Conformation of Spin-Labeled Tropomyosin in Reconstituted Muscle Thin Filaments in Response to Calcium Ion and Heavy Meromyosin†

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ABSTRACT: Tropomyosin (TM) exists in thermal equilibrium between a highly structured N state, a partially unfolded X state, and a completely unfolded D state, i.e., N ⇌ X ⇌ D. The strongly immobilized electron spin resonance (ESR) spectral component of spin-labeled TM corresponds to TM in the N state and the weakly immobilized component to TM in the X state below the main unfolding transition and to TM in the D state above this transition [Graceffa, P., & Lehrer, S. S. (1984) *Biochemistry 23*, 2606–2612]. The addition of actin, troponin (TN), and heavy meromyosin (HMM) to spin-labeled TM reduces the ratio of weakly to strongly immobilized labels, indicating a shift in the N ⇌ X ⇌ D equilibrium toward the N state. At 37 °C, for spin-labeled TM alone K (=X/N) > 1.0 with some TM in the D state, K = 0.8 for spin-labeled TM bound to actin, and K < 0.05 for spin-labeled TM bound to actin + TN ± Ca^{2+}, actin + HMM + TN ± Ca^{2+}, and actin + HMM. Thus, actin + TN dramatically shifts the TM structure to the N conformation with little further effect upon addition of Ca^{2+} or HMM. The temperature at which spin-labeled TM begins to dissociate from a protein complex was determined from the temperature dependence of the ESR spectra. This temperature increases in the following order upon complexation of spin-labeled TM to TN ± Ca^{2+} < actin ≪ actin + TN + Ca^{2+} < actin + TN − Ca^{2+} < actin + HMM + TN ± Ca^{2+} = actin + HMM.

Skeletal muscle consists of an interdigitating array of myosin thick and actin thin filaments which slide past each other during contraction. Rod-shaped tropomyosin (TM)[1] molecules bind end-to-end along the actin filament, each TM binding to seven actin monomers; troponin binds to actin and to the C-terminal half of TM. The sliding motion is due to the interaction between myosin and actin during the hydrolysis of ATP by myosin. Contraction is activated by the binding of Ca^{2+} to troponin which then relieves TM's inhibtion of the actomyosin ATPase activity (Ebashi et al., 1969). This is thought to involve a Ca^{2+}-sensitive change in position of TM on the thin filament (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973; Wakabayashi et al., 1975; Gillis & O'Brien, 1975). Myosin is also able to remove TM's inhibition of the ATPase activity (Bremel et al., 1972; Bremel & Weber, 1972; Lehrer & Morris, 1982). The modulations of TM's regulatory role by both myosin (Bremel et al., 1972; Bremel & Weber, 1972; Lehrer & Morris, 1982) and Ca^{2+} (Tawada et al., 1975; Grabarek et al., 1983) are cooperative phenomena, and TM's end-to-end interaction and flexibility are thought to play a crucial role in this cooperativity.

TM is composed of two parallel, coiled-coil α-helical polypeptide chains in a rod-shaped structure (Astbury et al., 1948; Holtzer et al., 1965; Caspar et al., 1969) with an α-helical content close to 100% at temperatures below 10 °C (Cohen & Szent-Gyorgi, 1957; Woods, 1969; Wu & Yang, 1976). The molecule unfolds with increasing temperature in two distinct stages (Woods, 1969; Satoh & Mihashi, 1972). The first stage, the pretransition, involves unfolding in the C-terminal half of TM, and the second stage, the main transition, involves the complete unfolding of the molecule (Woods, 1977; Lehrer, 1978; Pato & Smillie, 1978; Potekhin & Privalov, 1978). This has been described as an equilibrium between a highly structured, highly α-helical molecule, N, a partially unfolded (in the C-terminal half) intermediate state, X, and the completely denatured TM, D, i.e., N ⇌ X ⇌ D (Woods, 1976; Graceffa & Lehrer, 1980; Lehrer et al., 1981; Betteridge & Lehrer, 1983; Graceffa & Lehrer, 1984). The pretransition involves a loss of 15–30% α-helical content (Woods, 1976; Betteridge & Lehrer, 1983), and therefore, it is considered that TM is more flexible in the X state than in the N state (Woods, 1976; Lehrer, 1978; Lehrer et al., 1981). NMR (Edwards & Sykes, 1978) and X-ray crystallography (Phillips et al., 1979) studies of TM also indicate regions of flexibility in the C-terminal half of the molecule. Since it has been queried as to what role the N ⇌ X conformational equilibrium might play in TM's regulation of contraction (Woods, 1976; Lehrer, 1978; Lehrer et al., 1981), in this study the equilibrium constant, $K = X/N$, was measured for TM in reconstituted thin filaments in response to Ca^{2+} and heavy meromyosin (the two-headed myosin fragment).

The N ⇌ X equilibrium has been probed with a maleimide spin-label (with an opened succinimido ring) at cysteine-190, in the C-terminal half of TM (Graceffa & Lehrer, 1984). The ESR spectrum of spin-labeled TM consists of two spectral components corresponding respectively to labels that are strongly immobilized and those that are weakly immobilized. The former predominate at low temperatures and can be associated with TM molecules in the N state while the latter increase with increasing temperature and, below the main

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[1] Abbreviations: TM, tropomyosin; ATPase, adenosinetriphosphatase; TN, troponin; HMM, heavy meromyosin; MSL_0–MSL_4, series of maleimide spin-labels; MSL(II)TM, tropomyosin labeled with a maleimide spin-label whose succinimido ring was subsequently opened by alkaline incubation; ESR, electron spin resonance; A_w and A_s, amplitude of the low-field line of the weakly immobilized and strongly immobilized ESR spectral components, respectively; C_w and C_s, fractional concentration of the weakly immobilized and strongly immobilized spin-labels, respectively; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMPPNP, 5′-adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid.

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unfolding transition, can be associated with TM in the X state and above this transition with TM in the D state. The equilibrium constant $K = X/N$, determined from the ratio of the two types of labels, was 1.0 at 34 °C.

In the present work K was measured for spin-labeled TM in reconstituted thin filaments ± Ca^{2+} and ± heavy meromyosin. At 37 °C for spin-labeled TM in the presence of actin plus troponin the N ⇌ X equilibrium is shifted far to the left toward the N state, and the additional binding of Ca^{2+} and/or heavy meromyosin has little further effect. Thus, any flexibility of the TM molecule in the inhibiting or activating state is that of the N conformation, i.e., of a highly α-helical, highly structured rod.

Experimental Procedures

Proteins were prepared from rabbit leg and back muscles: TM by the method of Greaser & Gergely (1971), actin by the method of Spudich & Watt (1971), troponin (TN), by Dr. Edward P. Morris, by the methods of Ebashi et al. (1971) and van Eerd & Kawasaki (1973), myosin by the method of Nauss et al. (1969) modified to include 2 mM ATP in the Guba-Straub extraction medium, and chymotryptic heavy meromyosin (HMM) from myosin by the method of Weeds & Pope (1977). HMM is used because of its higher solubility compared to myosin.

TM was reduced by incubation for 2 h at room temperature in 20 mM dithiothreitol, 4 M guanidnium chloride, 2.5 mM Mops, and 1 mM EDTA, pH 7.5, and then exhaustively dialyzed vs. 0.1 M NaCl, 2.5 mM 2-(*N*-morpholino)ethanesulfonic acid, and 0.2 mM EDTA, pH 6.0. Reduced TM was labeled at cysteine-190 with a series of maleimide spin-labels (Syva) whose succinimido ring was subsequently opened by alkaline incubation by procedures described previously (Graceffa & Lehrer, 1984); it is referred to as MSL(II)TM. The labels are 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (MSL_0), the label used in the previous study (Graceffa & Lehrer, 1984), 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrolidinyloxy (MSL_1), 3-[(maleimidoethyl)carbamoyl]-2,2,5,5-tetramethyl-1-pyrolidinyloxy (MSL_2), 3-[(3-maleimidopropyl)carbamoyl]-2,2,5,5-tetramethyl-1-pyrolidinyloxy (MSL_3), and 3-[[2-(maleimidoethoxy)ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrolidinyloxy (MSL_4).

Binding of MSL(II)TM to actin was measured by sedimenting actin in a Beckman airfuge at 20 psi for 40 min at room temperature (25 °C) or in a 37 °C warm room. The supernatant and a control MSL(II)TM were then accurately diluted into 4 M guanidinium chloride, and their ESR spectra were recorded. In this solvent MSL(II)TM is completely denatured, and its ESR spectrum consists of three narrow lines corresponding to labels which are highly mobile (Graceffa & Lehrer, 1984). A comparison of the spectral amplitude of the denatured supernatant with that of the control yields the free MSL(II)TM from which the degree of binding can be calculated.

ATPase activity was measured at 33 °C for HMM + actin + TN + MSL_0(II)TM or reduced TM in a solution containing 0.05 M NaCl, 5 mM $MgCl_2$, 10 mM Mops, either 0.2 mM $CaCl_2$ or 0.2 mM EGTA, and 5 mM MgATP, pH 7.5. After incubation for 5 min, the reaction was stopped with an equal volume of 10% trichloroacetic acid and P_i determined by the method of Fiske & Subbarow (1925).

ESR spectra were recorded, and the amplitude ratio of the weakly immobilized ESR spectral component to the strongly immobilized component, A_w/A_s, of MSL_0(II)TM was measured as described previously (Graceffa & Lehrer, 1984). The concentration ratio of weakly to strongly immobilized spin labels, C_w/C_s, was determined from A_w/A_s from a calibration plot of C_w/C_s vs. A_w/A_s. The calibration plot was determined by first quantitatively decomposing an ESR spectrum of 50 μM MSL_0(II)TM, in 1 M NaCl, pH 7.5 at 33 °C, into strongly and weakly immobilized components and calculating C_w and C_s as perfomed previously (Graceffa & Lehrer, 1984). By recombining various proportions of the two spectral components, we then generated values of A_w/A_s and calculated the associated values of C_w/C_s. Some error will be introduced in this study in the calculation of C_w/C_s from this calibration at 33 °C since the spectra of interest were recorded at 37 °C. However, this error will be very small since spectral line shapes and line widths will not change very much over this small temperature range. Furthermore, since the effects observed in this study are large, any small error incurred would have no effect on the interpretation of the results.

Results and Discussion

The helix unfolding of spin-labeled TM, MSL_0(II)TM, in 1 M NaCl, pH 7.5, occurrs in two stages, the pretransition and the main unfolding transition (Graceffa & Lehrer, 1984). In this study MSL_0(II)TM was studied in 0.1 M NaCl and 5 mM $MgCl_2$, pH 7.5, ionic conditions closer to physiological conditions. In this solvent the unfolding profile was the same as in 1 M NaCl except for a small shift (2–3 °C) to a lower temperature for the main unfolding transition (not shown). In 1 M NaCl the temperature dependence of the ESR spectrum of MSL_0(II)TM also showed two stages which coincided with the two helix unfolding stages. As the temperature is increased, the amplitude ratio of the weakly immobilized spectral component to the strongly immobilized component, A_w/A_s, increases moderately in the pretransition temperature range and dramatically in the main unfolding temperature range (Graceffa & Lehrer, 1984). In 0.1 M NaCl and 5 mM $MgCl_2$, similar behavior is observed (Figure 1). Thus, since it was shown (Graceffa & Lehrer, 1984) that in 1 M NaCl the strongly immobilized spectral component corresponds to TM in the N state and that the weakly immobilized component corresponds to TM in the X state below the main unfolding temperature and to TM in the D state above this temperature, it is concluded that the same correspondence holds in 0.1 M NaCl and 5 mM $MgCl_2$.

The temperature at which the dramatic increase in A_w/A_s occurs for MSL_0(II)TM is shifted to higher values upon the addition of TN ± Ca^{2+}, actin, actin + TN ± Ca^{2+}, actin + HMM + TN ± Ca^{2+}, or actin + HMM (Figure 1). The degree of the shift increases in the order TN ± Ca^{2+} < actin ≪ actin + TN + Ca^{2+} < actin + TN − Ca^{2+} < actin + HMM + TN ± Ca^{2+} = actin + HMM. This is roughly the order of increasing binding of TM in the complex since it has been shown that TN (Drabikowski et al., 1968; Tanaka, 1972; Ishiwata, 1978; Wegner & Walsh, 1981) and HMM (Eaton, 1976) increase the binding of TM to actin and that TN − Ca^{2+} increases the binding more than TN + Ca^{2+} (Tanaka, 1972; Ishiwata, 1978; Wegner & Walsh, 1981). Thus, the temperature at which A_w/A_s increases steeply must represent the onset of dissociation of MSL_0(II)TM from the complex. Therefore, it appears that HMM strengthens the TM–actin binding more than does TN even though HMM alone has no affect on A_w/A_s (not shown) and does not bind to TM.

Since the regulatory function of TM is ultimately expressed in the presence of nucleotide, the temperature dependence of A_w/A_s was measured (at 0.05 M instead of 0.1 M NaCl) for MSL_0(II)TM + actin + HMM + TN ± Ca^{2+} in the presence and absence of 2.5 mM MgADP, 2.5 mM MgAMPPNP (nonhydrolyzable ATP analogue), or 15 mM MgATP (mea-

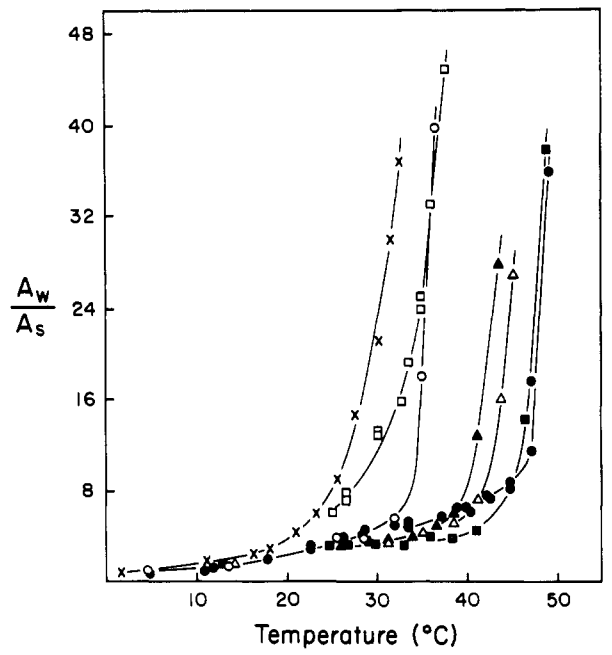

FIGURE 1: Temperature dependence of the amplitude ratio of weakly immobilized to strongly immobilized ESR spectral components, A_w/A_s, for MSL_0(II)Tm, 5.5 μM, plus alone (X), TN ± Ca^{2+} (□), actin (○), actin + TN + Ca^{2+} (▲), actin + TN − Ca^{2+} (△), actin + HMM + TN ± Ca^{2+} (●), and actin + HMM (■) all in 0.1 M NaCl, 5 mM $MgCl_2$, and 10 mM Mops, pH 7.5, plus either 1 mM EGTA or 0.2 mM $CaCl_2$. The molar ratio of MSL_0(II)Tm:TN:actin:HMM = 1:1.1:7.5:5.3. There was little change in these curves if the ratio of TN or actin to MSL_0(II)TM was increased up to 2-fold more. For HMM the same curves were obtained at an even lower HMM: MSL_0(II)TM ratio of 3.8.

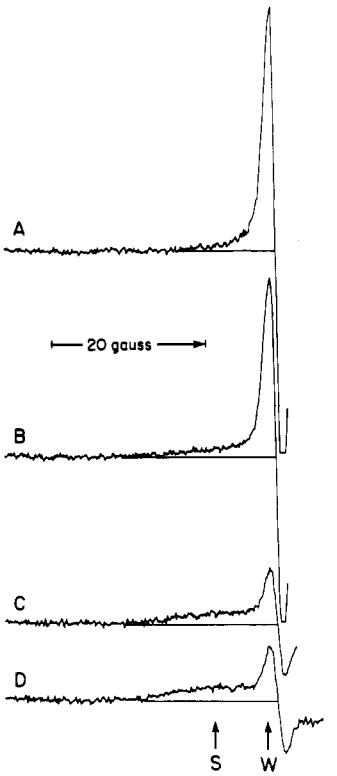

FIGURE 2: Low-field portion of the ESR spectrum of MSL_0(II)TM, 4.7 μM, plus (A) alone, (B) actin, (C) actin + TN + Ca^{2+}, and (D) actin + HMM all at 37 °C in 0.1 M NaCl, 5 mM $MgCl_2$, ±0.2 mM $CaCl_2$, and 10 mM Mops, pH 7.5. The molar ratio of MSL_0(II)-TM:TN:actin:HMM = 1:1:7:4.4. S and W indicate the position of the strongly and weakly immobilized spectral components, respectively.

sured only at 33 °C). From ATPase measurements it was determined that only 50% of the ATP was hydrolyzed in the presence of Ca^{2+} and much less in its absence during the recording of the ESR spectrum. [These two activities were the same when reduced unlabeled TM replaced MSL_0(II)TM, indicating that the label has little effect on TM's activity.] A_w/A_s values were unaffected by the presence of the nucleotides.

At each temperature, for each complex, there is a decrease in A_w and an increase in A_s, compared to MSL_0(II)TM alone (Figures 1 and 2), without any apparent change in the shape of either spectral component. More specifically, for the low-field line of each spectral component there is no change in position or in width, parameters which are sensitive to spin-label mobility [Figure 1 (Hsia & Piette, 1969); Figure 4 (Jost & Griffith, 1978)], upon binding of MSL_0(II)TM in any of the complexes studied. (The comparison of these parameters for the strongly immobilized component could only be made at ≤25 °C where there is a sufficient amount of this component.) This indicates that the mobility of the spin-label in the weakly and strongly immobilized states does not change significantly upon binding and that the change in A_w/A_s is due to an interconversion of labels between these two states. This is consistent with the nature of the N and X states being, for the most part, the same for TM alone and complexed in the thin filament and with the label not interacting directly with other thin filament proteins or HMM. Thus, it is reasonable to conclude that a decrease in A_w/A_s indicates a shift in the N ⇌ X ⇌ D equilibrium toward the N state and a stabilization of TM structure.

In order to quantitate the magnitude of this shift in equilibrium, the concentration ratio of weakly to strongly immobilized spin-labels (C_w/C_s) was determined from A_w/A_s of ESR spectra at 37 °C (representative spectra shown in Figure 2). To calculate A_w/A_s for bound MSL_0(II)TM, it is necessary to correct for any free MSL_0(II)TM. Thus, the binding of MSL_0(II)TM to actin, actin + TN ± Ca^{2+}, actin + HMM, and actin + HMM + TN ± Ca^{2+} was measured at 37 °C at the same conditions and protein concentrations as used for the spectra in Figure 2. The binding to actin alone was 60% and was between 85 and 90% in the other five complexes. A_w/A_s for bound MSL_0(II)TM is calculated by subtracting from the measured A_w and A_s the appropriate fraction of A_w and A_s, respectively, measured for MSL_0(II)TM alone. At 37 °C, for MSL_0(II)TM bound to actin A_w/A_s = 19 while for MSL_0-(II)TM bound in the other five complexes A_w/A_s was between 1.3 and 2.6. C_w/C_s, determined from A_w/A_s as described under Experimental Procedures, is 0.8 ± 0.15 for MSL_0(II)TM bound to actin and <0.05 for MSL_0(II)TM bound in the other five complexes.

MSL_0(II)TM in 1 M NaCl and at 37 °C is in the beginning of the main unfolding transition, and its structure can be described by the equilibrium N ⇌ X ⇌ D with $(X + D)/N$ = 2.5 and K ($=X/N$) > 1.0 (Graceffa & Lehrer, 1984). In 0.1 M NaCl and 5 mM $MgCl_2$ the equilibrium is somewhat further shifted to the right since A_w/A_s at 37 °C is greater in this solvent (Figure 1) than in 1 M NaCl (Graceffa & Lehrer, 1984). On binding to actin at 37 °C the MSL_0(II)TM structure changes to one adequately described by the equilibrium N ⇌ X, since it is assumed that the D state does not exist for bound TM, with K ($=C_w/C_s$) = 0.8 ± 0.15. Other work has also indicated that the binding of TM to actin shifts the N ⇌ X ⇌ D equilibrium to the left (Ishii & Lehrer, 1984). On binding to the other five complexes at 37 °C the MSL_0-(II)TM structure can be described by a further shift in the N ⇌ X equilibrium toward the N state with K < 0.05. Thus,

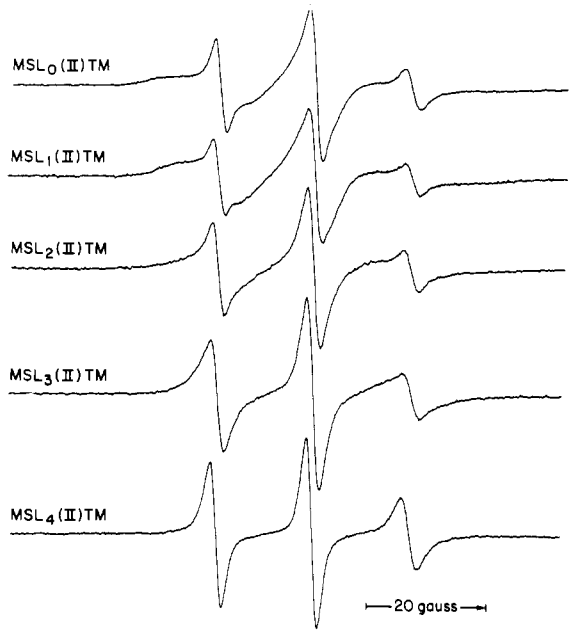

FIGURE 3: ESR spectra of tropomyosin labeled with maleimide spin-labels of increasing length, at 25 °C in 0.1 M NaCl, 5 mM $MgCl_2$, and 10 mM Mops, pH 7.5. $MSL_0(II)TM$ = 39 μM; $MSL_1(II)TM$–$MSL_4(II)TM$ = 13 μM. Label length of MSL_0–MSL_4 is 4.4, 5.7, 9.3, 10.5, and 12.9 Å, respectively.

while actin has a moderate effect on TM structure, actin + TN or actin + HMM stabilize TM conformational stucture dramatically. Since HMM alone does not affect A_w/A_s and does not bind to TM, it most probably influences TM's structure and TM–actin binding indirectly through its interaction with actin. A more direct interaction, however, cannot be ruled out since TM and myosin can come close to each other when bound to actin (Taylor & Amos, 1981; Wakabayashi & Toyoshima, 1981; Amos et al., 1982; Vibert & Craig, 1982; Tao & Lamkin, 1984).

TM was labeled with a series of maleimide spin-labels of increasing length in order to measure the depth of the cysteine-190 sulfhydryl group below the protein surface (Hsia & Piette, 1969; Zeidan et al., 1980) and to see if the labels interact directly with added proteins. The chemical structure of these labels is shown by Zeidan et al. (1980). The distance between the protein sulfhydryl group and the nitroxide ring for MSL_0–MSL_4 is 4.4, 5.7, 9.3, 10.5, and 12.9 Å, respectively (Zeidan et al., 1980). As the label length increases, the position of the low-field line of the strongly immobilized component shifts upfield and A_w/A_s increases [except for $MSL_1(II)$], indicating a generalized increase in label mobility (Figure 3). $MSL_3(II)$ is the shortest label to show only a weakly immobilized spectral component, suggesting that the nitroxide ring is at or beyond the protein surface where it is only slightly restricted in its motion. This would indicate that the sulfhydryl group is roughly 9.3–10.5 Å from the protein surface. This is consistent with the 10-Å radius of the TM rod (Holtzer et al., 1965; Caspar et al., 1969) and with a model that places cysteine-190 in the most interior location of the protein along the hydrophobic ridge between the two chains (McLachlan & Stewart, 1975).

When actin, actin + TN ± Ca^{2+}, or actin + HMM + TN ± Ca^{2+} are added to these MSL(II)TMs, at 25 °C as in Figure 1, there is a decrease in A_w/A_s for $MSL_0(II)TM$ as discussed above, a much smaller decrease in A_w/A_s for $MSL_1(II)TM$ without any change in line position or width, and no change at all for $MSL_2(II)TM$–$MSL_4(II)TM$. This lack of change is not due to a lack of binding since all MSL(II)TMs bound in all of these complexes to the extent of 83–95%. Thus, only labels below TM's surface and not those near to or beyond the surface are affected by added proteins. Another study has also shown very little interaction between a (fluorescent) probe at cysteine-190 and actin + TN (Lamkin et al., 1983). These results support the conclusion drawn above that the $MSL_0(II)$ label is not interacting directly with added proteins and that changes in its mobility are due to an indirect effect of these proteins on the conformation of TM.

In conclusion, it appears that tropomyosin bound to actin + TN is in the highly structured, highly α-helical N state and that this conformation is not significantly affected by the futher addition of Ca^{2+} and/or HMM. Thus, any flexibility of tropomyosin on the thin filament is due to the flexibility of a highly structured rod (N state) and not due to the flexibility about some unfolded region of the X state. Indeed X-ray crystallography studies indicate that tropomysoin has significant flexibility at 0–4 °C (Phillips et al., 1980; Phillips, 1984) where the N $\rightleftharpoons$ X equilibrium is shifted far to the left (Woods, 1976; Betteridge & Lehrer, 1983; Graceffa & Lehrer, 1984).

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500-MHz ^{1}H NMR Studies of Ragweed Allergen Ra5

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ABSTRACT: The solution conformation of short ragweed allergen Ra5, a protein of 45 amino acid residues cross-linked with four disulfide bridges, has been investigated by ^{1}H NMR spectroscopy at 500 MHz. The aromatic region, which contains resonances from three tyrosines and two tryptophans, has been partially assigned. Two tyrosines titrate with a pK of 10.2; a third tyrosine is buried under the tryptophan resonances, and its pK could not be determined. The two tryptophans reside in different microenvironments; the resonances of one are very similar to those found in random coil structures while the other has dramatically shifted peaks. Nuclear Overhauser effect (NOE) difference spectroscopy is used to define two distinct spin-diffusion systems for the aromatic residues and to further identify several methyl-containing amino acids involved in these systems. Assignments in the methyl region are based on selective decoupling, chemical shifts, NOE difference spectra, and 2-D J-resolved and 2-D J-correlated spectroscopy (COSY) methodology. A unique ring-current-shifted methyl doublet in the Ra5 spectrum titrates into the bulk methyl region with a pK of 10.2. Examination of the COSY map suggests that this resonance belongs to either leucine-1 or isoleucine-38. Chemical removal of the N-terminal leucine did not affect the ring-current-shifted methyl. Therefore, this unique resonance has been assigned to the methyl of isoleucine-38. With this assignment and the spin-diffusion behavior of the aromatic residues, it is possible to suggest disulfide assignments as well as specific structural features of Ra5 consistent with the toxin–agglutinin fold proposed by Drenth and co-workers [Drenth, J., Low, B. W., Richardson, J. S., & Wright, C. S. (1980) *J. Biol. Chem. 255*, 2652–2655].

In recent years there has been an increasing interest in the structure and activity of environmentally derived proteins that on inhalation or injestion provoke immediate allergic reactions in predisposed individuals [for reviews, see Marsh (1975), King (1976), and de Weck (1977)]. For the North American population, the most important single source of these provocative proteins, and the most studied, is the pollen of *Ambrosia elatior*, commonly known as short ragweed. This pollen

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