The Tropomyosin Domain Is Flexible and Disordered in Reconstituted Thin Filaments†

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ABSTRACT: We have used EPR spectroscopy to study the rotational motion and orientation of tropomyosin labeled with maleimide spin-label, in skeletal muscle fibers. Fibers depleted of intrinsic myosin, troponin, and tropomyosin were reconstituted with labeled tropomyosin. The 3−7 ns mobility of the labeled domains was only slightly (2-fold) inhibited by reconstitution into fibers. No motional changes were observed on addition of troponin, irrespective of the presence of Ca^{2+}; however, the binding of extrinsic myosin heads increased the rate of domain motion to that observed in solution. Orientational studies demonstrate a broad angular distribution of the labeled domain of tropomyosin, with respect to the fiber axis. Troponin reduces the orientational disorder, while the binding of Ca^{2+} to troponin partially reverses this ordering effect. Myosin S1 has no effect on the orientational distribution of tropomyosin. Overall, the observed changes are very small, implying a loose association of the probed domain of tropomyosin with the thin filament.

The conformational changes induced by the binding of Ca^{2+} to troponin (Tn)[1] are presumed to be propagated along the actin filament by tropomyosin (Tm), a regulatory muscle protein facilitating the interaction of the myosin heads with actin [for reviews, see El-Saleh *et al.* (1986) and Chalovich (1992)]. The molecular mechanism of this regulatory process is as yet not fully understood. On the basis of X-ray diffraction studies (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973) and electron microscopy (Wakabayashi *et al.*, 1975), a model has been proposed according to which the binding of Ca^{2+} to troponin triggers the movement of tropomyosin to a different position on the actin filament (McLachlan & Stewart, 1975; Parry, 1976; Phillips *et al.*, 1986). This model has been further substantiated by the EM reconstruction of Lehman *et al.* (1994). The movement of tropomyosin was found to precede crossbridge attachment and force development in the muscle, and to be independent of the binding of myosin heads to actin (Kress *et al.*, 1986). It seems that the Tm movement is not a "rigid body" reorientation, since no change was observed in the distance between Cys-374 of actin and Cys-190 of tropomyosin, on the binding of Ca^{2+} (Tao *et al.*, 1983; Miki, 1990).

The main goal of the present study was to correlate the dynamics of tropomyosin with the known mechanism of thin filament activation: Ca^{2+} and myosin head binding to the thin filament. A model involving the dynamics of tropomyosin has been proposed by Earley (1991), in which variations in the amplitude of tropomyosin vibrations drive the change in its radial position on actin. Tropomyosin is likely to exhibit considerable motion in the thin filament, since its crystal structure suggests a loose association with the thin filament, with interaction at only a few sites (Phillips *et al.*, 1986).

We have used EPR spectroscopy, a technique highly sensitive to protein dynamics and orientation, to investigate the rotational motion of tropomyosin, and the spin-labeled domain orientation within muscle fibers. We found that the domain mobility of tropomyosin is modulated in distinctive ways, depending upon whether the thin filament is activated by Ca^{2+} or by myosin binding. The orientational distribution was also found to respond differently to the two modes of activation, Ca^{2+} having a more pronounced effect than the myosin heads. However, the changes were subtle which suggests a loose coupling of tropomyosin orientation and dynamics with those of thin filament.

EXPERIMENTAL PROCEDURES

Preparations. Proteins were prepared from rabbit skeletal muscle according to standard methods: tropomyosin according to Greaser and Gergely (1971), troponin according to Potter (1982), and myosin S1 as described by Weeds and Pope (1977). Tropomyosin ($\alpha\beta$-Tm) was labeled at its cysteine residues (Cys-190 in the α- and β-chains, plus Cys-36 in the β-chain) with maleimide spin-label [MSL, *N*-(1-oxy-2,2,5,5-tetramethyl-4-piperidinyl)maleimide] by reacting a 20−30 μM solution with a 6 molar excess of MSL, for 6.5 h at room temperature, in 4 M guanidine hydrochloride, 20 mM imidazole, and 1 mM EDTA, pH 7.2. The solution was then dialyzed to remove unreacted spin-label and to renature the protein. The ratio of spin-label to protein was determined by the double integration of the EPR signal to

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[1] Abbreviations: MSL, *N*-(1-oxy-2,2,5,5-tetramethyl-4-piperidinyl)-maleimide; EPR, electron paramagnetic resonance; ST-EPR, saturation transfer electron paramagnetic resonance; S1, myosin subfragment 1; Tm, tropomyosin; Tn, troponin; LC, myosin light chains; HC, myosin heavy chains.

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give the number of spins, while protein concentration was measured by the Pierce BCA method (Pierce Chemical Co., Rockford, IL). Skeletal muscle fibers were depleted of intrinsic myosin, troponin, and tropomyosin (forming ghost fibers) by incubation with *Hasselbach–Schneider* solution (0.8 M KCl, 4 mM EGTA, 20 mM sodium phosphate, and 10 mM MgATP, pH 6.4) for 5–6 h at 4 °C. Reconstitution of the ghost fibers with MSL-Tm was performed by incubating the ghost fiber bundles with 20 μM MSL-Tm in a solution containing 130 mM KPr, 20 mM MOPS, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM NaN_3, pH 7.0 (rigor buffer), for 15 h at 4 °C. These same conditions were used for the reconstitution of MSL-Tm-containing fibers with troponin and myosin S1. Unbound proteins were removed by repeated washes with rigor buffer. The level of native tropomyosin removal, and subsequent reconstitution with Tm and Tn, was quantitated by SDS–PAGE electrophoresis (12% cross-linking). The gels were scanned using an 8 bit page scanner (Scanmaker II, Microtek), and the bands were integrated using software developed in our laboratory. The efficiency of the extraction of tropomyosin and troponin (estimated from TnC band) was 95% and 75%, respectively. After reconstitution with labeled tropomyosin and troponin, the protein levels were 72% and 92% of the original concentrations. The molar ratio of extrinsic S1 to actin in fibers was approximately 1:(3–4), as estimated by comparison to the band intensity of an equimolar S1–actin complex. The complexes were prepared by mixing a 4 molar excess of S1 over F-actin (50 μM) and by removal of free S1 by sedimentation in an Airfuge (Beckman) at maximum speed for 30 min. Myofibrils were prepared from reconstituted fibers by homogenization on ice with a Tissumizer, (Tekmar TR-10, for 8 15-s bursts at 75% power).

Labeled Tm was immobilized by ammonium sulfate precipitation (60%), or by cross-linking to DITC glass beads (isothiocyanato glass beads, average pore size 500 Å, G-4893; Sigma, St. Louis, MO). DITC beads (30 mg/mL) were reacted with 30 μM Tm in rigor buffer for 12 h at 4 °C. The beads were washed several times to remove unreacted protein.

EPR. EPR and saturation transfer EPR experiments were performed on a Bruker ECS-106 spectrometer (Bruker Instruments Inc., Billerica, MA) at 23 °C, as described in Fajer (1994). EPR spectra were recorded at 0.11 G microwave field and 1 G modulation amplitude, using a TM_{110} cavity modified to accept samples parallel to the static magnetic field, or a TE_{102} cavity for samples perpendicular to the magnetic field. The 2–3 reconstituted thin fiber bundles (each of ≈0.3 mm in diameter) were placed in a 50 μL glass capillary and held isometrically by surgical thread tied to the ends of the fibers. Rigor buffer was continuously flowed through the capillary at a rate of 0.2 mL/min. EPR spectra were analyzed using a microcomputer program developed in our laboratory (Fajer *et al.*, 1988).

The rotational correlation time (τ_r) for the Brownian motion of tropomyosin was calculated according to the equation of Freed (1976): $\tau_r = a(1 - S)^b$, where $a = 5.4 \times 10^{-10}$ s, $b = -1.36$, and $S = x/x_{max}$, where x and x_{max} are the spectral parameters of the experimental and rigid powder spectra, respectively. The spectral parameters used in the above equation are defined in Figure 2: hyperfine splitting ($2T_{eff}$); width of low-field (Δl) and high-field (Δh) resonances (full widths at half-height).

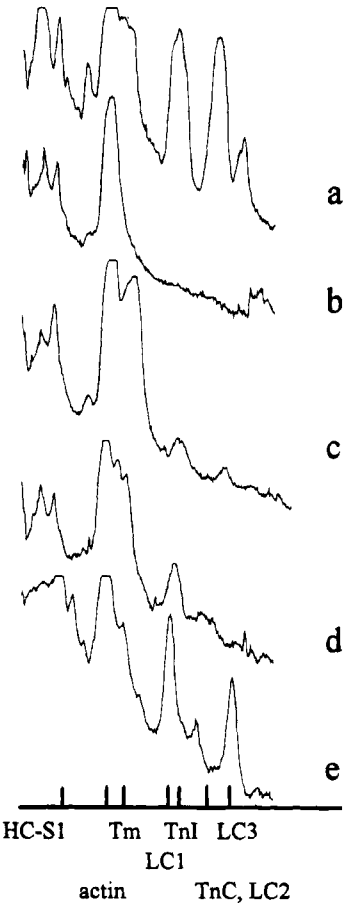

FIGURE 1: Density profiles of SDS gels of (a) glycerinated psoas fibers, (b) "ghost" fibers, (c) "ghost" fibers with reconstituted MSL-tropomyosin, (d) ghosts with MSL-Tm and troponin, and (e) ghosts with MSL-Tm and myosin S1.

RESULTS

Dynamics of MSL-Tropomyosin Solution. The labeling of rabbit skeletal tropomyosin with MSL resulted in the covalent modification of Cys-190 in the α- and β-chains and Cys-36 in the β-chain. The contribution of the MSL-labeled Cys-36 to the total labeled cysteines is about 17% since the α:β ratio in Tm is about 4:1 (Cummins & Perry, 1973). The labeling ratio of MSL to Tm (2.9) was higher than expected from a mixture of αα- and αβ-chains (2.4), suggesting the possible contribution of labeled Lys residues (Chao & Holtzer, 1975). The conventional EPR spectrum of MSL-Tm in solution is shown in Figure 2a. As the observed splitting ($2T_{eff}$ = 57.6 G) is more than 10 G smaller than the rigid limit for the label (68.5 G), there is considerable nanosecond motion. To ascertain that the observed motion is not due to librational motion of the probe with respect to the protein, the tropomyosin was cross-linked to DITC beads, or precipitated with ammonium sulfate (both standard methods for protein immobilization). Under these conditions, any observed motion will be due to the independent motion of the spin probe; the absence of motion can be taken as evidence for probe immobilization on the protein surface as it is very unlikely that the two different modes of immobilization would result in inadverent steric inhibition of probe mobility. The EPR spectra of cross-linked or precipitated tropomyosin display the characteristic "rigid-limit" powder spectrum (Figure 2b,c), with hyperfine splitting $2T_{eff}$ = 68.5 and 68.0 G, respectively (Table 1), comparing well with the rigid-limit values of 68.5 G obtained for im-

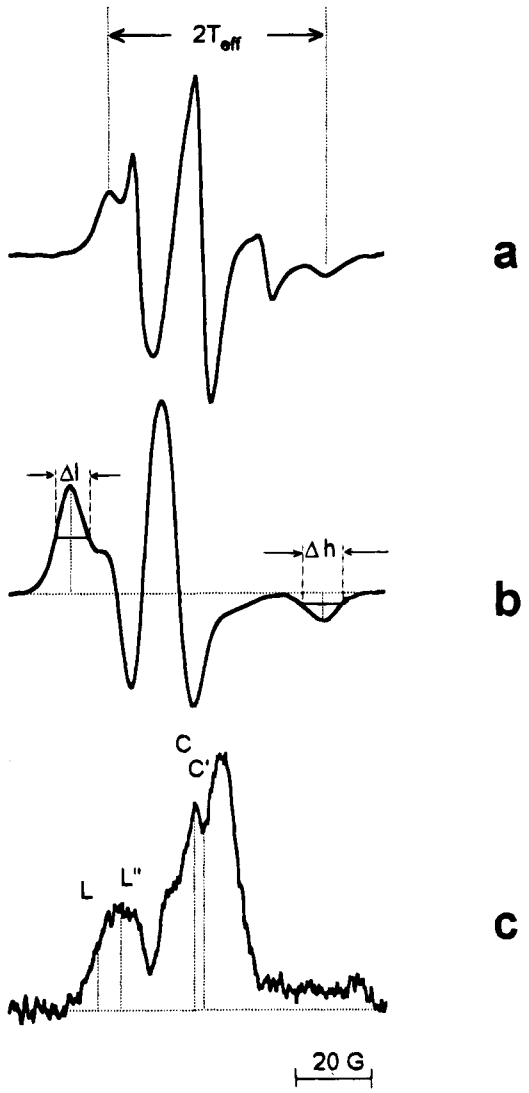

FIGURE 2: EPR spectra of MSL-labeled tropomyosin: (a) in solution; (b) precipitated with ammonium sulfate. (c) ST-EPR spectrum of precipitated MSL-Tm. [The hyperfine splitting ($2T_{eff}$) and the width of the low- and high-field resonances (Δl and Δh) are defined in (a) and (b), respectively.]

mobilized MSL-actin (Thomas *et al.*, 1979), 68.8 G for MSL-troponin C (Li & Fajer, 1994), 68.0 G for MSL-labeled Ca-ATPase (Hidalgo *et al.*, 1978), or 69.0 G for MSL-hemoglobin (Johnson, 1981). The absence of librational motion is further confirmed by the saturation transfer EPR line height ratios, $L''/L = 1.5$ $C'/C = 0.9$, which are at the rigid limit of the ST-EPR method (Thomas *et al.*, 1976). Since the rigid limit for conventional EPR is 0.1 μs, and for ST-EPR is 1−3 ms, we are confident that the spin probe is rigidly attached to the protein, and will faithfully report motions of the tropomyosin, either local (domain) or global.

In contrast to immobilized Tm, the spectrum of the protein in solution has the characteristic line shape of a motionally averaged species (Figure 2a). The powder line shape in which all possible orientations of the label are represented becomes narrower, since the anisotropy of the hyperfine interactions becomes averaged on the time scale of the EPR experiment. The extent of this averaging can be used to determine the rate of motional averaging (Freed, 1976). The rotational correlation time of tropomyosin in solution, as calculated from the spectrum in Figure 2a, varies between 3.2 and 6.6 ns, depending on the spectral feature being analyzed (Table 2). The spread of the calculated values is a reflection of the modeling of the motion as an isotropic Brownian rotation. Clearly, neither global motion of a long coiled-coil nor domain motions can be expected to follow such a simple model; the exhibited motion is highly anisotropic.

Reconstituted Myofibrils. Skeletal muscle fibers were depleted of intrinsic myosin, tropomyosin, and troponin (ghost fibers) using high salt conditions and then reconstituted by the addition of MSL-labeled tropomyosin. In order to avoid an orientational contribution to the EPR spectra from the fibers, the fibers were homogenized into randomly oriented myofibrils.

The spectrum of reconstituted tropomyosin shows noticeable changes over the solution spectrum (Figure 3a). The binding of the protein to the thin filaments resulted in an increase of $2T_{eff}$ from 57.6 to 60.1 G, accompanied by a 0.7−1.4 G increase in the width of the high- and low-field resonances (Table 1). The rotational correlation time of the reconstituted tropomyosin increased to 7.4−9.4 ns from 3.2−6.6 ns in solution (Table 2).

Table 1: Spectral Parameters of MSL-Tm

sample	$2T_{eff}$ (G)	Δl (G)	Δh (G)	n
solution	57.6 ± 0.3	7.2 ± 0.2	9.2 ± 0.3	6
DITC-glass beads immobilization	68.5 ± 0.3	9.9 ± 0.2	11.6 ± 0.2	2
ammonium sulfate precipitation	68.0 ± 0.5	9.9 ± 0.2	10.5 ± 0.2	2
reconstituted in ghost myofibrils (GMF)	60.1 ± 0.2	8.6 ± 0.2	9.9 ± 0.2	3
GMF + Tn/EGTA	60.3 ± 0.3	8.6 ± 0.2	9.9 ± 0.2	3
GMF + Tn/Ca^{2+}	60.6 ± 0.1	8.6 ± 0.2	10.0 ± 0.2	3
GMF + S1	58.2 ± 0.2	7.5 ± 0.1	9.6 ± 0.2	3

Table 2: Calculated Rotational Correlation Times of Tropomyosin[a]

sample	τ_r[b] (ns)	τ_r[c] (ns)	τ_r[d] (ns)	n
in solution	6.6 ± 0.3	3.2 ± 0.3	4.6 ± 0.8	6
reconstituted in ghost myofibrils (GMF)	9.4 ± 0.3	8.5 ± 1.8	7.4 ± 1.2	3
GMF + Tn/EGTA	9.7 ± 0.5	8.5 ± 1.8	7.4 ± 1.2	3
GMF + Tn/Ca^{2+}	10.2 ± 0.2	8.5 ± 1.8	7.9 ± 1.4	3
GMF + S1	7.1 ± 0.2	3.7 ± 0.2	5.9 ± 0.8	3

[a] τ_r was calculated for Brownian rotational motions according to the equation: $\tau_r = a(1 - S)^b$ where $a = 5.4 \times 10^{-10}$ s, $b = -1.36$, and $S = x/x'$. Errors are the standard deviations of n experiments. [b] $x = 2T_{eff}$, $x' = 2T_{max} = 68.5$ G. [c] $x = \Delta l$; $x' = \Delta l_{max} = 9.9$ G. [d] $x = \Delta h$; $x' = \Delta h_{max} = 11.6$ G.

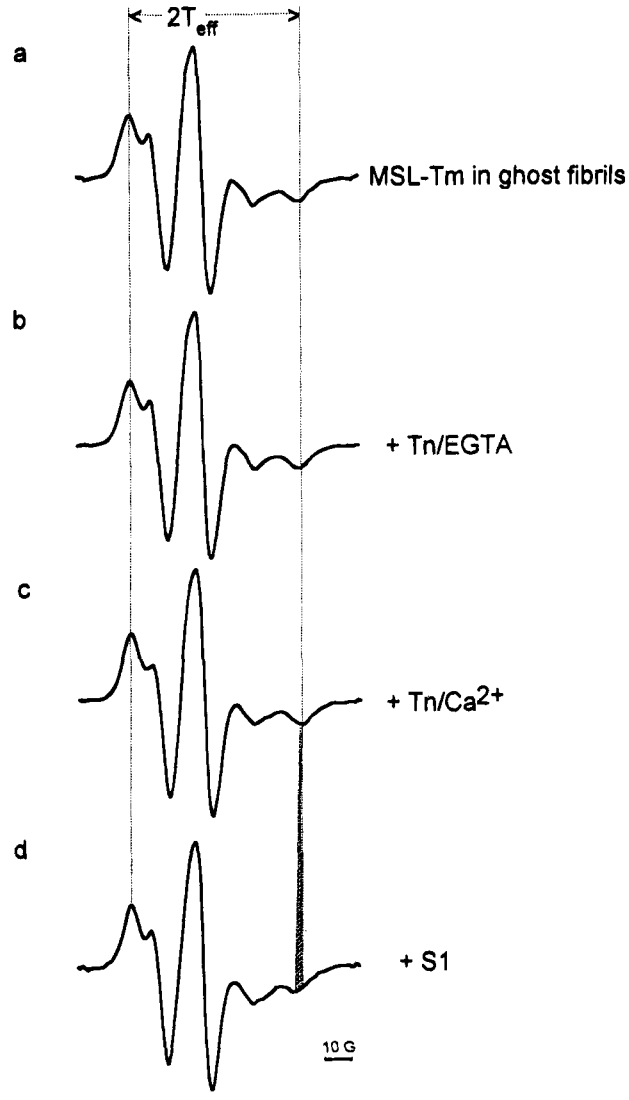

FIGURE 3: Spectra of MSL-tropomyosin in myofibrils (a), with added troponin (b), plus troponin and Ca^{2+} (c), and with myosin heads (d). The shaded area denotes the decrease of the hyperfine splitting on the addition of S1.

The binding of troponin to the reconstituted myofibrils did not change the mobility of the labeled domains of tropomyosin. The spectral line shape was the same as in the absence of troponin (Figure 3b,c). The values of the hyperfine splitting and the low- and high-field resonances remained constant (Table 1). There was also no effect on Ca^{2+} binding to troponin; τ_r changed by less than 5%, well within the experimental error (Table 2). However, there was a pronounced spectral change induced by the binding of myosin S1 to actin, in Tm reconstituted in ghost myofibrils (Figure 3d). The hyperfine splitting decreased by nearly 2 G, with a 1 G decrease in the line width (Table 1). These spectral changes indicate that the motional rate increased by 21−56%, depending upon the spectral parameter analyzed (Table 2).

Orientational Disorder of MSL-Tropomyosin Reconstituted in Skeletal Muscle Fibers. Muscle fibers oriented both perpendicular ($H_\perp$) and parallel ($H_\parallel$) to the static magnetic field (H) were used in the determination of tropomyosin orientational distribution. The spectral line shapes of tropomyosin bound to actin in ghost fibers shown in Figure 4 indicate a broad distribution of spins with respect to the fiber axis, reflecting the large amplitude of the nanosecond motion

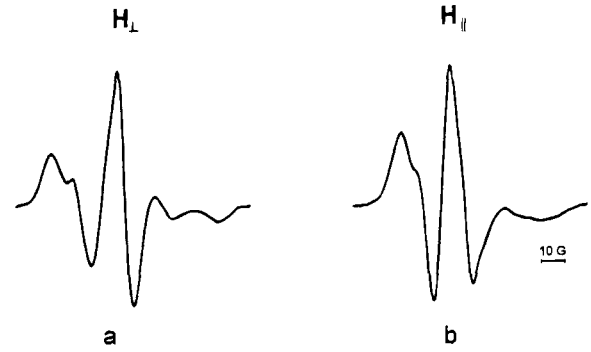

FIGURE 4: EPR spectra of MSL-Tm in oriented fiber bundles: (a) sample perpendicular to the magnetic field; (b) parallel to the field.

of the probed domain of tropomyosin as described above. The motion does not completely randomize the orientational distribution, since the perpendicular and parallel line shapes are different (10.5 G difference in hyperfine splitting), and are distinct from the spectrum of randomly oriented myofibrils.

(A) Effect of Troponin. The effect of troponin on the orientation of tropomyosin in fibers is shown in Figure 5. In the absence of Ca^{2+}, troponin decreased the hyperfine splitting of the $H_\parallel$ spectrum from 51.7 to 49.7 G, and the width of the high-field peak from 20.8 to 17.0 G (Table 3). Since troponin does not induce any motional changes, the changes in the parallel spectrum can be ascribed entirely to changes in orientational distribution. The decrease in the Δh value reflects the narrower orientational distribution of tropomyosin; the change of splitting implies a shift of the center of the broad distribution causing it to become more perpendicular to the fiber axis.

The absence of parallel changes in the $H_\perp$ spectrum is a reflection of the lower sensitivity of the perpendicular spectra to changes in orientational distribution (Table 3).

The determination of the absolute value of the reorientation is impeded by the presence of nanosecond motion. Current simulation algorithms consider orientational distribution in the absence of motion (Thomas & Cooke, 1980; Burghardt & Thompson, 1985; Fajer *et al.*, 1990; Fajer, 1994), and we are not aware of simulations incorporating both motion and orientation applicable to muscle symmetry.

The binding of Ca^{2+} to troponin partially reversed the effect of Tn on the angular distribution of tropomyosin reconstituted in fibers (Figure 5). The hyperfine splitting of the $H_\parallel$ spectrum increased to 51.6 G, i.e., to the level of $2T_{eff}$ characteristic for tropomyosin reconstituted in fibers in the absence of troponin. However, the width of the high-field resonance was 18.5 G as compared to 20.8 G for ghost fibers (Table 3), indicating smaller disorder. These differences indicate that tropomyosin in fibers exhibits three different orientational distributions, dependent on the presence of troponin and calcium.

(B) Effect of Myosin S1. As shown in Table 3, the binding of S1 to actin in fibers did not affect the $2T_{eff}$, Δl, or Δh parameters of the $H_\parallel$ spectrum of tropomyosin. This is somewhat surprising since there is an increase in motional narrowing observed in myofibrils (Table 2) and in the fibers oriented perpendicular to the field (Table 3). It is possible that there was a small change in the tropomyosin orientational distribution tilting the probe axis toward fiber axis which would result in the increase of the hyperfine splitting and would offset the decrease due to motional averaging. The reorientation would have to be small to leave $2T_{eff}$ un-

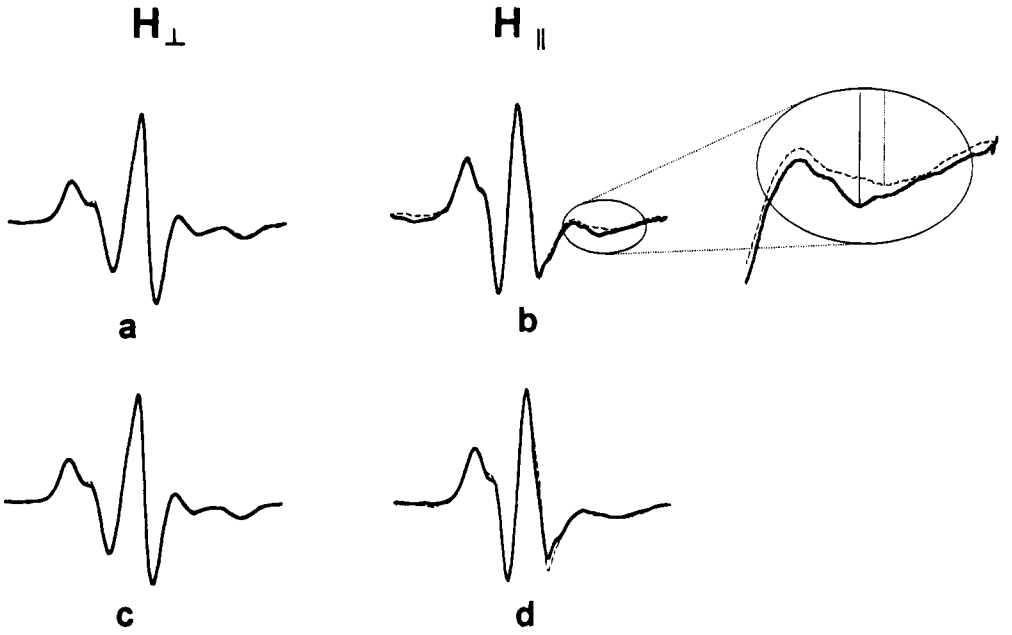

FIGURE 5: Effects of troponin addition on the perpendicular (left) and parallel (right) spectra of labeled tropomyosin. Top: troponin/EGTA; bottom: troponin and Ca^{2+}. Each spectrum is overlaid with the spectrum of MSL-Tm in the absence of troponin (dotted); the inset documents the small change of the $H_{||}$ spectrum on the addition of troponin.

Table 3: Spectral Parameters of MSL-Tm Reconstituted in Skeletal Ghost Fibers for Spectra Taken Perpendicular ($H_\perp$) and Parallel ($H_{||}$) to the Static Magnetic Field

	$H_\perp$				$H_{||}$			
sample	$2T_{eff}$	Δl	Δh	n	$2T_{eff}$	Δl	Δh	n
reconstituted in ghost fibers	62.2 ± 0.3	8.8 ± 0.1	8.6 ± 0.5	5	51.7 ± 0.1	8.7 ± 0.1	20.8 ± 0.4	4
+Tn/EGTA	62.2 ± 0.2	8.7 ± 0.3	9.4 ± 0.5	4	49.7 ± 0.5	8.2 ± 0.2	17.0 ± 0.2	3
+Tn/Ca^{2+}	61.8 ± 0.3	8.6 ± 0.2	9.4 ± 0.4	5	51.6 ± 0.4	8.6 ± 0.1	18.5 ± 0.1	3
+S1	61.1 ± 0.4	8.7 ± 0.1	9.4 ± 0.4	4	51.6 ± 0.1	8.6 ± 0.1	19.7 ± 0.5	3

changed. The quantitative estimate of the orientational change would require rigorous computational simulations of the orientation in the presence of nanosecond motion.

DISCUSSION

The dynamics study presented here is the first direct experimental investigation of the motion of tropomyosin in a reconstituted fiber system, and might be important for better understanding of the regulatory role of tropomyosin in muscle. We have detected small changes in the mobility of labeled tropomyosin induced by its interactions with actin, troponin, and myosin heads. In solution, the labeled domains of tropomyosin exhibit fast nanosecond motions, with a rotational correlation time of 3−7 ns. This mobility is decreased upon binding to actin filaments in "ghost" fibers. The addition of troponin to myofibrils reconstituted with tropomyosin does not change the mobility of tropomyosin domains, irrespective of the presence of Ca^{2+}. However, the rate of Tm motion does increase when myosin−S1 binds to the reconstituted thin filaments.

The orientational studies of tropomyosin reconstituted in fibers indicate a broad distribution of the probe with respect to the fiber axis. In the absence of Ca^{2+}, troponin decreases the angular disorder of tropomyosin relative to the fiber axis. The binding of Ca^{2+} to troponin partially reverses its effect on tropomyosin distribution, producing an orientation that is similar, but not identical, to the troponin-free system. No apparent change in Tm orientational distribution is observed upon the binding of extrinsic S1 to actin; thus, the angular distribution of the labeled domain of tropomyosin in fibers is affected by troponin and calcium, but is not significantly altered by S1. It is important to note, however, that the rotation about the long axis of the thin filament as postulated by the "steric block" model would remain undetected in EPR.

Relationship to Other Studies. The flexibility of tropomyosin, an α-helical coiled-coil protein, which is wound around the actin filaments in muscle, was postulated to be of importance for its regulatory function. The "simple harmonic" theory proposed by Earley (1991) attributes the regulatory mechanism of tropomyosin in the activation of muscle contraction to the protein's vibrations. It is postulated that the vibrational motion of Tm results in the protein's displacement from the periphery of the thin filament (an "off"-state) to the actin filament groove (an "on"-state). The degree of activation increases as the amplitude of vibrations increases. The forces driving the vibrations are thought to be associated with the interaction of the thin and thick filaments (electrostatic), hydrophobic forces between actin and Tm, and the forces induced by conformational changes in the troponin/tropomyosin complex following Ca^{2+} binding.

The experimental evidence for the proposed motions comes from the flexibility detected in isolated tropomyosin molecules. The EPR studies of labeled tropomyosin by Graceffa and Lehrer (1984) noted that the measured correlation time is a function of the temperature as well as the structure of the label used. The MSL label revealed a temperature-dependent mobility of 50 ns at 0.5 °C, decreasing to 9 ns at 20 °C, the latter value consistent with the τ_r = 6.6 ns observed here at 24.5 °C (Table 2). Opening of the succinimido ring under alkaline conditions (MSL-II label)

resulted in a weakly immobilized signal ($\tau_r = 1$ ns) sensitive to the local conformational changes accompanying the melting pretransition of tropomyosin's coiled-coil. Our confirmation of these original results is of particular significance since the immobilization experiments (Figure 2) excluded the possibility of independent motion of the label. In addition to EPR studies, fluorescence anisotropy decays of labeled tropomyosin also indicated substantial mobility. Wahl *et al.* (1978) observed at least two different rates of decay, corresponding to different modes of motion. The faster rate, $\tau_r = 4.5$ ns, compares well with the flexibility observed here (see Table 2). The slower, 50−500 ns motions most likely are either rotations about the long axis of a cylinder or the flexure of the long chain. Tropomyosin can be modeled as a prolate ellipsoid ($a = 40$ nm; $b = 1$ nm); the utilization of the Perrin equations (Cantor & Schimmel, 1980) gives a correlation time for the rotation about the long axis $\tau_r = 16\pi\eta ab/18kT = 27$ ns. If the cylinder is flexible, as expected for an elongated molecule, the flexure time is given by $\tau_r = (5.53 \times 10^{-3})\pi\eta a^4/[\lambda_k T \ln(a/b)]$, where λ is the persistence length (Ookubo *et al.*, 1976). For a persistence length of 130 nm as estimated by Hvidt *et al.* (1983), the flexure time is 22 ns, too close to the estimated rotation about the long axis to be resolved.

Interestingly, this tropomyosin flexibility persists in Tm crystals as manifested by the variation of the X-ray temperature factors as a function of residue position along the Tm molecule (Phillips *et al.*, 1980, 1986) and is little inhibited by association with the thin filament. Fixing at least one end of the protein to the actin filament can be expected to result in a 59-fold increase of τ_r, if the persistence length is constant (Hvidt *et al.*, 1982). Since the rate of Tm motion in fibers is only half of that in solution (Table 2), either the domain motions observed here have little to do with the bending motions or the association with actin is very loose. The subtle increase of the correlation time might reflect the stabilization of a coiled-coil structure in the melting pretransition range (25 °C) as observed previously by Graceffa (1985). The contribution of the "weakly immobilized" component of the MSL-II spin-label was decreased in the presence of actin.

There is also little effect of troponin on these domain motions, despite the fact that Tn interacts with tropomyosin in the region of its labeled domains (Flicker et al., 1982). The absence of any significant effects of troponin on the domain motions was also observed by Wahl *et al.* (1978). It could well be that troponin interacts very loosely with the domains containing the labeled cysteines (Ishii & Lehrer, 1991). The absence of the effect on Tm mobility by troponin binding is contrasted with the 1.3−2.3-fold increase of the motional rate of tropomyosin, caused by the attachment of the myosin heads to the thin filaments (Table 2). Since actin flexibility does not change upon myosin binding (Ostap & Thomas, 1991; Ostap *et al.*, 1992; Naber *et al.*, 1993; Borovikov *et al.*, 1993), it is unlikely that the observed change in Tm motion is coupled to actin flexibility.

Equally important is the orientational distribution of Tm. The EPR spectra indicate a large disorder of the average angle of the probe with respect to the fiber axis (Figure 4); this disorder is modulated by Ca^{2+} binding to troponin. The angular distribution of Tm in the presence of Tn/Ca^{2+} matches that in the absence of troponin, in agreement with a recent report from X-ray diffraction studies (Poole *et al.*, 1994). Interestingly, we did not observe any significant effect of myosin S1 on the orientational distribution of tropomyosin, as might be expected if the two proteins compete for the same site on actin. The absence of an effect of S1 on the linear dichroism of labeled Tm reconstituted in myofibrils was recently reported (Szczesna & Lehrer, 1992), but the linear dichroism does not distinguish between the average orientation and disorder, which makes any conclusions about orientational distribution equivocal. EPR, with its spectral resolution of orientations, is capable of distinguishing between these two parameters, which affect the spectral line shape in an independent manner (Thomas & Cooke, 1980). The identity of the EPR line shapes translates directly into the identity of orientational distribution (Fajer, 1994); thus, even without spectral simulations we can state that the orientation of Tm is little affected by myosin head binding.

It is clear that the mechanism of Tm regulation has to be considered in terms of both dynamics and orientational transitions of Tm on actin. It appears that the two different ways of thin filament regulation, the binding of Ca^{2+} and the binding of myosin S1, have different effects on the protein's behavior. The binding of myosin S1 to fibers modulates the motion of Tm, while the binding of Ca^{2+} to troponin affects the tropomyosin distribution within the thin filament.

The present results are consistent with the general framework of the steric blocking hypothesis, where Ca^{2+} binding to troponin moves Tm from its blocking position on actin, allowing myosin heads to interact with the thin filament. We found that the binding of Ca^{2+} and the binding of S1 are distinct in terms of a change in the Tm orientational distribution within reconstituted fibers. Transition from the "low [Ca^{2+}] state" to the "high [Ca^{2+}] state" resulted in an increase of the angular distribution of Tm with respect to the actin axis. However, we were not able to quantify this change in terms of the average orientation of Tm on actin in both states of the thin filament due to computational difficulties of combining nanosecond motion with orientational distribution.

During past years there has been a lot of interest in clarifying the various relationships between the components of the regulatory apparatus in muscle. The steric blocking model was followed by the two-state allosteric/cooperative binding theory (Hill *et al.*, 1980; Green & Eisenberg, 1980) according to which S1 (not Ca^{2+}) binding is required in order to switch the thin filament from the "off" to the "on" state. The S1-induced change of the thin filament conformation was directly confirmed by fluorescence studies with the label at Cys-190 of Tm (Lehrer & Ishii, 1988; Ishii & Lehrer, 1990, 1993). More than two states of thin filament regulation were subsequently proposed by Lehrer and Morris (1982), Babu and Gulati (1988), and McKillop and Geeves (1993) and incorporated in the schematic model of Lehrer (1994). The model combines aspects of the steric theory with the later cooperative models of regulation. According to this model, the binding of Ca^{2+} to troponin results in the dissociation of the Tn-I subunit of Tn from actin and leads to the activation of Tm. Further changes occur when the myosin heads bind and cause Tm to move from the "off" to the "on" state. Our data agree with the main idea of this model, with Ca^{2+} triggering the change of Tm orientational distribution on actin, and myosin heads changing its dynamic state within the thin filament. Our findings are also in accord with the theory of Earley associating the regulatory mech-

anism of Tm in muscle with its vibrational motion. As the amplitude of Tm motion increases, the molecule decreases in its effective length, causing its displacement on actin. What we observe is that Ca^{2+} changes the amplitude of Tm motion, since the angular distribution increases on addition of Ca^{2+}. The rate of Tm motion is clearly regulated by its binding to actin and subsequently by the binding of myosin S1 to actin. Interestingly, S1 increases this rate almost to the value characteristic for the motion of Tm in solution. The presence of the fast motion as well as the disorder of the labeled domain indicates a loose coupling of the probed domains on the tropomyosin and the thin filament. Preliminary X-ray diffraction data from the actin−Tm gels and the fibers stretched beyond overlap suggest an electrostatic interaction between Tm and actin (Poole *et al.*, 1994). Such an interaction could be expected to place few steric constraints on the tropomyosin domain movement observed here. Further studies are needed to correlate the observed changes in Tm motion with the activation in muscle.

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