$ values (Table I). Nonetheless, the introduction of calcium ions does not cause any further variation in the intensity of the fluorescence spectra for these mutants.

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The addition of Tn ($-\text{Ca}^{2+}$) affects the degree of accessibility of the fluorescent probes in a position-dependent manner. The accessibility of the probes in $\alpha$-bands was either unchanged (positions 90 and 101) or significantly reduced (94 and 261) by
the presence of Tn in the absence of Ca\(^{2+}\) (Fig. 7). In contrast, all probes positioned in \(\beta\)-bands became either more accessible (positions 107, 111, and 122) or did not change (276 and 278) upon the addition of Tn in the absence of Ca\(^{2+}\) (Fig. 7). These results are consistent with a role for Tn in fixing Tm into position in the absence of calcium, bringing \(\alpha\)-band residues into closer contact with the actin filament while at the same time positioning \(\beta\)-band residues away from the actin filament.

The relatively small changes observed for \(\beta\)-band mutants 5OH276W and 5OH278W upon troponin binding (Ca\(^{2+}\)) should be interpreted in the light of the fact that this region probably interacts with troponin (53). Thus a significant exposure of the probes with respect to the actin filament may be balanced by partial burial by troponin.

The addition of Ca\(^{2+}\) ions to Tm-actin-Tn results in dramatic changes in 5-OHW accessibility, and, again, the nature of the change is dependent on the localization of the probes to either \(\alpha\)- or \(\beta\)-bands. Ca\(^{2+}\) binding to Tn causes all four \(\alpha\)-band positions tested to become more accessible to quenching by acrylamide (increased \(K_{SV}\) values) while at the same time causing all \(\beta\)-band residues to become less accessible to the quencher (reduced \(K_{SV}\) values)(Fig. 7 and Table I). These results powerfully illustrate the mechanism by which Tn positions the Tm molecule over the actin filament in a Ca\(^{2+}\)-dependent manner and are consistent with the model proposed by McLachlan and Stewart (23) in which the \(\alpha\)-bands are used to bind to actin in the absence of Ca\(^{2+}\), whereas the \(\beta\)-band residues are involved in binding in the presence of Ca\(^{2+}\). We note that in the current three state model for the thin filament (13–15, 20) Tm occupies very similar positions in the absence of Tn and in the presence of Tn plus Ca\(^{2+}\). We, however, observed differences in the \(K_{SV}\) values in these two states. These data are not necessarily inconsistent with one another: the overall Tm positions may be similar in the two states but may differ in terms of flexibility (22, 52) and in terms of relative orientation of Tm and actin surfaces. Thus, upon Ca\(^{2+}\) binding, the Tn complex may actually promote Tm repositioning/reorientation on actin and not simply passively release it to assume its Tn-free preferred position/orientation.

DISCUSSION

To facilitate interpretation of the quenching results, a few considerations should be made regarding the probe environments in the Tm and thin filament structures. First, due to the parallel, in-register coiled-coil structure of the Tm molecule, each mutant has two 5-OHW residues, one in each polypeptide
Tropomyosin: The α- and β-Band Hypothesis Revisited

chain placed at diametrically opposed positions on the coiled-coil solvent-exposed surface (Fig. 2). The microenvironments of each 5-OHW residue are therefore equivalent or pseudo-equivalent in the absence of other proteins, but this symmetry is broken upon binding to actin. Second, probes localized to α/β repeat III (90, 94, 101, 107, 111, and 122; Fig. 2A and D) are not expected to make direct contact with Tn. Due to the rotational symmetries of the actin filament and the Tm coiled-coil, for these six mutants one of the probes is most probably accessible to solvent and quencher at all times, whereas the exposure of the other should be a function of the extent of its interaction with the actin surface. Third, as Tn makes contacts with the C-terminal half of Tm, the three mutants with 5-OHW probes in the C-terminal α/β repeat VII (261, 276, and 278; Fig. 2B, C, E, and F) could theoretically have both their probes partially buried, one by actin and the other by Tn (specifically the extended N-terminal domain of TnT3). Finally, positions 276 and 278 could be expected to be even less accessible to quencher in reconstituted filaments, because they are localized in the head-to-tail overlap region where complex, though as yet structurally uncharacterized, interactions involving two Tm dimers, the actin filament and TnT are taking place (33, 50, 51). Note that, in the recently determined crystal structure of a C-terminal fragment of skeletal muscle Tm, the 22 C-terminal residues splay apart due to the formation of a tail-to-tail dimer with a symmetry-related molecule (45) (Fig. 2, C and F). Evidence that this separation of Tm α-helices takes place in solution has been presented in NMR studies of a covalently linked Tm C-terminal dimer (46). The physiological significance of this splaying is not yet clear, but it is possible that it may reflect the structure of the C terminus in complex with the Tm N terminus, TnT, and/or actin.

Our results indicate that the α- and β-bands originally identified on the basis of a 14-fold periodicity (2, 23, 53) in the Tm primary sequence are indeed related to a differential binding of this molecule to the actin filament in the different states of skeletal muscle contraction. The results suggest that the introduction of Ca2+ to fully regulated thin filaments causes residues placed within α-bands to move away from the actin filament while residues within β-bands are moved closer the actin filament. Note that the −Ca2+ and +Ca2+ states of the thin filament probably do not correspond to two specific and stable conformations but are rather most likely best described by different equilibrium mixtures of two or three (or more) states whose relative populations are modulated by Ca2+ binding and/or the binding of myosin motor domains (11, 21, 22, 25, 26, 54, 55). We may nevertheless associate the α-bands with the principal interactions that occur in the “blocked” position, whereas the β-bands may be associated with the maintenance of the “closed” (and perhaps the “open”) position. The acrylamide quenching results are therefore most readily interpreted in terms of shifts in this equilibrium triggered by the binding of Ca2+ rather than reflecting structural features of pure and distinct conformational states. The factors that affect the accessibility of the 5-OHW probes may be more complex than a simple direct effect of the Tm-actin interface. No doubt, more data regarding the disposition of Tm-actin interfaces in different thin filament states is necessary. Nevertheless, all the nine mutants tested without exception follow the proposed pattern and are consistent with a sensible and testable regulation hypothesis.

Tm movement across the F-actin surface could in principle occur through a variety of mechanisms. Two simple and distinct mechanisms, “sliding” and “rolling,” are illustrated in Fig. 8. In a purely sliding mechanism (Fig. 8A) the Tm coiled-coil maintains the same face pointed toward the actin filament axis as it binds to different sites on the actin surface. This mechanism would predict minimal changes in solvent exposure of Tm residues in response to Ca2+, although non-systematic and therefore not easily foreseeable changes could be expected at specific sites. On the other hand, in a rolling mechanism (Fig. 8B) the Tm coiled-coil rotates about its own local axis and different faces of the coiled-coil surface are used to interact with actin. A rolling mechanism is consistent with our data, which reveals systematic solvent exposure changes across specific stretches of the Tm surface. With the limited number of mutants used, we cannot precisely determine the magnitude of rotation of the Tm coiled-coil in response to Ca2+, but the fact that all nine positions tested follow the pattern predicted for a 90° rotation suggests that this is a good first estimate. Quenching analysis of a greater number of Tm fluorescent mutants could in principle map out the periodic nature of the Tm-actin interaction in more detail and could possibly detect discontinuities in this periodicity such as kinks or twists. It should be noted that, as the α- and β-bands are displaced both azimuthally and axially along the Tm structure, the α- and β-band binding sites on actin should also be displaced both axially and azimuthally. At the moment, we cannot determine the phase of Tm binding to actin; that is whether all residues in one α/β repeat (as defined previously (23)) bind to a single actin monomer or whether binding is, for example, half-staggered (56) in which the α-band binds to one monomer and the β-band binds to another.

Tm rotation does not on its own imply rolling: Tm could simply rotate while remaining in the same position. As Ca2+-induced Tm movements have been observed (13–19), rotation leading to rolling is strongly favored. If Tm does roll on the surface of the actin-filament without slipping or sliding, then the rotation angle (−90°) and the radius of the coiled-coil (between −9 and 14 Å) may be used to estimate a translation of between 14 and 22 Å on the surface of the actin monomer. These values are consistent with models of Ca2+-induced Tm shifts on regulated thin filaments (11, 13–15, 19).

How does Ca2+ binding to Tn induce Tm rolling across actin? Our data favor an active role for Tn in positioning the Tm molecule in both the absence and in the presence of Ca2+.
because the accessibility profiles of both states are significantly different from that observed for Tm plus actin in the absence of Tn. Ca$^{2+}$-induced changes in the Tn complex may induce Tm rolling by applying a torque to the C-terminal half of Tm via the extended and helical N-terminal domain of TnT and/or may be influenced by Ca$^{2+}$-dependent interactions with TnI and the globular N-terminal domain of TnT with the central portion of Tm (6, 11, 20, 27, 51, 57). The specific mechanism of signal transduction from Tn to Tm and actin remain to be elucidated.

The recently resolved structure of the ternary Tn complex (9) should provide valuable leads for a first generation of high resolution model building.

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Ca\(^{2+}\)-induced Rolling of Tropomyosin in Muscle Thin Filaments: THE α- AND β-BAND HYPOTHESIS REVISITED

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