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Dynamic Equilibrium between the Two Conformational States of Spin-Labeled Tropomyosin†

Philip Graceffa* and Sherwin S. Lehrer

ABSTRACT: Tropomyosin was labeled with a maleimide nitroxide spin-label attached to cysteine-190 via a succinimido ring which was subsequently opened by incubation at alkaline pH. Electron spin resonance (ESR) spectra showed a temperature-dependent equilibrium, below the main unfolding transition of tropomyosin, between labels which were restricted in their motion (strongly immobilized), predominating at low temperatures, and those which were highly mobile (weakly immobilized), predominating at higher temperatures. These label states were associated with two protein states from a comparison of the ESR spectral changes with the thermal unfolding profile of tropomyosin. The strongly immobilized labels were associated with the completely folded molecule and the weakly immobilized labels with a partially unfolded (in the cysteine-190 region) state which is an intermediate in the thermal unfolding of tropomyosin. A spectral subtraction technique was used to measure the concentration ratio of strongly and weakly immobilized labels from which an equilibrium constant, K, was determined at different temperatures. A linear van't Hoff plot was obtained, indicating that the spin-labeled protein is in thermal equilibrium between these two conformational states with $\Delta H = 17$ kcal/mol, $\Delta S = 56$ cal/(deg·mol), and $K = 1.0$ at 34 °C. An upper limit of 10^7 s^{-1} for the conformational fluctuation was estimated from the shapes and separation of the two ESR spectral components. In contrast to the label with the opened succinimido ring, the spin-label with an intact succinimido ring remained strongly immobilized on the protein, indicating that in the partially unfolded state the molecule retains structure in the cysteine-190 region.

Tropomyosin, a regulatory muscle protein, is composed of two parallel, coiled-coil α-helical polypeptide chains in a rod-shaped structure with a high α-helical content (Caspar et al., 1969). Rabbit skeletal tropomyosin contains two types of chains, α and β, with an α/β ratio of roughly 4/1 (Cummins & Perry, 1973) which are combined to form α-α and α-β tropomyosin molecules (Eisenberg & Kielley, 1974; Yamaguchi et al., 1974; Lehrer, 1975). Both chains have a very similar amino acid sequence of 284 residues, with the α chain having one cysteine residue at position 190 in the C-terminal half of the molecule and the β chain containing cysteine residues at positions 190 and 36 (Mak et al., 1980). The two chains of tropomyosin are in register since they can form an interchain disulfide bond at cysteine-190 (Johnson & Smillie, 1975; Lehrer, 1975; Stewart, 1975).

Although tropomyosin has generally been considered to be a rigid rod, evidence has accumulated which shows that tropomyosin has regions of lower stability which are thought to impart some degree of flexibility to the molecule. The molecule unfolds, with increasing temperature or concentration of denaturant, in two distinct stages (Woods, 1969; Pont & Woods, 1971; Satoh & Mihashi, 1972). The first stage, the pretransition, appears to involve unfolding of the C-terminal half of the molecule, and the second stage, the main transition, involves the complete unfolding of the molecule (Chao & Holtzer, 1975; Woods, 1977; Betcher-Lange & Lehrer, 1978; Lehrer, 1978; Pato & Smillie, 1978; Potekhin & Privalov, 1978). Woods (1976) has described this as an equilibrium between the native molecule, N, a partially unfolded (in the C-terminal half) intermediate state, X, and the completely denatured tropomyosin, D; i.e., $N \rightleftharpoons X \rightleftharpoons D$. Studies of the fluorescence of probes attached to cysteine-190 (Graceffa &

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Lehrer, 1980; Betteridge & Lehrer, 1983), of the NMR of histidine-153 (Edwards & Sykes, 1980), and of differential scanning calorimetry of tropomyosin (Williams & Swenson, 1981; Potekhin & Privalov, 1982) have provided more direct evidence for multistate thermal equilibria. Furthermore, it has been suggested that the X state has its chains separated in the cysteine-190 region (Graceffa & Lehrer, 1980; Lehrer et al., 1981). X-ray crystallography of tropomyosin has also demonstrated a loosening of the structure in the C-terminal half of the molecule with increasing temperature (Phillips et al., 1979).

The electron spin resonance (ESR)[1] spectrum of a nitroxide spin-label is very sensitive to the label's motion and thus possibly to conformational changes in the local environment of a protein to which the label is attached. In the present work, rabbit skeletal tropomyosin was spin-labeled at cysteine-190 with the aim of probing the conformational change involved in the equilibrium between completely folded and partially unfolded tropomyosin (N $\rightleftharpoons$ X). A maleimide spin-label was employed whose succinimido ring either was left intact (type I) or was opened, subsequent to labeling, by incubation at alkaline pH (type II). A similar type II maleimide fluorescence label on tropomyosin was very effective in monitoring this equilibrium (Graceffa & Lehrer, 1980; Lehrer et al., 1981).

Two ESR spectral components were observed for type II spin-labeled tropomyosin, corresponding to strongly and weakly immobilized labels. The strongly and weakly immobilized spin-labels could be associated with the N and X protein states, respectively, since the relative contribution of the two spectral components changed over the temperature range of the pretransition. The fraction of protein in each of these states was quantitated by measuring the fraction of strongly and weakly immobilized labels. Thus, thermodynamic data for N $\rightleftharpoons$ X could be determined. The type II label becomes highly mobile upon proceeding from the N to the X state, indicating a loosening of the structure in the cysteine-190 region of tropomyosin in the X state. However, the cysteine-190 region in the X state retains structure since the type I spin-label remained strongly immobilized on the protein throughout the pretransition. Thus, the partial unfolding of tropomyosin in the X state may involve a limited loosening up over an extended region which includes cysteine-190. Since tropomyosin is in thermal equilibrium between two conformational states of roughly equal concentration in the physiological temperature range, this dynamic fluctuation might have a functional role.

Experimental Procedures

Preparation and Spin-Labeling of Tropomyosin. All procedures were carried out at 4 °C unless otherwise stated. Rabbit skeletal TM was prepared and reduced with dithiothreitol at 37 °C as described by Lehrer (1975), and excess dithiothreitol was removed by low-salt dialysis vs. 2 mM Mops–0.1 mM EDTA, pH 7.5, to maintain TM in the reduced state (Lehrer & Morris, 1982). The sulfhydryl content of reduced TM, determined according to Ellman (1959), was 2.3 ± 0.1 SH/TM.

(*A*) *MSL(I)TM.* TM was labeled with the maleimide nitroxide spin-label 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (MSL) (Syva, Palo Alto, CA) by reacting reduced TM with 4.6 MSL/TM for 15 h at room temperature in 4.5 M GdmCl, in which TM is completely denatured (Pont & Woods, 1971), 20 mM Mes, and 1 mM EDTA, pH 6.0. The MSL was initially dissolved in a small volume of ethanol. MSL(I)TM was then dialyzed vs. 1 M NaCl, 5 mM Mes, and 1 mM EDTA, pH 6.0.

(*B*) *MSL(II)TM.* MSL(I)TM was converted to MSL(II)TM by alkaline incubation for 15 h at room temperature in 4.5 M GdmCl, 40 mM Mops, and 1 mM EDTA, pH 8.5, whereby the succinimido ring is opened either by aminolysis (Wu et al., 1976; Betcher-Lange & Lehrer, 1978) or by hydrolysis (Knight, 1979). MSL(II)TM was then dialyzed vs. 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5.

In order to check for spin-labeling at amino acid residues other than cysteine, TM cysteines were blocked prior to reaction with MSL. TM cysteines were blocked by reacting TM with 20 mM 5,5′-dithiobis(2-nitrobenzoate) in 4.1 M GdmCl, 0.7 M NaCl, 10 mM Mops, and 0.7 mM EDTA, pH 7.85, for 15 h at room temperature and then dialyzing vs. 1 M NaCl, 5 mM Mes, and 1 mM EDTA, pH 6.0. This blocked TM was reacted with MSL as in the preparation of MSL(I)TM.

TM was cross-linked by disulfide formation at cysteine-190 by Cu^{2+}-catalyzed $K_3Fe(CN)_6$ oxidation (Bridgart et al., 1973). TM was incubated at room temperature for 6 h in the presence of 5 mM $K_3Fe(CN)_6$, 1 μM $CuSO_4$, 1 M NaCl, and 5 mM Mops, pH 7.5, and then dialyzed vs. 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5. Sodium dodecyl sulfate gel electrophoresis of this oxidized TM showed only bands corresponding to TM dimers, indicating that the TM was fully cross-linked (Lehrer, 1978).

TM spin-labeled only at cysteine-190 with the MSL(II) label was prepared as follows. TM was blocked at cysteine-36 by reacting disulfide-cross-linked TM with 0.6 *N*-ethylmaleimide/TM in 4 M GdmCl, 0.5 M NaCl, 2.5 mM Mops, and 0.5 mM EDTA, pH 7.5, for 15 h at room temperature. Dithiothreitol (20 mM) was then added to react with excess *N*-ethylmaleimide and to reduce the cross-link at cysteine-190. After 3 h at room temperature, the sample was dialyzed vs. 1 M NaCl, 5 mM Mes, and 1 mM EDTA, pH 6.0. This cysteine-36-blocked TM was then reacted with MSL as in the preparation of MSL(I)TM and then incubated at alkaline pH to open the succinimido ring as in the preparation of MSL(II)TM.

(*C*) *Degree of Labeling.* The concentration of TM was determined from the optical density at 277 nm (Lehrer, 1978), and the concentration of spin-labeled TM was determined by the Lowry method using unlabeled TM as a standard. The concentration of MSL in MSL(I)TM was determined by comparing the amplitude of its ESR signal in the denaturing solvent 4 M GdmCl, 10 mM Mes, and 1 mM EDTA, pH 6.0, to that of a standard solution of MSL(I)TM in the same solvent. Both samples exhibit the same three-line spectrum characteristic of highly mobile labels. The standard solution of MSL(I)TM was prepared, by the same procedure used to prepare MSL(I)TM, by reacting a known weighed quantity of MSL with reduced TM that had a slight excess of SH content over MSL content to ensure complete reaction. A high excess of SH was avoided since it may have reduced the nitroxide to a diamagnetic hydroxylamine (Morrisett & Drott, 1969). The degree of labeling could then be determined from

[1] Abbreviations: ESR, electron spin resonance; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; MSL, 3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy; TM, tropomyosin; MSL(I)TM, TM spin-labeled with MSL; MSL(II)TM, succinimido ring of MSL(I)TM opened by alkaline incubation; W and S, weakly immobilized and strongly immobilized ESR spectral components, respectively; 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.

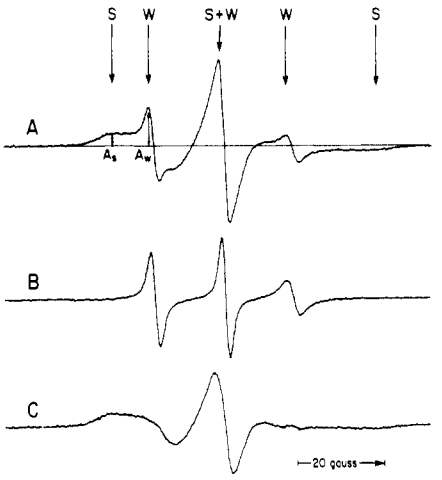

FIGURE 1: Decomposition of ESR spectra into weakly immobilized (W) and strongly immobilized (S) components by the spectral subtraction technique. The arrows indicate the positions of the individual lines for each component. All spectra were recorded at the same power and gain settings. (A) ESR spectrum at 17 °C of MSL(II)TM at 3.3 mg/mL in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5. This spectrum represents a composite of both W and S spectral components. A_s and A_w respectively show the amplitude of the low-field line of the S and W spectra. (B) ESR spectrum at 17 °C of MSL(II)TM at 2.5 mg/mL in 4 M GdmCl, 0.5 M NaCl, 2.5 mM Mops, and 0.5 mM EDTA, pH 7.5. This spectrum consists only of a W spectral component. (C) Spectrum A minus 0.703 spectrum B. This resulting spectrum consists of only an S spectral component.

the protein and label concentrations.

Circular Dichroism. To follow the thermal unfolding of TM's helical structure, the ellipticity at 222 nm was measured on a Cary 60 instrument. The ellipticity values were normalized at 10 °C since at this temperature there was no difference in circular dichroism between labeled and unlabeled TM. The temperature was monitored with a calibrated thermistor probe inserted into the sample.

ESR Spectroscopy. ESR spectra were recorded on a Varian E-109 X-band spectrometer, with a rectangular cavity, operating at 100-kHz field modulation and interfaced with a Nicolet 1074 digital computer. The spectrometer was also equipped with a Varian Dewar and temperature-controlling unit which maintained the temperature to within ±0.5 °C. Temperature was monitored with a calibrated thermistor probe inserted into the sample which was contained in a small flat cell (Wilmad Glass, Buena, NJ). Spectra were run at 2-G modulation amplitude and at a power level of 10 mW or less. In some cases, especially for spectra recorded at higher temperatures, a power level as low as 1 mW was used in order to avoid any power saturation.

Determination of the Fractional Concentration of Weakly Immobilized (C_w) and Strongly Immobilized (C_s) Spin-Labels by Spectral Subtraction. The ESR spectrum of MSL(II)TM in 1 M NaCl (Figure 1A) shows two spectral components corresponding to spin-labels with two different mobilities. The labels with greater mobility are referred to as weakly immobilized (W) and those with more restricted motion as strongly immobilized (S) by convention (Boeyens & McConnell, 1966). [See Figure 1 of Hsia & Piette (1969) and Figure 4 of Jost & Griffith (1978) for ESR spectral shape as a function of spin-label mobility.] The ESR spectrum of MSL(II)TM accurately diluted into 4 M GdmCl (Figure 1B), where tropomyosin is completely denatured (Pont & Woods, 1971), only shows spectral components corresponding to weakly immobilized labels. These two spectra were stored in the digital computer, and increasing fractions of the weakly immobilized spectrum of Figure 1B were subtracted from the composite spectrum of strongly and weakly immobilized components of Figure 1A until, by visual inspection, the smooth strongly immobilized spectrum of Figure 1C was obtained. This technique is very accurate (to within 2%) since one is subtracting a very sharp spectrum from a broad one (Jost & Griffith, 1978). Thus, the spectrum of denatured MSL(II)TM in 4 M GdmCl simulates quite well the weakly immobilized spectrum of MSL(II)TM in 1 M NaCl. From the fraction of the Figure 1B spectrum which was subtracted from the Figure 1A spectrum to obtain the Figure 1C spectrum, one can calculate C_w and thus C_s ($=1 - C_w$) for MSL(II)TM in 1 M NaCl. Since the width and possibly the line shape of the weakly immobilized spectrum of MSL(II)TM in 1 M NaCl and in 4 M GdmCl changed with temperature, it was necessary to subtract the spectrum of denatured MSL(II)TM from the spectrum of MSL(II)TM in 1 M NaCl recorded at the same temperature in order to produce a smooth strongly immobilized spectrum. At each temperature, the ESR spectra of MSL(II)TM in 1 M NaCl and in 4 M GdmCl were recorded at the same power setting. The same ESR cell was used for MSL(II)TM in both solvents and was always oriented identically in the cavity.

A further correction to C_w, and thus to C_s, was necessary since the spectra of MSL(II)TM were recorded in different solvents (1 M NaCl or 4 M GdmCl) in which there may be different amounts of microwave power absorbed with a concomitant difference in signal amplitude. In order to make this correction, MSL was dissolved, at equal concentrations, in both the 1 M NaCl and 4 M GdmCl solvents, and the ESR spectra were recorded as a function of temperature at a 0.5-mW power setting. In both solvents, the ESR spectrum consisted of three narrow lines, which is characteristic of freely tumbling nitroxide spin-labels. The spectral line widths were the same in both solvents whereas the ratio of the spectral amplitude in the 1 M NaCl solvent, A_{na}, to that in the 4 M GdmCl solvent, A_g, increased monotonically from 1.0 at 0 °C to 1.4 at 57 °C. At each temperature, the corrected value for C_w is the value as determined above multiplied by A_g/A_{na} and will be the value used in the remainder of the paper.

Results

Thermal Unfolding of Spin-Labeled Tropomyosin by Circular Dichroism. The helix unfolding profile of MSL(I)TM is very similar to that of reduced tropomyosin whereas the MSL(II) probe perturbs the protein structure somewhat as indicated by a decrease of ellipticity, at temperatures greater than 10 °C (Figure 2). The small loss of structure of MSL(II)TM is due to the presence of the label and not the labeling procedure since reduced tropomyosin put through the labeling procedure in the absence of the spin-label showed no loss of ellipticity. However, the general features of the profile of MSL(II)TM are very similar to those of reduced tropomyosin. As the temperature is increased from 0 °C, there is a moderate loss of ellipticity up to 35 °C, for MSL(II)TM, or up to 40 °C, for MSL(I)TM and reduced tropomyosin, above which there is a sharp drop in ellipticity. The initial change is referred to as the pretransition and the subsequent change as the main unfolding transition.

Temperature Effects on the ESR Spectra of Spin-Labeled Tropomyosin. The ESR spectra of MSL(I)TM and MSL(II)TM show two spectral components (Figures 3 and 4, respectively) corresponding to strongly immobilized (S) and weakly immobilized (W) spin-labels (see Experimental Procedures). The relative contribution of the S and W labels changes with temperature such that the former dominates at low temperatures and the latter at higher temperatures. The

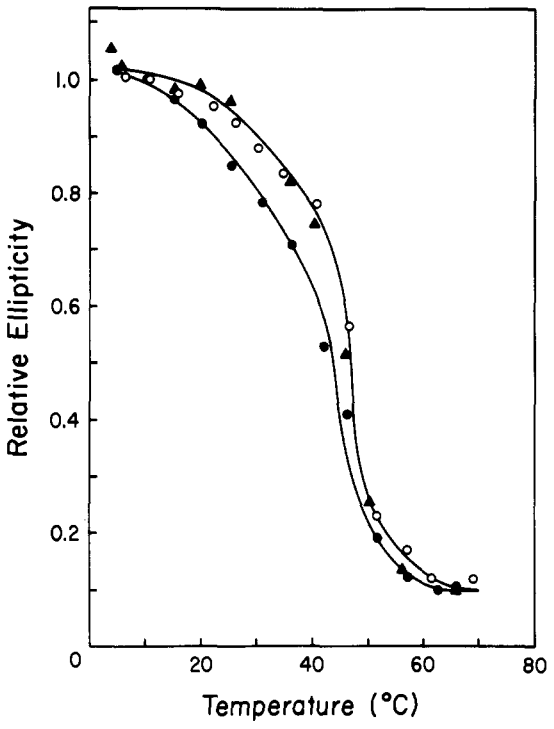

FIGURE 2: Thermal melting profile of reduced TM (○), MSL(I)TM (▲), and MSL(II)TM (●) all at 0.04 mg/mL in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5. The ellipticity at 222 nm is normalized to the value at 10 °C.

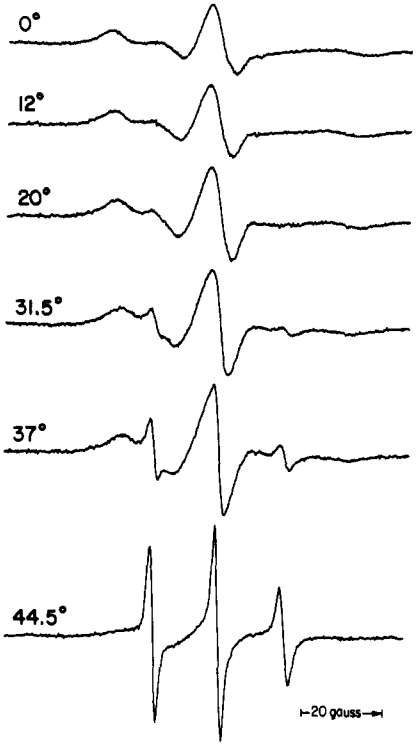

FIGURE 3: Temperature dependence of the ESR spectrum of MSL-(I)TM at 2.5 mg/mL in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5. The gain and power settings are different at each temperature.

temperature effects on the spectra were reversible except when samples were kept at 50 °C or higher for at least 30 min. The temperature dependence of the amplitude ratio, A_w/A_s (determined as shown in Figure 1), of the two spectral components for MSL(I)TM and MSL(II)TM is quite different (Figure 5). For MSL(I)TM, the value of A_w/A_s remained low and did not increase much in the pretransition temperature range. Above 40 °C, a dramatic increase in A_w/A_s conincided with

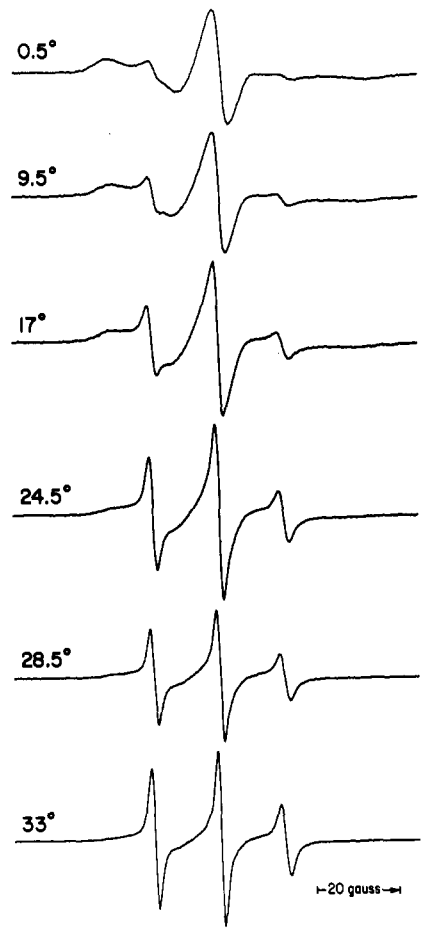

FIGURE 4: Temperature dependence of the ESR spectrum of MSL-(II)TM at 3.3 mg/mL in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5. The gain and power settings are different at each temperature.

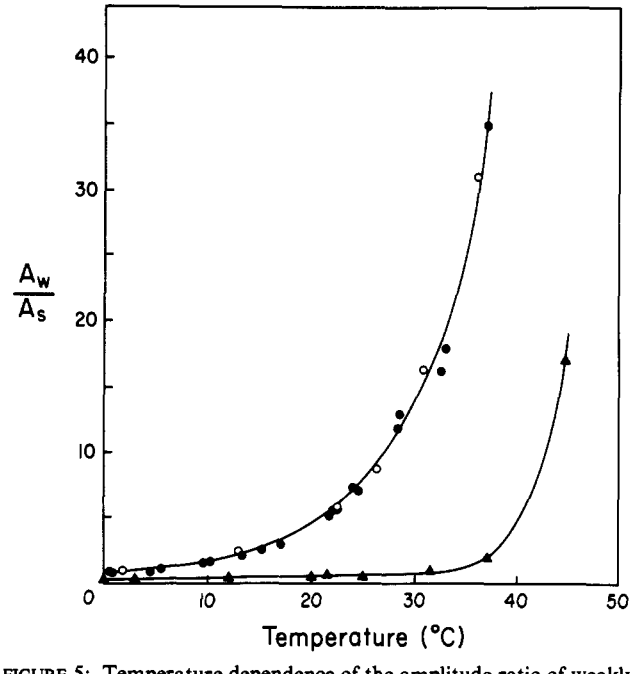

FIGURE 5: Temperature dependence of the amplitude ratio of weakly immobilized to strongly immobilized ESR spectral components, A_w/A_s, for three different preparations of MSL(II)TM (●), TM with *N*-ethylmaleimide at cysteine-36 and MSL(II) at cysteine-190 (○), and MSL(I)TM (▲), all in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5.

the main unfolding transition. Therefore, for MSL(I)TM the strongly immobilized spectrum corresponds to tropomyosin in a native configuration, i.e., N + X states (see the introduction),

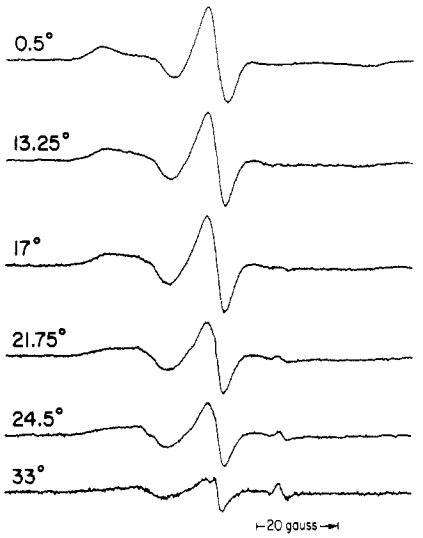

FIGURE 6: Temperature dependence of the strongly immobilized spectral component, obtained by the spectral subtraction technique, of MSL(II)TM in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5.

and the weakly immobilized spectrum corresponds to tropomyosin in the completely unfolded state, D.

For MSL(II)TM, A_w/A_s increases moderately from 0 to 35 °C during the pretransition of the molecule and then increases much more sharply above 35 °C during the main unfolding transition. This suggests that the type II spin-label probes the structural changes in tropomyosin during the pretransition. In order to investigate these changes in more detail and because of limitations in the use of A_w/A_s to estimate the ratio of spin-label in the two states, a different technique was used to obtain the fraction of spin-label in the strongly immobilized and weakly immobilized spin-label states of MSL(II)TM.

Thermal Dependence of the Fraction of Labels in the Two Spin-Label States of MSL(II)TM. Generally, changes in the amplitude ratio A_w/A_s have been used to monitor conformational changes in spin-labeled proteins. However, this has several important uncertainties especially when studies are done at varying temperatures which may affect spectral line widths and line shapes. The concentration of spins is proportional to $k_s A w^2$ where k_s is a constant which depends on the spectral line shape and A and w are, respectively, the amplitude and the width of the line (Poole, 1967). Since, for example, k_s can change by a factor of 3.5 on going from Gaussian to Lorentzian line shapes (Poole, 1967) and since the line width is squared, large errors may result from ignoring these parameters. Furthermore, since the line widths of weakly immobilized spectra are much narrower than those of strongly immobilized spectra, the amplitude ratio distorts the actual relative contribution of the two components. Finally, the overlap between the W and S spectral components (Figure 1) and the small values of A_s frequently encountered can led to an important error in determining A_w/A_s.

We therefore determined the fractional concentration of weakly immobilized, C_w, and strongly immobilized, C_s, spin-labels of MSL(II)TM in 1 M NaCl by a spectral subtraction technique (Experimental Procedures). In this procedure, increasing amounts of the weakly immobilized spectrum, simulated by MSL(II)TM in 4 M GdmCl, were subtracted from the composite spectrum of strongly immobilized and weakly immobilized spectral components of MSL(II)TM in 1 M NaCl to obtain a smooth spectrum representing the strongly immobilized component. C_w could then be determined from the amount of weakly immobilized spectrum subtracted, and C_s

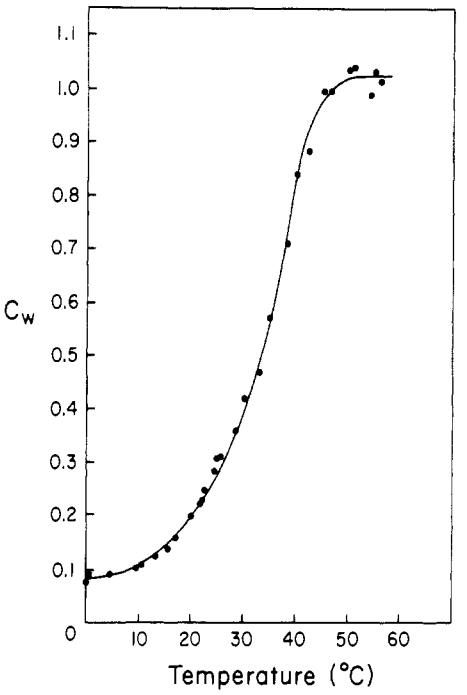

FIGURE 7: Temperature dependence of the fraction of weakly immobilized spin-labels, C_w, for two different preparations of MSL-(II)TM in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5.

was calculated from the relationship $C_s = 1 - C_w$. The ESR spectra of MSL(II)TM in 1 M NaCl at various temperatures are shown in Figure 4, and some resulting strongly immobilized component spectra are shown in Figure 6. The strongly immobilized component spectra are, in general, smooth and typical strongly immobilized spectra (Hsia & Piette, 1969; Jost & Griffith, 1978) as especially seen in the low-field region. As temperature increases, the spectra become less smooth in the middle and high-field regions of the spectrum. This can be explained, for the most part, by a slight difference in the nitrogen splitting constant, i.e., the separation between spectral lines, of the weakly immobilized spectrum in 1 M NaCl as compared to in 4 M GdmCl. Thus, all three lines of the weakly immobilized specta could not be precisely aligned during the subtraction procedure, and we therefore chose to align the low-field lines with the result that this region of the difference spectrum is always smooth.

C_w increased from 0.08 at 0 °C to 0.95 at 43 °C with very little change occurring above this temperature (Figure 7) while only 50% of the helical content of MSL(II)TM was lost over the same temperature range (Figure 2). This behavior indicates that a major part of the change in the ESR spectra with temperature occurs in the pretransition, confirming the conclusions drawn from the temperature dependence of A_w/A_s (Figure 5). Furthermore, support for the validity of the spectral subtraction procedure is given by the fact that C_w levels off close to 1.0 at temperatures greater than 45 °C where only weakly immobilized spectra are observed (Figure 7).

A van't Hoff plot of log (C_w/C_s) vs. $1/T$ (Figure 8) was linear from 11 to 34 °C, in the pretransition temperature range, which indicates that the spin-label at cysteine-190 is in thermal equilibrium between two states, with an equilibrium constant $K = C_w/C_s$. The enthalpy difference, ΔH, between these two states can be calculated from the slope of this plot and together with K yields the entropy difference, ΔS [ΔH = 17 kcal/mol, ΔS = 56 cal/(deg·mol), and at 34 °C, K = 1.0]. The rather large entropy change indicates that the two spin-label states can be associated with two protein states. The strongly immobilized spin-label state can be identified with

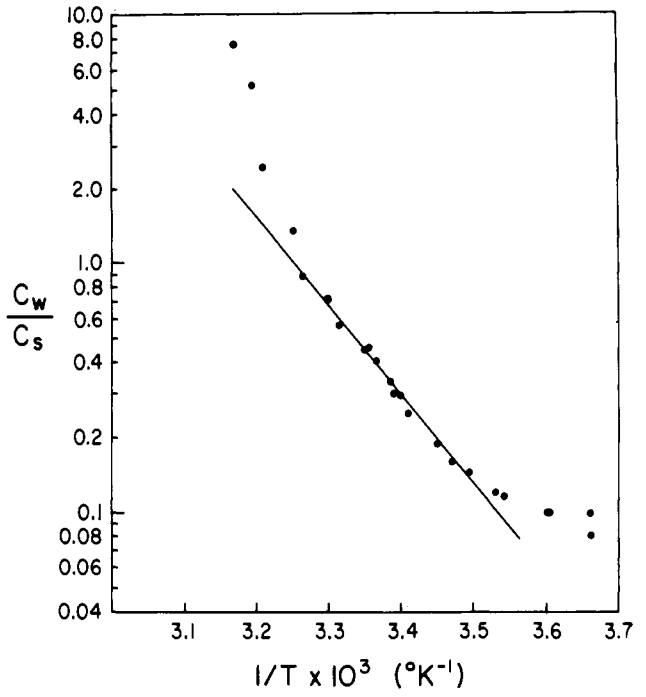

FIGURE 8: van't Hoff plot of the concentration ratio of weakly immobilized to strongly immobilized spin labels, C_w/C_s, for two different preparations of MSL(II)TM in 1 M NaCl, 5 mM Mops, and 1 mM EDTA, pH 7.5. A straight line, from which ΔH is determined, is drawn through the points in the 10–35 °C temperature range.

tropomyosin's completely folded state, N (see the introduction), and the weakly immobilized spin-label state with tropomyosin's partially unfolded (in the cysteine-190 region) state, X.

Above 34 °C, the van't Hoff plot shows a sharp increase in slope which coincides with the onset of the sharp main unfolding transition of the molecule (Figure 2). Thus, above this temperature, the weakly immobilized spectral component corresponds to a mixture of the X and D states of tropomyosin. Below 11 °C, the van't Hoff plot shows a decrease in slope which suggests a transition in tropomyosin structure prior to the pretransition. In this lower temperature range, the mobility of the strongly immobilized spin-labels has become more immobile since the low- and high-field lines have become more sharp and more separated (Shimshick & McConnell, 1972) compared to the 11–35 °C range (Figure 6). Although this also suggests a change in tropomyosin structure prior to the pretransition, a temperature effect on spin-label mobility irrespective of protein structural changes cannot be entirely ruled out below 11 °C.

Characterization of the Specificity of Spin-Labeling. The degree of labeling for several samples of maleimide-spin-labeled tropomyosin, MSL(I)TM, was 2.2 ± 0.2 MSL/TM, essentially equivalent to the SH content of tropomyosin. When MSL was reacted with tropomyosin whose SH groups were blocked with 5,5′-dithiobis(2-nitrobenzoate), the degree of labeling was 0.02, an upper limit since some small fraction of SH groups might not have been blocked. Thus, tropomyosin's SH groups were completely and specifically spin-labeled under the labeling conditions used here, i.e., 4.5 M GdmCl, which maximizes cysteine exposure, and pH 6.0, which minimizes lysine reactivity (Means & Feeney, 1971). These labeling conditions also minimized the amount of the weakly immobilized ESR spectral component for MSL(I)TM (Figure 3) that previous studies (Tonomura et al., 1969; Chao & Holtzer, 1975) observed when labeling was done at pH 7–8. These higher pH values result in considerable label incorporation into lysines, which show only weakly immobilized spectra (Chao & Holtzer, 1975).

Spin labeling of tropomyosin occurs at both cysteine-190 (2.0/TM) and cysteine-36 (0.3/TM). The changes observed in the ESR spectra, discussed above, are primarily reflective of the cysteine-190 labels (87% contribution) since tropomyosin which had *N*-ethylmaleimide at cysteine-36 and the type II spin-label at cysteine-190 showed the same temperature dependence of its ESR spectrum as was found for tropomyosin with spin-labels at both cysteines, i.e., MSL(II)TM (Figure 5).

Discussion

The thermal unfolding of tropomyosin is thought to take place primarily in two distinct stages (see the introduction), resulting in a pretransition prior to the main unfolding transition. This can be represented by N ⇌ X ⇌ D where N is the low-temperature completely folded state, X is a partially unfolded (in the C-terminal half of the molecule) state at intermediate temperatures, and D is the completely unfolded molecule at high temperature. The temperature-dependent ESR spectra of tropomyosin spin-labeled at cysteine-190, located in the C-terminal half of the molecule, are consistent with this scheme. Furthermore, in contrast to previous studies, these spectra allowed us to quantitate the concentration of protein in the N and X states as a function of temperature and thereby determine thermodynamic parameters for N ⇌ X.

Tropomyosin was labeled with a maleimide spin-label whose succinimido ring was subsequently opened, MSL(II)TM. MSL(II)TM was in equilibrium, over the pretransition temperature range, between two states: one, favored at low temperatures, in which the label was restricted in its motion on the protein and another, increasing at higher temperatures, in which the label was highly mobile and thus indicative of some unfolding of the tropomyosin structure in the cysteine-190 region of the molecule. The spin-label in the latter state was as mobile as MSL(II)TM completely denatured in 4 M GdmCl. These two spin-label states were identified with the N and X protein states, respectively, from a comparison of the ESR spectra with the helix melting profiles. From the linear van't Hoff plot of the concentration ratio of these two states, we determined enthalpy and entropy differences for the transition from the N to the X state to be 17 kcal/mol and 56 cal/(deg·mol), respectively. The midpoint of the N ⇌ X transition, i.e., where $K = 1.0$, at 34 °C agrees with the literature values in the range of 30–35 °C for the pretransition of unlabeled tropomyosin under the conditions used in this study (Woods, 1976; Betteridge & Lehrer, 1983). However, some deviation of these thermodynamic values from those for unmodified tropomyosin is to be expected since the spin-label somewhat perturbs the helix unfolding.

The ΔH and ΔS values determined here for the pretransition are about 6% of these parameters for the complete unfolding of tropomyosin at 25 °C (Privalov, 1982). If the partial unfolding in the X state corresponded to a complete unfolding in the cysteine-190 region, it would be expected to involve the complete unfolding of about 6% × 284 residues = 17 residues per chain of tropomyosin. However, the spin-label with an intact succinimido ring on tropomyosin, MSL(I)TM, remained strongly immobilized on tropomyosin throughout the pretransition melting region, suggesting that although there is unfolding in the cysteine-190 region of tropomyosin in the X state this region still maintains a degree of structure. Thus, it is reasonable to conclude that a more extensive region than 17 residues per chain of tropomyosin is involved in the partial unfolding. This is consistent with X-ray studies of tropomyosin

crystals which show a disorder in structure over a considerable portion of the C-terminal half of the molecule (Phillips et al., 1979).

One can estimate an upper limit to the frequency with which tropomyosin oscillates between the N and X states from the ESR spectrum of MSL(II)TM. Since the strongly immobilized and weakly immobilized spectral components are well resolved, the protein must remain in each state for the length of at least one rotation of the spin-label in that state. More precisely, MSL(II)TM must oscillate between these two spin states at a rate which is slower than the inverse of the sum of the rotational correlation times of the label in each of these states. One can get a rough approximation of the rotational correlation times in these states by comparing their spectral shape (Figures 1B and 6) to those of a model spin-label with varying rotational correlation times as shown by Hsia & Piette (1969). This comparison indicates that the rotational correlation time of the strongly immobilized labels is roughtly 50 ns, and that of the weakly immobilized labels is about 3 ns. Thus, the oscillation rate is slower than about 10^7 s^{-1}. A less descriptive but more rigorous calculation of this rate can be made by use of the following equation: rate $<< 2\pi\Delta\nu$, where $\Delta\nu$ is the frequency difference between the low-field lines of the two spectral components. From this equation, rate $<< 2 \times 10^7$ s^{-1}.

In conclusion, tropomyosin is in a dynamic equilibrium between two conformational states of roughly equal concentration close to the physiological temperature range. Thus, this equilibrium could well play a role in tropomyosin's function of regulating muscle contraction, especially since this equilibrium takes place in the C-terminal half of the molecule where troponin binds (Ohtsuki, 1974). The initial events of regulation are triggered by the binding of Ca^{2+} to troponin (Ebashi et al., 1969). The maleimide spin-label with an open succinimido ring appears to be a good label to probe for conformational changes in tropomyosin in reconstituted Ca^{2+}-regulated contractile systems.

Registry No. MSL, 5389-27-5; cysteine, 52-90-4.

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