Regulatory Properties of Recombinant Tropomyosins Containing 5-Hydroxytryptophan: Ca^{2+}-Binding to Troponin Results in a Conformational Change in a Region of Tropomyosin outside the Troponin Binding Site†

Chuck S. Farah*,‡ and Fernando C. Reinach‡,§

Departamento de Bioquímica, Instituto de Química, and Howard Hughes Medical Institute, Universidade de São Paulo, Av. Prof. Lineu Prestes 748, Bloco da Química Fina, São Paulo, SP, CEP 05508-900, Brazil

Received November 30, 1998; Revised Manuscript Received May 17, 1999

ABSTRACT: We have introduced tryptophan codons at different positions of the chicken α-tropomyosin cDNA (Monteiro, P. B., Lataro, R. C., Ferro, J. A., and Reinach, F. C. (1994) *J. Biol. Chem. 269,* 10461−10466) and employed a trp auxotrophic *Escherichia coli* strain to express the proteins in media containing either normal tryptophan, 5-hydroxytrptophan, or 7-azatryptophan. The fluorescence of these latter two tryptophan analogues is excitable at 312−315 nm at which the natural fluorescence of other thin filament proteins (actin, troponin) is not excited. The recombinant tropomyosins have tryptophans or analogues located at amino acid positions 90, 101, 111, 122, or 185 of the protein, all on the external surface of the tropomyosin coiled-coil (positions "c" or "f" of the hydrophobic heptad repeat). The first four mutations are located within the third actin-binding zone of tropomyosin, a region not expected to interact directly with troponin or with neighboring tropomyosin molecules in muscle thin filaments, while position 185 is located in a region that has been implicated in interactions with the globular domain of troponin. The fluorescence intensity of the mutant containing 5-hydroxytryptophan at position 122 (5OH122W) is sensitive to actin binding and sensitive to Ca^{2+}-binding to thin filaments reconstituted with troponin. Assuming that the globular domain of troponin binds to a site between residues 150 and 190 of tropomyosin, the distance between the troponin-binding site and the fluorescent probes at position 122 can be estimated to be 4.2−10.2 nm. While X-ray diffraction and electron micrograph reconstitution studies have provided evidence of Ca^{2+}-induced changes in tropomyosin's interactions in the thin filament, their resolution was not sufficient to distinguish between changes involving the whole tropomyosin molecule or only that region directly interacting with troponin. Here we provide a clear demonstration that Ca^{2+}-binding to troponin results in a conformational change in a region of tropomyosin outside the troponin binding site which is probably associated with a changed interaction with actin.

Tropomyosin (Tm[1]) is a dimeric coiled-coil protein which polymerizes along the long-pitch helix of the thin filaments in muscle and nonmuscle cells. Each coiled-coil dimer interacts with seven consecutive actin monomers along one strand of the actin filament. Tm is an essential component in the troponin-based regulation of the actomyosin interaction in skeletal and cardiac muscle (*1*−*4*), is necessary for the cooperative binding of myosin subfragment 1 (S1) motor domains to actin (*5*), and can activate or inhibit the actomyosin subfragment 1 (acto-S1) ATPase, depending on the actin:S1 ratio (*6*, *7*). Muscle tropomyosin's affinity for actin is highly sensitive to ionic strength and to the presence of troponin (*8*). The NMR structure of a coiled-coil dimer of a chimeric polypeptide containing the first 15 amino acids of muscle tropomyosin has recently confirmed that the coiled-coil structure probably extends right up to the amino terminus (*9*).

A statistical analysis of the chemical nature of the solvent-exposed residues of tropomyosin (*10*−*12*) revealed seven pseudorepeats (see Figure 1) in which the solvent-exposed residues at specific positions have a tendency to be positive, hydrophobic, or negatively charged. It has been hypothesized that each pseudorepeat of approximately 40 amino acids may represent individual actin monomer binding sites and that specific residues may be alternatively utilized in the presence and in the absence of Ca^{2+} (*11*, *12*). Similarly, it has been proposed that regulation of the actomyosin interaction via Ca^{2+}-binding to troponin is mediated by changing the equilibrium of tropomyosin binding between two or more alternate sites along F-actin (*13*−*15*). This idea has recently

† This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional de Pesquisa (Brazil) and the Howard Hughes Medical Institute.

* To whom correspondence should be addressed. E-mail: chuck@iris1.iq.usp.br. Tel: 011-55-11-818-3312. Fax: 011-55-11-815-5579.

‡ Instituto de Química.

§ Howard Hughes Medical Institute.

[1] Abbreviations: Tm, tropomyosin; S1, myosin subfragment 1; NMR, nuclear magnetic resonance; DTT, dithiothreitol; IPTG, isopropylthio-β-D-galactoside; pCa, −log[Ca^{2+}]; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycolbis(β-amino ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ATP, adenosine 5′-triphosphate; ASTm, recombinant chicken tropomyosin with a dipeptide alanine−serine N-terminal fusion; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DAB-Mal, (4-(dimethylamino)phenyl)azophenyl-4-maleimide.

10.1021/bi982813u CCC: $18.00
Published on Web 07/22/1999

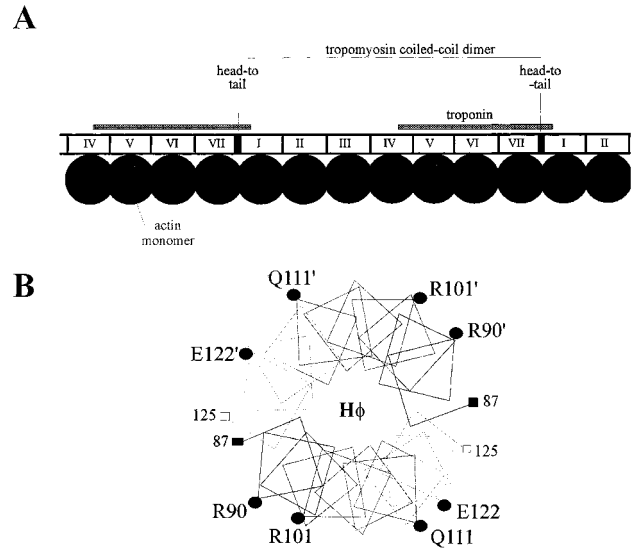

FIGURE 1: Design of fluorescent tropomyosin mutants. (A) Schematic diagram of tropomyosin's interactions in the thin filament showing the seven (I−VII) pseudorepeats first identified by MacLachlan and Stewart (*11*). Four of the tropomyosin mutants used in this study (90W, 101W, 111W and 122W) are located in repeat III. The fifth mutant (185W) is located in repeat V. Note that, originally, a 14-fold periodicity in the tropomyosin primary sequence was observed (*11*) in which each 7-fold repeat was divided into two pseudoequivalent bands (α and *β*) with each band consisting of a predominantly negatively charged zone of 12 residues and a hydrophobic/positvely charged zone of 8 residues. The statistical and structural significance of this 14-fold periodicity has been questioned, and when one considers the azimuthal as well as linear positions of tropomyosin residues in the coiled-coil structure, a more significant 7-fold periodicity (with maxima corresponding roughly to the α bands) has been found (*12*). Tropomyosin regions believed to be involved in interactions with troponin (*1*, *38*) are indicated by solid horizontal bars. (B) Localization of the fluorescent probes in the coiled-coil structure. A model (*63*) of the peptide backbone (C_α positions) of repeat III of tropomyosin (residues 87−125) is shown with the amino terminal end of the α-helices above the plane of the page. The C_α positions of residues 90, 101, 111, and 122 on each α-helical polypeptide chain of the coiled-coil are shown as large circles. These residues are located in solvent-exposed positions f, c, f, and c, respectively, of the coiled-coil heptad repeat. Mutant 185W (not shown) corresponds to position c in the heptad repeat. Hydrophobic residues at positions a and d of the heptad repeat are found at the interface between the two α helices at the center of the coiled-coil (H*ϕ*).

gained support from kinetic studies (*16*, *17*), analysis of electron micrographs of regulated thin filaments in the presence and absence of Ca^{2+} or myosin motor domains (*18*, *19*), and model-building studies based on electron microscope and X-ray diffraction data (*20*−*23*). Due to the lack of high-resolution data, the above modeling studies have assumed that the tropomyosin coiled-coil shifts between binding sites on actin essentially as a rigid rod, in each state making seven semiequivalent contacts with actin monomers. In most cases, head-to-tail contacts between neighboring tropomyosin molecules have been used to explain the tropomyosin-dependence of a variety of cooperative phenomena associated with thin filament assembly, Ca^{2+}-binding, and binding to myosin (*6*, *7*, *24*−*27*). Recent studies have suggested that at least some cooperative interactions between regulatory units in the thin filament are transmitted through actin and not through tropomyosin or troponin (*4*, *24*, *28*, *29*). Though tropomyosin's conformational flexibility has long been recognized (*12*, *30*), the possibility that different regions within a single tropomyosin molecule may bind actin with some degree of independence has been considered in only a small number of cases (*12*, *31*, *32*). The structural basis of tropomyosin-based cooperativity has been difficult to investigate due to the paucity of sites on tropomyosin amenable to specific modification with chemical probes.

Table 1

name of mutant tropomyosin	wild-type amino acid	pseudorepeat[a]	posn in heptad repeat
5OH90W	Arg90	III	f
5OH101W	Arg101	III	c
5OH111W	Gln111	III	f
5OH122W	Glu122	III	c
5OH185W	Leu185	V	c

[a] 5-Hydroxytryptophan residues are located in either the third (III) or fifth (V) 7-fold pseudorepeat of tropomyosin as defined in ref *11*.

We have shown that a recombinant tropomyosin containing a dipeptide amino-terminal fusion (ASTm) exhibits regulatory and physical properties similar to that of the muscle protein (*33*). In this study we introduced unique tryptophan codons at five different positions in the chicken skeletal tropomyosin cDNA with the dipeptide fusion (wild-type tropomyosin does not contain tryptophan). The mutant proteins were expressed using a trp auxotrophic *Escherichia coli* strain (*34*) to produce five ASTm mutants containing 5-hydroxytrptophan or 7-azatryptophan residues whose fluorescence is excitable at 312−315 nm (*35*−*37*) at which the natural fluorescence of other thin filament proteins (actin, troponin) is not excited. The recombinant tropomyosins have 5-hydroxytryptophans located at positions 90, 101, 111, 122, or 185 of the protein, all of which are on the external surface of the tropomyosin coiled-coil (positions *c* or *f* of the hydrophobic heptad repeat; ref *10*). The first four mutants all have 5-hydroxytrptophans located within the third pseudorepeat of tropomyosin (Figure 1 and Table 1). This region is not expected to interact directly with troponin nor with neighboring tropomyosin molecules (*38*). We also produced mutant 5OH185W in which the 5-hydroxytryptophan is in the fifth pseudorepeat (Table 1), a region expected to interact with the globular domain of troponin (Figure 1; *1*−*4*, *38*).

We studied the regulatory and fluorescence properties of these mutant proteins. We found the following: (i) All the mutant tropomyosins bind actin. (ii) All the 5-hydroxytryptophan-containing mutants, except for 5OH90W, inhibit the actomyosin(S1) ATPase. (iii) While 5OH90W does not inhibit the actomyosin(S1) ATPase, the same mutant with a tryptophan residue at position 90 (90W) inhibits the ATPase in a manner similar to ASTm. (iv) Only the 5OH122W mutant's fluorescence intensity is significantly sensitive to actin binding. Actin binding to this tropomyosin causes a 40% increase in fluorescence intensity with little or no change in λ_{max}. (v) The fluorescence of mutant 5OH122W is sensitive to Ca^{2+}-binding to thin filaments reconstituted with troponin. The Ca^{2+}-dependent fluorescence change is not observed in the absence of actin. This is a clear and direct demonstration that Ca^{2+}-binding to troponin results in a conformational change (probably a modified actin−tropomyosin interaction) outside the troponin-binding site. The fluorescence intensity of thin filaments reconstituted with 5OH185W is not affected by troponin ($\pm Ca^{2+}$) binding. (vi) Ca^{2+} has previously been shown to bind cooperatively to

vertebrate thin filaments (*27*, *39*) and the fluorescence change in mutant 5OH122W during Ca^{2+} titrations was significantly cooperative with a Hill coefficient of 2.2 and an apparent association constant of 1.4×10^6 M^{-1} ($pCa_{50} = 6.14$).

EXPERIMENTAL PROCEDURES

Recombinant and Muscle Proteins. Tryptophan codons were created at codons 90, 101, 111, 122, and 185 of the chicken fast skeletal tropomyosin cDNA (*33*) by oligonucleotide-mediated site-directed mutagenesis (*40*). The mutated cDNAs were transferred to the pET-3a expression vector (*41*). Recombinant tropomyosins with a dipeptide Ala-Ser amino-terminal fusion were expressed and purified as described (*33*) with the following modifications: (1) The host strain for protein expression was the trp auxotroph CY(DE3)pLysS (*34*, *41*; R. B. Quaggio and F. C. Reinach, unpublished). (2) Transformed bacterial cells were grown in minimal media containing 200 mg/L carbenicillin, 200 mg/L chloramphenicol succinate, and 100 mg/L L-tryptophan up to $A_{600} = 0.8-1.0$. For expression with tryptophan, 0.4 mM IPTG was added and the cells were incubated for 3 h at 37 °C. For expression with 5-hydroxytryptophan or 7-azatryptophan, cells were collected by centrifugation and transferred to minimal media (lacking tryptophan) containing antibiotics plus 0.4 mM IPTG. After 15 min of incubation at 37 °C, 200 mg/L L-5-hydroxytryptophan or 50 mg/L D,L-7-azatryptophan (Sigma) was added and the cells were incubated for 3 h at 37 °C. (3) The ammonium sulfate precipitation was omitted. (4) A 30−140 mM NaCl gradient was used in the DEAE-cellulose chromatography. We estimated the incorporation of 5-hydroxytryptophan or 7-azatryptophan into the recombinant proteins to be >90% (i.e., less than 10% tryptophan) on the basis of fluorescence excitation spectra (comparing emissions collected at 330 and 450 nm for 7-azatryptophan) and emission spectra (comparing excitations at 280 and 315 nm for both analogues). Furthermore, if no tryptophan or analogue is added to the media after cellular resuspension (15 min after addition of IPTG; see above), no recombinant protein is expressed. Actin (*42*) was purified from chicken pectoralis major and minor muscles. Myosin subfragment 1 (S1) was prepared by the chymotryptic digestion of chicken muscle myosin (*43*). Recombinant chicken fast skeletal muscle TnI, TnT, and TnC were expressed and purified as described (*44*, *45*). Troponin complexes were reconstituted as described (*45*) except that a 2-fold excess of TnC was used. Regulated thin filaments containing tropomyosin mutant 5OH122W for use in fluorescence assays were reconstituted in the following manner: Actin (7 μM), 5OH122W (1.5 μM), and troponin (3 μM) were combined in 25 mM MOPS (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, and 3.3 mM DTT and incubated on ice for 1 h. Thin filaments were sedimented 150 000*g* for 20 min at 4 °C. Supernatants containing excess tropomyosin, troponin, and TnC were discarded, and the pellets, containing stoichiometric amounts of actin, tropomyosin, and troponin, were resuspended in 1.5 volumes of the above buffer plus 0.5 mM EGTA (i.e. [actin] = 4.6 μM). We observed that the cooperativity of the Ca^{2+}-induced fluorescence change was dependent on the reconstitution of the thin filament with an initial stoichiometric excess of tropomyosin and troponin as described above, presumably to ensure saturation of F-actin with these components. Tobacman and Sawyer (*27*) also reported a necessity of a stoichiometric excess of troponin in order to observe cooperativity in the Ca^{2+}-induced fluorescence change of thin filaments containing TnC labeled with 2-(4-iodoacetamidanilo)naphthalene-6-sulfonic acid.

Actin-Binding Cosedimentation Assay. The standard assay consisted of combining 7 μM actin (diluted approximately 25-fold from a stock solution containing 25 mM MOPS (pH 7), 50 mM NaCl, and 1 mM MgATP) and 1 μM tropomyosin in the buffer described in Figure 4A and centrifuged at 150 000*g* for 10 min at 25 °C. Proteins in the mixtures before centrifugation and in the supernatants and pellets after centrifugation were analyzed by SDS−PAGE.

Actomyosin(S1) Mg^{2+}-ATPase Measurements. The 15 μM actin, 0.5 μM S1, and tropomyosin (concentration indicated in the figure) were combined in 25 mM MOPS (pH 7), 40 mM NaCl, 3.5 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM DTT. Reaction was initiated by the addition of 2.5 mM ATP. After 15 min, liberated inorganic phosphate was determined (*46*).

Fluorescence Spectra. Spectra were obtained using a Hitachi F4500 fluorometer (5 nm excitation and 5 or 10 nm emission slit widths, scan speed = 60 nm/min). Spectra were not corrected for nonideal responses of the instrument. Emission spectra were excited at 312 or 315 nm. Unless otherwise stated, assay conditions were 25 mM MOPS (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. As the filamentous form of actin scatters incident light in fluorescence experiments, when necessary this light-scattering background was subtracted from the overall signal to obtain the net tropomyosin fluorescence signal. In all of the figures only the net fluorescence signal is shown. For a mixture containing 1 μM 5OH122W and 7 μM actin, light scattering due to actin represents less than 15% of the intensity (λ_{ex} = 315 nm, λ_{em} = 335 nm) as shown in Figure 4A. Troponin (1 μM) does not scatter light under these conditions nor does it increase the light scattering of F-actin. Least-squares analysis (using SigmaPlot v3.01, Jandel Scientific) of the Ca^{2+}-dependent changes in the fluorescence of reconstituted thin filaments is described in the legend of Figure 5.

RESULTS AND DISCUSSION

Recombinant Tropomyosin Mutants Containing Tryptophan, 5-Hydroxytrptophan, and 7-Azatryptophan. Figure 1A shows a schematic model of tropomyosin's interactions with actin and troponin (*10*−*12*, *38*). The seven putative (see figure legend and ref *12*) actin-binding pseudorepeats in the tropomyosin primary sequence are shown. The expressed mutants have tryptophans or 5-hydroxytryptophans placed at positions 90, 101, 111, 122, and 185. A mutant containing 7-azatryptophan at position 122 was also produced. In the wild-type protein, these residues are Arg, Arg, Gln, Glu, and Leu, respectively. The tryptophan residues are located in the solvent-exposed positions f (mutants 90W, 111W) or c (mutants 101, 122, 185) of the hydrophobic heptad repeat (Figure 1B; Table 1). Since tropomyosin is a parallel homodimeric in-register coiled-coil (*10*, *47*), each dimer contains two tryptophans in equivalent positions on opposite sides of the coiled-coil surface (Figure 1B). Mutants 90W, 101W, 111W, and 122W were chosen so as to evenly cover the coiled-coil circumference of the molecule and to test which regions of the third pseudorepeat (a region not expected to be in direct contact with troponin (*38*)) are involved in binding to actin (Figure 1B). In the absence of

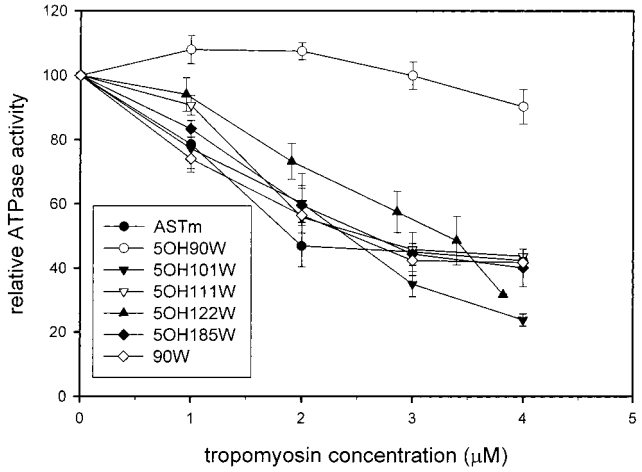

FIGURE 2: ATPase inhibition by recombinant tropomyosins. The acto-myosin(S1) Mg^{2+}-ATPase rate was determined as a function of recombinant tropomyosin concentration. Conditions: 15 μM actin; 0.5 μM myosin subfragment-1 (S1); 40 mM MOPS (pH 7); 40 mM NaCl; 3.5 mM $MgCl_2$; 1 mM DTT; 2.5 mM ATP; recombinant tropomyosin concentration indicated in the figure. A value of 100% corresponds to the Mg^{2+}-ATPase rate in the absence of tropomyosin. Symbols for each recombinant tropomyosin are indicated in the figure.

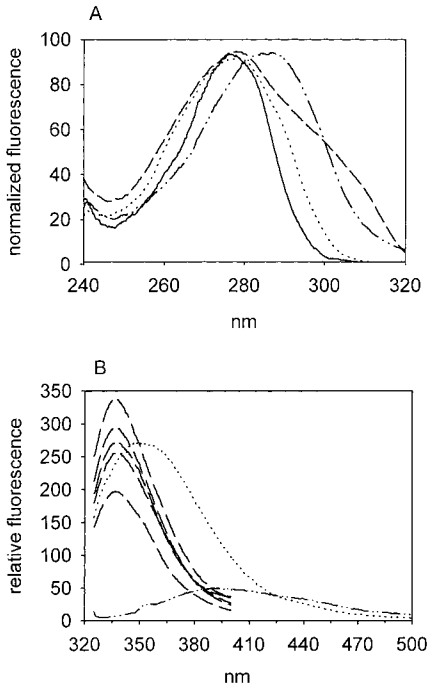

FIGURE 3: Comparison of fluorescence spectra of recombinant tropomyosins. (A) Normalized excitation spectra. The fluorescence emission was collected at 330 nm (ASTm), 330 nm (122W), 350 nm (5OH122W), and 390 nm (7N122W). Lines: ASTm, —; 122W, - - -; 5OH122W, — —; 7N122W, — - -. (B) Emission spectra. Excitations for the 5-hydroxtryptophan and 7-azatryptophan containing proteins were at 315 nm. Excitation for 122W was at 295 nm. Due to the different excitation wavelengths, the emission intensity (at λ_{max}) of 122W was normalized to that of 5OH122W. Lines: 122W, - - -; 7N122W, — - -; in order of increasing intensity, 5OH101W, 5OH111W, 5OH122W, 5OH185W, 5OH90W, — —. Conditions: 0.5 μM tropomyosins in 25 mM MOPS (pH 7.0); 50 mM NaCl; 5 mM $MgCl_2$; 1 mM DTT.

other proteins, the chemical environments of corresponding residues on each strand of a parallel homodimeric coiled-coil are expected to be equivalent, though asymmetry has been observed for a pair of asparagine residues at equivalent positions in the NMR structure of a coiled-coil homodimer (*48*). However, in the presence of tropomyosin-binding proteins (i.e. actin) this symmetry would most likely be broken and each α-helical strand may be expected to suffer a unique chemical environment.

Actomyosin(S1) Mg^{2+}-ATPase Measurements. ASTm inhibits the actomyosin(S1) Mg^{2+}-ATPase with the same efficiency as that of chicken skeletal muscle tropomyosin (*33*). We tested the ATPase inhibitory activity of all the recombinant tropomyosins and found that all except for 5OH90W were very similar to that observed for ASTm. Figure 2 shows the inhibition curves for ASTm and all the 5-hydroxytryptophan containing tropomyosins. ASTm inhibits the ATPase to approximately 50% of the activity observed in its absence at an ASTm:actin ratio of 1:7 (Figure 2). 5OH101W, 5OH111W, 5OH122W, and 5OH185W all displayed similar inhibitory properties (Figure 2). On the other hand, mutant 5OH90W did not inhibit the ATPase significantly even when present at a 2-fold stoichiometric excess with actin (Figure 2). Under the conditions of the ATPase assay, 5OH90W is bound stoichiometrically to actin (below). These inhibition and actin-binding results were observed with two different protein preparations of 5OH90W. Interestingly, the same mutant expressed in the presence of normal tryptophan (90W) inhibited the ATPase in a manner similar to ASTm (Figure 2), indicating that the anomalous behavior of 5OH90W is in part due to the presence of the 5-hydroxy group and not solely to the presence of the indole moiety at position 90. The other recombinant tropomyosins containing tryptophan (101W, 111W, 122W, 185W) or 7-azatryptophan (7N122W) showed inhibitory properties similar to that observed for ASTm (data not shown).

Actin-Binding. We tested the ability of the recombinant tropomyosins to bind to actin in cosedimentation assays. At micromolar protein concentrations, the recombinant tropomyosins containing either normal tryptophan, 5-hydroxytryptophan, or 7-azatryptophan residues were able to bind to actin (data not shown and see Figure 4B). This indicates that the mutations did not result in any drastic change in tropomyosin structure which affects its interaction with actin. Local changes in tropomyosin secondary (α-helical) or tertiary (coiled-coil) structure are unlikely due to the placement of all the trp residues on the exterior solvent-exposed surface of the coiled-coil structure (Figure 1). Some changes in particular actin−tropomyosin contacts however cannot be ruled out and in the case of 5OH90W seem likely. This mutant binds actin but not in a manner which inhibits the acto-S1 Mg^{2+}-ATPase. The introduction of a 5-hydroxytryptophan residue at position 90 seems to dislocate the equilibrium between thin filament conformational states toward one which promotes the actomyosin interaction (see below).

Fluorescence Excitation and Emission Spectra. Figure 3A compares the normalized excitation spectra of tropomyosins containing no tryptophan (ASTm), normal tryptophan at position 122 (122W), 5-hydroxytryptophan (5OH122W), or 7-azatryptophan (7N122W). This figure demonstrates the utility of using modified tryptophan to study protein interactions in complex systems. ASTm, which does not possess any trp residues, exhibits an excitation spectrum with λ_{max} = 277 nm due to its six tyrosine residues per polypeptide chain. The 122W mutant has a λ_{max} = 278 nm and a shoulder that extends its spectrum into higher wavelengths as expected

for a tryptophan-containing protein. 5OH122W also has a $\lambda_{max} = 278$ nm but a much more prominent shoulder which extends beyond 300 nm. The excitation spectrum of 7N122W is significantly blue-shifted with a λ_{max} of 287 nm. Only 5OH122W and 7N122W have significant fluorescence when excited above 305 nm as expected (*35*−*37*).

Figure 3B compares the emission spectra of 122W and 7N122W with the emission spectra of all the 5-hydroxytryptophan-containing mutants. While the fluorescence intensity of each 5-hydroxytryptophan mutant varies over a 2-fold range, the λ_{max} of their spectra show very little variation (all 337 nm ± 1 nm). This is expected as all the mutants have their probes positioned on the solvent-exposed surface of the coiled-coil (Figure 1B). There was also very little variation in the emission λ_{max} of the tryptophan-containing proteins (349 nm, Figure 3B and data not shown) which was only slightly blue-shifted from the λ_{max} of tryptophan in water, again consistent with the expected solvent-exposed environments of these probes. The λ_{max} of 7N122W was well red-shifted with respect that of tryptophan and 5-hydroxytryptophan, but the relative fluorescence intensity at λ_{max} was much less than that observed for 122W and 5OH122W. The relatively low fluorescence intensity exhibited by 7N122W is consistent with previous observations for 7-azaindole and 7-azatryptophan in water (*37*). In the hydrophobic interior of proteins or in nonaqueous solvents, this probe's quantum yield has been observed to increase by a factor of 10 or more (*49*, *50*). The photophysics of tryptophan, 5-hydroxytryptophan, and 7-azatryptophan incorporated at position 122 of tropomyosin have been investigated and will be published elsewhere.

Actin-Induced Fluorescence Change. In the presence of at least 45−60 mM monovalent salt (Figure 4A) or in the presence of millimolar amounts of Mg^{2+} (Figure 4C), the addition of 7 molar equiv of actin to mutant 5OH122W resulted in an increase in fluorescence intensity of approximately 40%. Below 45 mM NaCl, no fluorescence intensity change was observed (Figure 4A). As expected, the maximal fluorescence change is reached at an actin:tropomyosin ratio of 7:1 (data not shown). Actin did not induce comparable changes in the fluorescence intensities of the other four tropomyosins under these conditions (less than 10% change, data not shown).

The binding of muscle tropomyosin to actin has previously been shown to be sensitive to ionic strength (*8*). To investigate whether the fluorescence change is due the bimolecular association of 5OH122W with actin or due to a salt-dependent conformational change of a preformed 5OH122W−actin complex, we performed cosedimentation experiments at various ionic strengths. Actin binding as monitored by cosedimentation was not observed in the absence of both monovalent and divalent cations while binding was observed in the presence of either 45 mM NaCl (Figure 4B) or 5 mM $MgCl_2$ (data not shown). When the NaCl concentration was 30 mM or lower, no actin-binding was observed (Figure 4B), consistent with the conclusion that the fluorescence change in 5OH122W observed between 30 and 45 mM NaCl (Figure 4A) is due to a bimolecular association with actin. It is still possible that at concentrations between 30 and 45 mM NaCl a weak acto−5OH122W complex may form followed by an unimolecular isomerization at higher ionic strengths, but at the moment we have no evidence for this distinction. It should be mentioned, however, that tropomyosin molecules tethered to actin at only one end have been observed under specific conditions in the

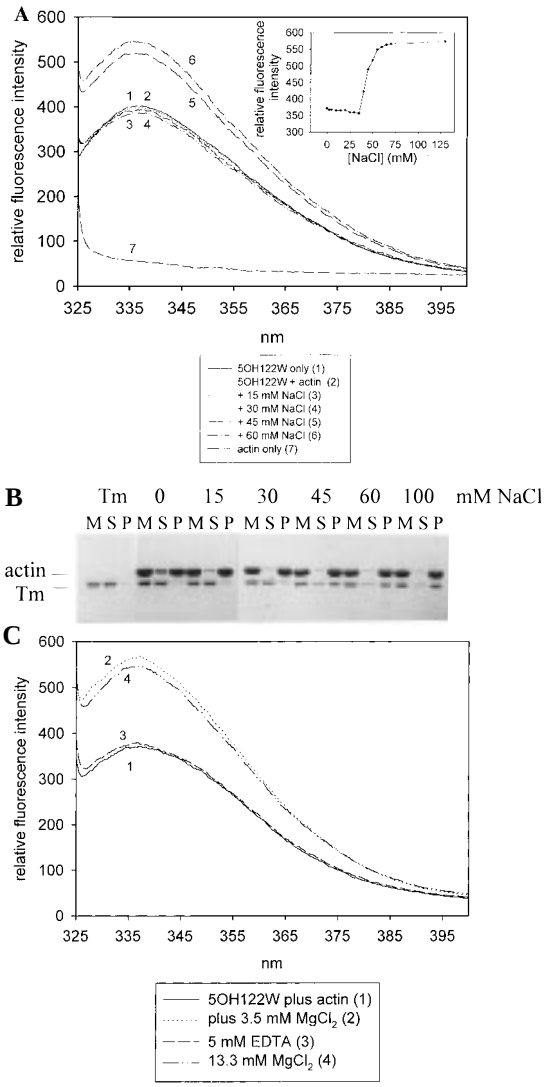

FIGURE 4: Actin-induced fluorescence change in 5OH122W. (A) Fluorescence emission spectra of 5OH122W in the presence of actin at varying concentrations of NaCl in the absence of divalent metal ions (315 nm excitation). In the absence of actin, the emission spectra is independent of [NaCl] (data not shown). In all cases the light scattering of the buffer (not shown) and the light scattering due to actin (indicated in figure) were subtracted. Inset: Fluorescence emission intensity at 335 nm as a function of [NaCl]. Note that the fluorescence intensity increases when [NaCl] > 35 mM. This increase has been correlated to tropomyosin binding to actin (see part B). Conditions: 1 μM tropomyosin; 7 μM actin (where indicated); 25 mM MOPS (pH 7.0); 1 mM EDTA; 1 mM DTT; 1 mM ATP; NaCl at the indicated concentrations. Lines are defined at the bottom. (B) Binding of 5OH122W to F-actin at different NaCl concentrations. After each NaCl addition in part A, aliquots were analyzed for actin binding in cosedimentation assays as described in Material and Methods and analyzed by SDS−PAGE: M = mixture before centrifugation; S = supernatant; and P = pellet after centrifugation; Tm = 5OH122W only. Note that the cosedimentation of 5OH122W with actin occurs at 45, 60, and 100 mM NaCl but not at 0, 15, or 30 mM NaCl. Therefore the fluorescence change observed in part A corresponds to binding to F-actin. (C) $MgCl_2$ dependence of acto-5OH122W emission spectrum in the absence of monovalent metal ions (315 nm excitation). Conditions: 0.5 μM tropomyosin; 3.5 μM actin; 25 mM MOPS (pH 7.0); 1 mM DTT. $MgCl_2$ and EDTA were added sequentially to the final concentrations as shown at the bottom.

solid state by electron microscopy (*51*). A reasonable assumption is that the formation of such a complex would not be expected to affect the steady-state fluorescence of a probe located in the middle of the tropomyosin molecule unless there exists a mechanism by which a conformational change at the end of the tropomyosin molecule is transmitted to its center.

The positions of tryptophan analogues in the four tropomyosin mutants (5OH90W, 5OH101W, 5OH111W, and 5OH122W; Figure 1) were designed to test which region of the third pseudorepeat are involved in binding to actin and participate in tropomyosin function. Though the existence of actin-induced changes in fluorescence lifetimes or anisotropy was not investigated in these mutants, only 5OH122W presented a fluorescence signal whose intensity was sensitive to actin binding. On the other hand, the ATPase data also implicate residue 90 in interactions important for tropomyosin function. Since the distance between residue 90 and 122 is approximately 4.8 nm, the approximate diameter of an actin monomer, it is unlikely that all the positions of the probes in mutants 90W, 101W, and 111W are located between actin subunits. It may be that even though tropomyosin follows the actin filament along its full length, only a small number of its residues are involved in stereospecific interactions with actin, as has been suggested (*12*).

The mutant with 7-azatryptophan at position 122 (7N122W) has a relatively low fluorescence intensity as expected from its solvent-exposed position (Figure 1B). The low quantum yield of 7-azatryptophan in aqueous solvents may increase by over 1 order of magnitude in alcoholic or aprotic solvents or when buried in the hydrophobic interior of proteins (*37*, *49*, *50*, *52*). The fluorescence intensity of 7N122W increased slightly (approximately 15%) upon addition of actin (data not shown). The 7N122W fluorescence intensity was of the same order of magnitude as the actin light scattering, which decreases the utility of this mutant protein in studies with reconstituted thin filaments.

Ca^{2+} Sensitivity of the Fluorescence in the Presence of Troponin. The addition of troponin ($-Ca^{2+}$) to mixtures of actin and the recombinant tropomyosins caused only small (less than 10% changes in the fluorescence signals of any of the mutants (data not shown), and only in the case of 5OH122W did the fluorescence change significantly in response to Ca^{2+}. In the case of 5OH122W, the addition of troponin in the absence of Ca^{2+} caused a 5% decrease in fluorescence intensity (data not shown). The addition of Ca^{2+} to acto−5OH122W−Tn resulted in a 10% increase in fluorescence intensity with no significant change in λ_{max} (Figure 5A). In the absence of actin, this Ca^{2+}-dependent fluorescence intensity change was not observed (data not shown). Figure 5A shows the emission spectra of a typical Ca^{2+} titration between pCa 7.6 and pCa 4.6. Figure 5B shows the data from four Ca^{2+} titrations of thin filaments reconstituted from actin, troponin, and 5OH122W tropomyosin. Inspection of the data in Figure 5B indicates a significant degree of cooperativity in the Ca^{2+}-induced change in tropomyosin structure detected by the 5-hydroxytryptophan probe. Attempts to fit the data to a noncooperative model in which it is assumed that the Ca^{2+}-binding events responsible for the fluorescence are equivalent and that the fluorescence change caused by Ca^{2+}-binding to the first regulatory site is equal to that caused by Ca^{2+} binding to the second regulatory site were not at all satisfactory (dotted line in Figure 5B).

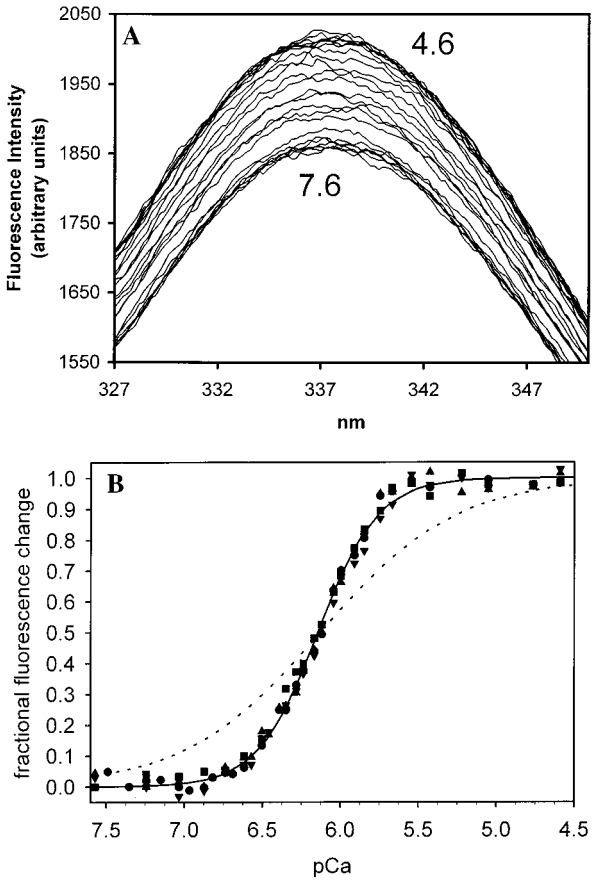

FIGURE 5: Ca^{2+}-induced fluorescence change in reconstituted thin filaments containing 5OH122W. (A) Emission spectra from a typical Ca^{2+} titration of reconstituted thin filaments containing 5OH122W tropomyosin. The intensity increases approximately 10% upon increasing the [Ca^{2+}] from pCa 7.6 to pCa 4.6. The total fluorescence intensity (area) between 327 and 345 nm was used to calculate the fractional fluorescence change at each pCa plotted in part B. Conditions: Thin filaments were reconstituted as described in Materials and Methods and, after sedimentation, resuspended in 25 mM MOPS (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, and 3.3 mM DTT. $CaCl_2$ was added to achieve the desired pCa (ref *64*). Excitation was at 312 nm. (B) At each pCa, the fractional maximal fluorescence change is shown for four independent titrations (symbols). The solid line shows curve fittings based on cooperative models of Ca^{2+} binding. First, the data were fit to the Hill equation $\{\Delta F/\Delta F_{max} = (K'[Ca^{2+}])^n/(1 + (K'[Ca^{2+}])^n)\}$, where K' is an apparent binding constant and n is the Hill coefficient (*39*). In this case the curve fitting (solid line) gives values of $K' = 1.39\ (\pm 0.05) \times 10^6\ M^{-1}$ and $n = 2.22\ (\pm 0.09)$. Another curve fitting was performed on the basis of the cooperative model of Tobacman and Sawyer (*27*) (indistinguishable from the solid line). In this model, $\Delta F/\Delta F_{max} = 2K_0[Ca^{2+}]/\{Y((1 - K_0[Ca^{2+}])^2 + 4K_0[Ca^{2+}]/Y)^{1/2}(1 - K_0[Ca^{2+}] + ((1 - K_0[Ca^{2+}])^2 + 4K_0[Ca^{2+}]/Y)^{1/2}\}$, where K_0 is the Ca^{2+} affinity of an isolated tropomyosin/troponin unit and Y is a cooperativity parameter describing the fold-increase in affinity of sites adjacent to already occupied sites. Fitting the data according to this model gives $K_0 = 1.37\ (\pm 0.05) \times 10^6\ M^{-1}$ and $Y = 5.8\ (\pm 0.5)$. As pointed out by Tobacman and Sawyer (*27*), the cooperativity parameters in the two models are related ($n = Y^{1/2}$). The dotted line is a curve fitting based on a model assuming that the probe is sensitive to noncooperative Ca^{2+}-binding to independent and equivalent Ca^{2+}-specific sites of troponin C. Here we assume that the fluorescence change caused by Ca^{2+}-binding to the first regulatory site is equal to that caused by Ca^{2+} binding to the second regulatory site, and an apparent association constant of $1.4\ (\pm 0.6) \times 10^6\ M^{-1}$ is obtained.

Two curve fittings based on cooperative models of Ca^{2+} binding were carried out (solid line in Figure 5B). First, fitting the data to the Hill equation gave an apparent association constant (K') of 1.4 ($\pm$0.05) $\times$ 10^6 M^{-1} and a Hill coefficient (n) of 2.22 ($\pm$0.09). This Hill coefficient is comparable to those reported for Ca^{2+}-binding to the thin filament monitored via fluorescent probes attached to troponin C (*27*, *39*). In those studies, Hill coefficients between 1.5 and 1.9 were observed. Another curve fitting of the data (indistinguishable from the solid line in Figure 5B) was performed on the basis of the cooperative model of Tobacman and Sawyer (*27*). Here, the Ca^{2+} affinity of an isolated tropomyosin/troponin unit (K_o) and the cooperativity parameter (Y) describing the fold-increase in affinity of sites adjacent to already occupied sites were found to be 1.37 ($\pm$0.05) $\times$ 10^6 M^{-1} and 5.8 ($\pm$0.5), respectively (Figure 5B).

The apparent equilibrium constants and cooperativity parameters obtained from these models are similar to those observed for Ca^{2+}-binding to the regulatory sites in reconstituted thin filaments (*27*, *39*, *53*, *54*). In these studies Ca^{2+}-binding to the thin filament was measured directly or by fluorescence changes in labeled troponin C, while in the present study we detected fluorescence changes in tropomyosin at a site not directly involved in Ca^{2+}-binding. As has been frequently pointed out by others, Ca^{2+}-based cooperativity in the thin filament may occur at various levels: between Ca^{2+}-binding sites in a single TnC subunit, between Ca^{2+}-binding sites in neighboring troponin complexes along the thin filament, between Ca^{2+} binding and conformational changes in thin filament structure, between Ca^{2+} binding and myosin binding, and between Ca^{2+} binding and the Mg^{2+}-ATPase rate (reviewed in ref *4*). All of these cooperative phenomena are most likely linked to varying degrees due to the molecular interactions between thin filament components and are enhanced even further by myosin (*39*). The fact that the Hill coefficient we measured is approximately 2 (the number of regulatory Ca^{2+}-binding sites per troponin/tropomyosin unit) could be due to cooperativity between the two regulatory Ca^{2+}-binding sites within a single TnC subunit or due to cooperative changes between neighboring thin filament regulatory units (see also refs *4* and *39*). In any case, the cooperative change detected by the probe suggests that Ca^{2+}-binding to troponin is directly coupled to a conformational change in tropomyosin (probably affecting its interaction with actin), independent of myosin binding with implications for models of thin filament regulation (see below).

Due to the absence of high-resolution data of the troponin−tropomyosin complex, we must consider the possibility that the probe sensitivity to Ca^{2+} is due to Ca^{2+}-induced changes in a direct interaction between the probe at position 122 and troponin. The distance between cys^{133} of 1,5-IAEDANS -modified TnI and cys^{374} of DAB-Mal-modified actin has been shown to decrease by approximately 1.5 nm upon Ca^{2+} dissociation from troponin (*55*), and TnI has been cross-linked to cys^{190} of tropomyosin (*56*). In the latter study, the site of attachment on TnI was not identified and Ca^{2+}-dependent effects on cross-linking yield were not investigated. Some studies have implicated an extended region of the carboxyl-terminal half of troponin TnI (residues 96−156) in binding to TnC in the presence of Ca^{2+} and to actin−tropomyosin in the absence of Ca^{2+} and to be responsible for Ca^{2+}-sensitive inhibition of the actomyosin ATPase (*1*−*4*, *45*, *57*−*59* and references therein). If this region adopts an α-helical conformation, as expected from its primary structure (ref *60* and Farah and Reinach, unpublished observations), then in the absence of Ca^{2+} it would be just long enough to extend the distance from the troponin binding site at residues 150−190 (*38* and *56*) to the probe at position 122. Such an extended helix would probably not be unambiguously identified in electron micrographs or low-resolution X-ray diffraction studies of reconstituted thin filaments (reviewed in ref *23*). To date, the only crystal structure of the troponin−tropomyosin complex is that of White et al. (*38*) which delimited the troponin globular domain head binding site on tropomyosin to residues 150−180 in the absence of Ca^{2+}, conditions which weaken interactions between TnI and TnC and which would favor interactions between troponin and tropomyosin. As this structure had a resolution of only 17 Å (the length of an 11 amino acid stretch of α-helix), this site could extend anywhere from residue 139 to residue 191 on tropomyosin. This still does not include the probe position at residue 122. Therefore, the evidence present in the literature to date leads us to rule out a mechanism of direct contact between troponin and the probe to explain the Ca^{2+} sensitivity of the probe fluorescence.

Assuming that the globular domain of troponin binds to a site between residues 150 and 190 of tropomyosin (*1*, *38*, *56*), the distance between the troponin-binding site and the fluorescent probe at position 122 can be estimated to be between 4.2 and 10.2 nm. This is a clear demonstration that Ca^{2+}-binding to troponin results in a conformational change that propagates to a region of tropomyosin outside the troponin binding site. As the Ca^{2+}-dependent change was not observed in the absence of actin, it is likely that the 5-hydroxytryptophan probe is detecting Ca^{2+}-induced modifications in the actin−tropomyosin interaction at or near residue 122. All other probes detecting Ca^{2+}-induced conformational changes have been covalently attached to cysteine-190, which is implicated in direct binding to troponin (see for example ref *17* and references cited within). While X-ray diffraction and electron micrograph reconstitution studies have provided evidence of Ca^{2+}-induced changes in tropomyosin's interactions within the thin filament (*18*−*23*), the resolution in those studies was not sufficient to distinguish between changes involving the whole tropomyosin molecule or only regions in direct contact with troponin. Our results are consistent with the previously suggested idea that troponin regulates the nature of tropomyosin's contacts with actin monomers not in direct contact with troponin, thereby simultaneously modulating the accessibility/affinity of seven binding sites for myosin (*14*−*17*).

Miki et al. (*61*) did not detect any Ca^{2+}-dependent change in fluorescence energy transfer efficiency between probes located on skeletal tropomyosin (positions 87 or 190) and actin (positions 41, 61, or 374 or the nucleotide binding site) in thin filaments reconstituted with troponin. The large number of pairwise donor−acceptor pairs tested suggested that tropomyosin did not change its orientation with respect to actin. These authors proposed that Ca^{2+}-induced changes in troponin's interactions with actin and Tm may cause distortions in the actin filament without necessarily changing tropomyosin's position with respect to actin. The results of

Miki et al. are consistent with some studies (*62*) but contradict evidence of tropomyosin movements of greater than 10 Å obtained by low-resolution X-ray diffraction and three-dimensional reconstruction by electron microscopy (*18*−*22*, but see ref *23*). Our results do not resolve this discrepancy but do indicate that Ca^{2+}-binding changes the chemical environment around residue 122 of tropomyosin (outside the troponin binding site) and that this change is cooperative. Whether this change is associated with physical displacements large enough to be detected in fluorescence energy transfer experiments is not known. In any case, it seems that the probe at position 122 is sensitive to specific interactions with actin which change upon Ca^{2+}-binding since other probes close to residue 122 (positions 90, 101, 111) and within the troponin-binding site (position 185) did not show significant actin- or Ca^{2+}-dependent fluorescence intensity changes.

One can imagine a variety of mechanisms which could result in this change, independent of whether tropomyosin translates significant distances across the F-actin surface in response to Ca^{2+}. For example, Ca^{2+}−troponin-induced conformational changes in actin, in tropomyosin, or in the interaction interface between the two proteins could affect the microenvironment of the probe. Regarding possible changes in actin−Tm interactions, the region of tropomyosin containing residue 122 could be switching between bound and unbound states, could be alternating between two distinct and separate binding sites on actin, or could be utilizing different residues to bind to the same region of actin in different manners.

Structural, kinetic, and model-building studies over the past decade have emboldened some to add atomic details to the original steric-blocking model of tropomyosin−troponin-based regulation of the actomyosin interaction. Present-day regulatory models normally refer to three possible thin-filament conformational states (*16*, *17*, *22*, *23* and references cited): (i) One is a "blocked" state in which tropomyosin makes contacts with subdomains 1 and 3 of actin, thereby blocking stereospecifc interactions with myosin. This conformation is stabilized by troponin in the absence of Ca^{2+}. (ii) Another is a "closed" state which describes the position of tropomyosin on actin in the presence of Ca^{2+}−troponin but in the absence of strong actomyosin cross-bridges. Here tropomyosin has moved 10−20 Å across subdomain 3 toward the groove of the actin helix. This conformation exposes a subset of actin sites for stereospecific "weak" interactions with myosin but blocks isomerization to "strong" actomyosin contacts. (iii) In the "open" state, tropomyosin has moved further away from the myosin binding site, thereby allowing strong actomyosin interactions to occur. The effect of Ca^{2+} in this scheme is to shift the equilibrium from the "blocked" state to the "closed" and "open" states. Binding of myosin heads shifts the equilibrium further toward the open state, thereby explaining the cooperativity of acto-S1 binding in the presence of tropomyosin (*5*−*7*). Squire and Morris (*23*) have recently critically reviewed the structural evidence for tropomyosin movements across the actin filament surface in response to Ca^{2+} and myosin binding. They point out that the structural data to date cannot distinguish between three-state models and "dynamic two-state" models. Nor can the structural data distinguish between models containing pure states or shifting equilibria between mixtures of states. This applies to our data as well. We do not know whether the three different fluorescence intensities we observed without troponin and with troponin ± Ca^{2+} represent (a) three "pure" or "stable" structural states of the thin filament, (b) three different mixtures of two structural states, or (c) three different mixtures of three structural states. Furthermore, it appears that the X-ray data (*20*−*23*) and microscopy data (*18*, *19*) can be equally explained by various combinations of modeled conformational changes in tropomyosin, troponin, and actin induced by Ca^{2+} and/or myosin binding. Squire and Morris (*23*) suggest that, in the absence of myosin, Ca^{2+}-induced structural changes observed in thin filaments or nonoverlap regions of the sarcomere could be explained equally well by movements in tropomyosin or (and) troponin. In this sense, the Ca^{2+}-induced fluorescence change which we observe in the absence of myosin support models (*18*, *21*) which include some change in tropomyosin environment caused by Ca^{2+} alone (i.e. independent of myosin), even in regions not in direct contact with troponin.

In this report we have presented some of the fluorescence properties of five recombinant tropomyosins containing 5-hydroxytryptophans at unique positions in the solvent-exposed surface of its coiled-coil structure. Similar to the free 5-hydroxytryptophan amino acid in water (*36*), these proteins present an absorption shoulder between 300 and 320 nm which is not observed in normal tryptophan and which may be used to selectively excite their fluorescence in the presence of other muscle proteins. Only mutant 5OH122W presented a fluorescence signal whose intensity was significantly sensitive to actin binding. In this mutant the fluorescent residue is located in the third of tropomyosin's seven pseudorepeats. The fluorescence of this probe was also sensitive to Ca^{2+}-binding to troponin. These results provide direct evidence for transmission of the Ca^{2+}-binding signal to regions of tropomyosin not in direct contact with troponin.

ACKNOWLEDGMENT

We thank Deodoro Oliveira for assistance in sequencing of some of the mutant tropomyosins.

REFERENCES

1. Ohtsuki, I., Maruyama, K., and Ebashi, S. (1986) *Adv. Protein Chem. 38*, 1.
2. Zot, A. S., and Potter, J. D. (1987) *Annu. Rev. Biophys. Chem. 16*, 535.
3. Farah, C. S., and Reinach, F. C. (1995) *FASEB J. 9*, No. 9, 755.
4. Tobacman, L. S. (1996) *Annu. Rev. Physiol. 58*, 447.
5. Greene, L. E., and Eisenberg, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2616.
6. Williams, D. L., Greene, L. E., and Eisenberg, E. (1988) *Biochemistry 27*, 6987.
7. Lehrer, S. S., and Morris, E. P. (1982) *J. Biol. Chem. 257*, 8073.
8. Eaton, B. L., Kominz, D. R., and Eisenberg, E. (1975) *Biochemistry 14*, 2718.
9. Greenfield, N. J., Montelione, G. T., Farid, R. S., and Hitchcock-DeGregori, S. E. (1998) *Biochemistry 37*, 7834.
10. McLachlan, A. D., and Stewart, M. (1975) *J. Mol. Biol. 98*, 293.
11. McLachlan, A. D., and Stewart, M. (1976) *J. Mol. Biol. 103*, 271.
12. Phillips Jr, G. N., Fillers, J. P., and Cohen, C. (1986) *J. Mol. Biol. 192*, 111.

13. Haselgrove, J. C. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 341.
14. Huxley, H. E. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 361.
15. Parry, D. A. D., and Squire, J. M. (1973) *J. Mol. Biol. 75*, 33.
16. McKillop, D. F. A., and Geeves, M. A. (1993) *Biophys. J. 65*, 693.
17. Geeves, M. A., and Lehrer, S. S. (1994) *Biophys. J. 67*, 273.
18. Lehman, W., Craig, R., and Vibert, P. (1994) *Nature 368*, 65.
19. Lehman, W., Vibert, P., Uman, P., and Craig, R. (1995) *J. Mol. Biol. 251*, 191.
20. Lorenz, M., Poole, K. J. V., Popp, D., Rosenbaum, G., and Holmes, K. C. (1995) *J. Mol. Biol. 246*, 108.
21. Poole, K. J. V., Holmes, K. C., Evans, G., Rosenbaum, G., Rayment, I., and Lorenz, M. (1995) *Biophys. J. 68*, 348s.
22. Holmes, K. C. (1995) *Biophys. J. 68*, 2s.
23. Squire, J. M., and Morris, E. P. (1998) *FASEB J. 12*, 761.
24. Pan, B.-S., Gordon, A. M., and Luo, Z. (1989) *J. Biol. Chem. 264*, 8495.
25. Brandt, P. W., Diamond, M. S., and Schachat, F. H. (1984) *J. Mol. Biol. 180*, 379.
26. Brandt, P. W., Roemer, D., and Schachat, F. H. (1990) *J. Mol. Biol. 212*, 473.
27. Tobacman, L. S., and Sawyer, D. (1990) *J. Biol. Chem. 265*, 931.
28. Hill, L. E., Mehegan, J. P., Butters, C. A., and Tobacman, L. S. (1992) *J. Biol. Chem. 267*, 16106.
29. Butters, C. A., Willadsen, K. A., and Tobacman, L. S. (1993) *J. Biol. Chem. 268*, 15565.
30. Szczesna, D., and Fajer, P. G. (1995) *Biochemistry 34*, 3614.
31. Hill, T. L., Eisenberg, E., and Greene, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186.
32. Zou, G., and Phillips, G. N., Jr. (1994) *Biophys. J. 67*, 11.
33. Monteiro, P. B., Lataro, R. C., Ferro, J. A., and De Castro Reinach, F. (1994) *J. Biol. Chem. 269*, 10461.
34. Drapeau, G. R., Brammar, W. J., and Yanofsky, C. (1968) *J. Mol. Biol. 35*, 357.
35. Negrerie, M., Bellefeuille, S. M., Whitham, S., Petrich, J. W., and Thornbourg, R. W. (1990) *J. Am. Chem. Soc. 112*, 7421.
36. Hogue, C. W. V., Rasquinha, I., Szabo, A. G., and MacManus, J. P. (1992) *FEBS Lett. 310*, 269.
37. Rich, R. L., Gai, F., Lane, J. W., Petrich, J. W., and Schwabacher, A. W. (1995) *J. Am. Chem. Soc. 117*, 733.
38. White, S. P., Cohen, C., and Phillips, G. N., Jr. (1987) *Nature 325*, 826.
39. Grabarek, Z., Grabarek, J., Leavis, P. C., and Gergely, J. (1983) *J. Biol. Chem. 258*, 14098.
40. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367.
41. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60.
42. Pardee, J. D., and Spudich, J. A. (1982) *Methods Enzymol. 85*, 164.
43. Margossian, S. S., and Lowey, S. (1982) *Methods Enzymol. 85*, 55 (Abstract).
44. Quaggio, R. B., Ferro, J. A., Monteiro, P. B., and Reinach, F. C. (1993) *Protein Sci. 2*, 1053.
45. Farah, C. S., Miyamoto, C. A., Ramos, C. H. I. R., Da Silva, A. C. R., Quaggio, R. B., Fujimori, K., Smillie, L. B., and Reinach, F. C. (1994) *J. Biol. Chem. 269*, 5230.
46. Heinomen, J. K., and Lahti, R. J. (1981) *Anal. Biochem. 113*, 313.
47. Smillie, L. B. (1979) *Trends Biochem. Sci. 4*, 151.
48. Junius, F. K., O'Donoghue, S. I., Nilges, M., Weiss, A. S., and King, G. F. (1996) *J. Biol. Chem. 271*, 13663.
49. Wong, C.-Y., and Eftink, M. R. (1997) *Protein Sci. 6*, 689.
50. Smirnov, A. V., English, D. S., Rich, R. L., Lane, J., Teyton, L., Schwabacher, A. W., Luo, S., Thornburg, R. W., and Petrich, J. W. (1997) *J. Phys. Chem. 101*, 2758.
51. Mabuchi, K. (1996) *J. Struct. Biol. 116*, 278.
52. Hogue, C. W. V., and Szabo, A. G. (1993) *Biophys. Chem. 48*, 159.
53. Bremel, R. D., and Weber, A. (1972) *Nat. New Biol. 238*, 97.
54. Rosenfeld, S. S., and Taylor, E. W. (1985) *J. Biol. Chem. 260*, 252.
55. Tao, T., Gong, B.-J., and Laevis, P. C. (1990) *Science 247*, 1339.
56. Tao, T., Scheiner, C. J., and Lamkin, M. (1986) *Biochemistry 25*, 7633.
57. Malnic, B., Farah, C. S. and Reinach, F. C. (1998) *J. Biol. Chem. 273*, 10594.
58. Pearlstone, J. R., and Smillie, L. B. (1995) *Biochemistry 34*, 6932.
59. Hodges, R. S., Van Eyk, J. E., and Tripet, B. (1997) *J. Mol. Biol. 271*, 728.
60. Olah, G. A., and Trewhella, J. (1994) *Biochemistry 33*, 12800.
61. Miki, M., Miura, T., Sano, K.-I., Kimura, H., Kondo, H., Ishida, H., and Maeda, Y. (1998) *J. Biochem. 123*, 1104.
62. Tao, T., Lamkin, M., and Lehrer, S. S. (1983) *Biochemistry 22*, 3059.
63. Whitby, F. G., Kent, H., Stewart, F., Stewart, M, Xie, X., Hatch, V., Cohen, C., and Phillips, G. N., Jr. (1992) *J. Mol. Biol. 227*, 441.
64. Bers, D., Patton, C., and Nuccitelli, R. (1994) *Methods in Cell Biology*, Vol. 40, Chapter 1, Academic Press, New York.

BI982813U