Spin-Label Studies of Tropomyosin†

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ABSTRACT: Studies are reported on nitroxide spin-labeled tropomyosin. The labels attach to sulfhydryl groups and to amino groups. The amino spins are highly mobile, the sulfhydryl much less so. Spin count studies show an average of ~0.5 labeled sulfhydryl/tropomyosin molecule and only ~0.15 labeled amino group/molecule. The spectra are used to study the denaturation of tropomyosin by guanidine hydrochloride. The information obtained reveals the course of denaturation at sites near the sulfhydryl group. It is found that these sites are more susceptible to guanidine than the bulk of the molecule; denaturation at the sulfhydryl sites is complete by 1.5 *M* guanidine, whereas optical studies indicate the molecule as a whole is not completely denatured until the concentration reaches 3.5 *M*. Spectra are also shown of tropomyosin fibers oriented variously with respect to the applied magnetic field. Strong orientation effects are seen and these indicate that the sulfhydryl-attached spins (but not the amino-attached spins) have a definite orientation in the fiber. Interpretation of the spectra reveals that the normal to the nitroxide plane is inclined to the fiber axis at an angle of 50°. Circular dichroism studies in the tyrosine region also reveal drastic changes with guanidine denaturation, confirming the idea that denaturation produces pronounced increase in mobility at the β carbon (as in the sulfhydryl case). A strong negative band existing only in helical tropomyosin at pH's where the tyrosines are uncharged appears to be due to interaction of tyrosines with the helical backbone, whereas the appearance of a strong positive CD band at 250 nm at high pH (~11) seems to be ascribable to interaction between the charged phenolic groups and the dissymmetric backbone α-carbon atom.

In the denaturation of a protein, a relatively rigid, highly specific conformation of the macromolecule is transformed into a random coil, characterized by a great deal of motional freedom about the covalent bonds of the backbone and perhaps also the side chains. The electron spin resonance (ESR) spectrum of a free radical molecule is extremely sensitive to molecular motion. For that reason, the spin-labeling technique, in which a free radical is bonded to a macromolecule, can be used to probe such conformation changes.

Many physical properties change in protein denaturation and can be used to monitor the process. The spin-labeling technique possesses the advantages of sensitivity to the motion itself, i.e., to the dynamics, rather than to, for example, the mean molecular size and asymmetry, as in viscosity; and of probing *local* motional changes at the site of attachment of the labels rather than motion of the molecule as a whole.

The muscle protein tropomyosin is important not only biologically, because it plays a regulatory role in muscle contraction (Ebashi and Kodama, 1965, 1966), but also structurally, because it is a rod-like, double α helix (Holtzer et al., 1965). Tropomyosin denaturation, therefore, is unusual in that it bears a closer resemblance to the well-described helix–coil transition of synthetic polypeptides (Zimm and Bragg, 1959) than does the denaturation of typical globular proteins. Furthermore, a tropomyosin molecule has only about three sulfhydryl groups (Hodges et al., 1972; Cummins and Perry, 1973) so the possibility exists of spin-labeling it at a very limited number of sites and of studying the local denaturation of those sites. Finally, tropomyosin is a fibrous protein and can be obtained in highly oriented form, which has effects on the spectrum of attached labels that facilitate interpretation of the ESR spectra and provide information on the orientation, if any, of the label. For these reasons we have performed, and report here, nitroxide spin-label studies of the guanidine hydrochloride (Gdn·HCl)[1] denaturation of tropomyosin. Studies of the ESR spectra of some oriented samples are included and some supporting circular dichroism (CD) spectra are reported as well.

As we will encounter several different varieties of ESR spectra, it might be well to review here their origins (Hamilton and McConnell, 1968). A small spin-label molecule, when dissolved in media of low viscosity, undergoes rapid translational and rotational motion which averages the magnetic environment. The result is a spectrum of three narrow lines arising from transitions (between electronic Zeeman levels) for which the nuclear magnetic moment of the nitroxide nitrogen is respectively parallel, perpendicular, and antiparallel to the external magnetic field.

If, on the other hand, a spin-label molecule is specifically oriented and frozen in space, as in a crystal for example, the spin sees one of three unique magnetic environments, depending, again, on the orientation of its nitrogen nuclear spin. Thus, again, three sharp lines would appear, albeit shifted from their positions in the liquid (Libertini and Griffith, 1970). If each spin is, instead, attached to a very slowly moving body, as in a glass, the sample consists of a mixture of spins with various spatial orientations, each orientation contributing three differently shifted sharp lines. The resulting observation is of the sum of all these lines and has the appearance of a "powder" spectrum (Itzkowitz, 1967).

If, finally, the spins are situated on oriented molecules, with some, but limited, freedom, the spectrum will, like the crystal spectrum, consist of three lines but these will not be sharp, and, furthermore, the spectrum will depend upon the

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[1] Abbreviations used are: guanidine hydrochloride (Gdn·HCl); dithiothreitol (DTT); *p*-chloromercuribenzoate (Cl-HgB_2O).

orientation of the sample relative to the applied magnetic field (Jost et al., 1971). In the course of this paper we will be discussing all three types, which we shall refer to for short as loose, tight, and oriented spins.

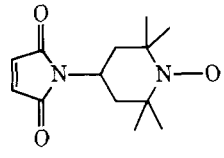
Experimental Methods

Protein Preparation. Tropomyosin was prepared from rabbit skeletal (thigh and back) muscle by the method of Bailey (1948) with slight modifications (Noelken, 1962; Ebashi and Kodama, 1965). Reagents used were as described earlier (Frederiksen and Holtzer, 1968).

Reducing and Blocking of Sulfhydryl Groups. Reduction of oxidized sulfhydryl groups was accomplished by adding a 50-fold (molar basis) excess of dithiothreitol (DTT) to 1% tropomyosin solution in 5 *M* Gdn·HCl at 4°. After 12 hr, the solution was dialyzed exhaustively in a glove bag under nitrogen atmosphere vs. $(Gdn{\cdot}HCl)_{5.0}$ $(KCl)_{0.64}$ $(K[PO_4])_{0.064}$ (7.2).[2]

In some instances it is desirable to block the reduced sulfhydryl groups to prevent them from reacting with the spin-label. This can be accomplished by using *p*-chloromercuribenzoate (Cl-HgB_zO), *N*-ethylmaleimide, or iodoacetamide. To be certain, we usually employed Cl-HgB_zO *and* one of the other two reagents. The molar concentration of each blocking reagent was about 20 times that of the protein. The blocking reaction was allowed to proceed for 15 hr at 4° in a medium containing 5 *M* Gdn·HCl to ensure complete exposure of sulfhydryl groups to the blocking reagent. The solution was then dialyzed *vs.* the solvent of interest to remove excess blocking reagent.

Spin-Labeling. Labeling was performed with *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)maleimide (**1**), which was



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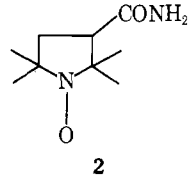
graciously supplied by Professor H. McConnell. In the labeling reaction the hydrogen from an -SH or $-NH_2$ group and the sulfur or nitrogen add across the carbon-carbon double bond of the label (Roberts and Rouser, 1958). Denatured tropomyosin was reduced with DTT and the DTT dialyzed out as described above. Sufficient solid label was added to bring the molar concentration of label to 5–10 times that of the protein and the labeling reaction was allowed to proceed for 8 hr at 25°. Dialysis then followed.

For determination of spin concentration, a primary standard is needed. We used 2,2,5,5-tetramethyl-3-carbamidopyrrolidin-1-oxyl (**2**). This compound was synthesized



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[2] $K[PO_4]$ is a mixture of K_2HPO_4 and KH_2PO_4. The frequent necessity of referring to complex aqueous solvent media makes shorthand notation desirable. We therefore designate such solvents by writing the chemical formula (or name) of each component (omitting water) with its molarity as a subscript, followed by parenthetical specification of the pH (Holtzer et al., 1965).

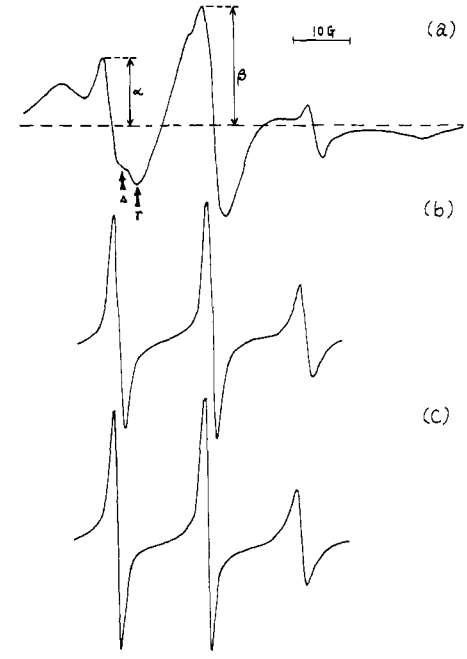

FIGURE 1: (a) ESR spectrum of tropomyosin labeled in the denatured state (i.e., in 5 *M* Gdn·HCl), then renatured by dialysis vs. $(KCl)_{1.0}(K[PO_4])_{0.1}$ (7.3). See Discussion for meaning of Greek leters. (b) ESR spectrum of sulfhydryl-blocked tropomyosin labeled in the denatured state (5 *M* Gdn·HCl), then renatured by dialysis vs. $(KCl)_{1.0}(K[PO_4])_{0.1}$ (7.3). (c) ESR spectrum of tropomyosin labeled and left in the denatured state (4 *M* Gdn·HCl).

from 2,2,5,5-tetramethylpyrrolidine-3-carboxamide following Rozantzev and Krinitzkaya (1965). After recrystallization from hexane–dioxane, the melting point was 172–173°; the reported value is 174°.

Preparation of Tropomyosin Fibers. The method of Caspar et al. (1969) was used. The protein solution was dialyzed against the $(NaHCO_3)_{0.01}(MgCl_2)_{0.005}$ solvent. After centrifugation at 40,000 rpm for 12 hr in the Spinco L-2, the supernatant was discarded and the pellet removed with a spatula. A sharp-tipped glass rod was used to pull the fiber out of the pellet. The fiber was then laid onto the central plate of the Teflon ESR cell (see below) with the fiber axis perpendicular to the cell axis for the actual measurements.

Physical Measurements. ESR experiments were done using the Varian E3 spectrometer. For the oriented fiber studies, flats were machined onto a cylindrical Teflon rod and several fibers laid on the flats with their axes perpendicular to the cell axis. Thus, by changing the angle about the rod axis, the fiber axis could be arranged parallel, perpendicular, or at intermediate angle to the applied magnetic field. In one experiment, the fiber axis was parallel to the cell axis in order to verify that the resulting spectra are independent of orientation about the cell axis. A close-fitting glass tube was used as a cover so the fibers would not dry out during the time spectra were recorded.

Circular dichroism spectra were measured at room temperature with a Durrum-Jasco Model J-20 spectrophotometer.

Results

The Position and Extent of Labeling. The first questions one wishes to answer about a labeled protein are simple but fundamental: where are the labels and how many are on a molecule? Figure 1a shows the ESR spectrum of labeled

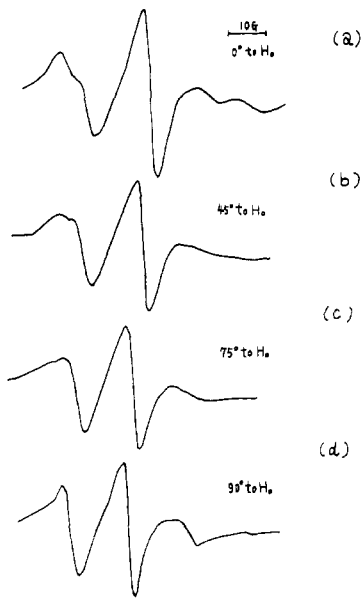

FIGURE 2: ESR spectra of labeled tropomyosin fibers at various orientations. (a) Fiber axis parallel to the external field; (b) fiber axis at 45° to the external field; (c) fiber axis at 75° to the external field; (d) fiber axis perpendicular to the external field.

Table I: Spin Concentration in Labeled Tropomyosin.

Molar spin/molar protein concn during labeling	6	15
Molar protein concn	8.1×10^{-5}	8.3×10^{-5}
Total molar spin concn	5.4×10^{-5}	5.4×10^{-5}
Molar concn of amino labels	1.2×10^{-5}	1.5×10^{-5}
Molar concn of sulfhydryl labels	4.2×10^{-5}	3.9×10^{-5}

tropomyosin to be the sum of two types: loose spins and tight spins. It is well known that maleimide spin-label will attach to both sulfhydryl and amino groups (Griffith and McConnell, 1966). Figure 1b, the spectrum of a tropomyosin that was labeled after the sulfhydryl groups were blocked, shows only loose spins. Clearly, then, in the unblocked protein the loose spins are labeled amino groups while the tight ones are labeled sulfhydryls.

Denaturation alters the spectrum of unblocked tropomyosin. As seen in Figure 1c, only loose spins are apparent, indicating that both sulfhydryl and amino labels are mobile in the randomly coiled molecule.

The spin concentration in the unblocked, denatured protein is relatively easy to determine. In general, the spin concentration of a mobile spin is given by (Swartz et al., 1972)

$$C_{PS} = [(\Delta H_p)^2 \Delta h_p / (\Delta H_2)^2 \Delta h_2] C_2 \qquad (1)$$

wherein C_{PS} is the molar concentration of spins on protein, C_2 the molar concentration of standard spin (i.e., **2**), ΔH_p is the peak-to-peak width of the $M = 1$ band of the protein, Δh_p is the corresponding peak-to-peak height, ΔH_2 and Δh_2 are the corresponding quantities for the $M = 1$ band of the standard spin spectrum. The spin concentration calculated from eq 1 and the spectrum of Figure 1c gives the total concentration of labeled aminos plus labeled sulfhydryls and is given in Table I for two different ratios of spin-label to protein in the labeling process.

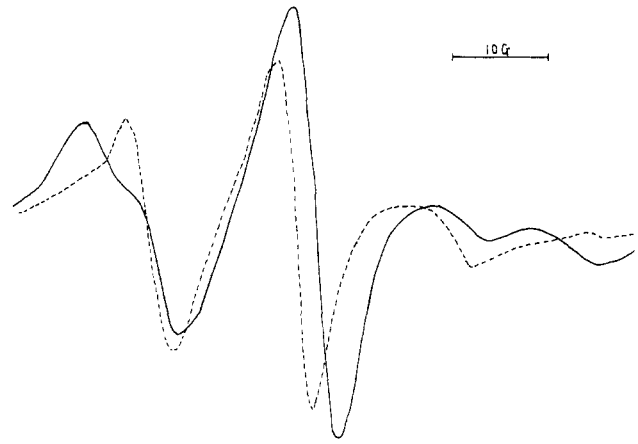

FIGURE 3: ESR spectra of spin-labeled, oriented tropomyosin fibers taken at the same frequency. Spectra are for fiber axis parallel (—) and perpendicular (- - -) to external field.

Estimation of the number of spins on aminos from Figure 1a is somewhat less straightforward since the spectra of loose spins and tight spins overlap. We proceed as follows. The peak-to-peak width of the mobile spins is independent of their concentration and therefore can be determined from the spectrum of sulfhydryl-blocked protein (Figure 1b). The peak-to-peak height can be determined from Figure 1a itself since the first half of the $M = 1$ band of the mobile spins stands relatively clear of the immobile spin spectrum. We therefore took this half-peak height and multiplied by two. Equation 1 is then used as before. These results also appear in Table I. It is apparent from Table I that the extent of labeling is very small; there is an average of a little more than one spin for every two tropomyosin molecules, comprising about 0.5 sulfhydryl label per molecule and 0.15 amino label. Furthermore, somewhat surprisingly, the total incorporation of label is independent of the initial ratio of label to protein; we have no explanation for this puzzling observation.

ESR Spectra of Oriented Samples. ESR spectra of oriented fibers of labeled tropomyosin are shown in Figure 2 for various angles of orientation of the fiber axis with respect to the applied magnetic field. The spectra differ markedly from angle to angle, reflecting pronounced orientation of the spins attached to tropomyosin molecules in the fiber.

Orientation exerts its effects only on the tight (sulfhydryl) spins. We have taken spectra for tropomyosin whose sulfhydryls had been blocked prior to labeling. The spectra for these mobile (amino) spins are independent of orientation of the fiber axis (Chao, 1975). Strong alignment of tropomyosin rods thus does not restrict the motion of the loose spins appreciably.

The ease with which oriented specimens are produced can be appreciated from the results of another experiment in which the tropomyosin pellet was simply stroked onto the ESR cell plate. Definite orientational effects are still observed (Chao, 1975).

In calculations to be presented below, of various quantities for these orientation-dependent spectra, it is desirable to employ spectra taken at exactly the same frequency. We also display, therefore, in Figure 3, spectra for parallel and perpendicular orientations taken at the same frequency.

CD Spectra. Since the sulfhydryl groups are located at the β carbon position, the spin-label studies probe the molecular motion at that point. Since the phenolic groups of

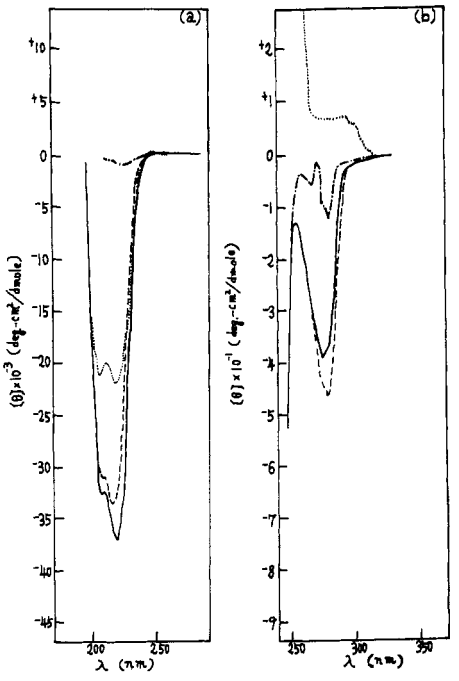

FIGURE 4: CD spectra of tropomyosin in various media. Protein concentration is 0.46%. (---) $(Gdn{\cdot}HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}$ (6.8); (···) $(KCl)_{1.0}$ (11.3); (- - -) $(KCl)_{1.0}$ (6.8); (—) $(KCl)_{1.0}$ (2.3). (a) Backbone region. Each done in 0.1-mm cell. (b) Tyrosine region. Each done in 1-cm cell.

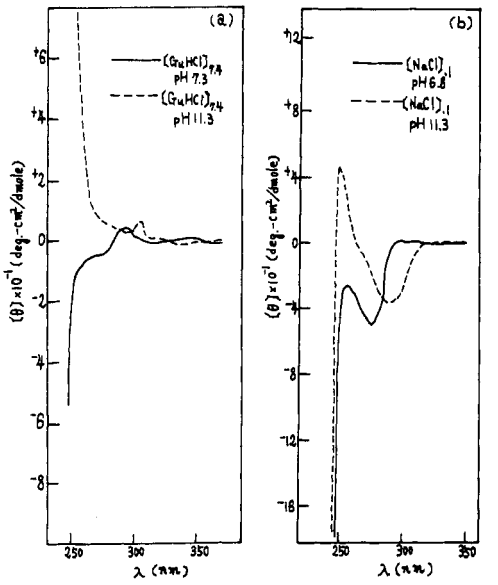

FIGURE 5: CD spectra of the paramyosin tyrosine region. (a) Effect of pH on 0.15% protein in Gdn·HCl. Conditions as marked; 1-cm cell. (b) Effect of pH on 0.15% protein in NaCl. Conditions as marked; 1-cm cell.

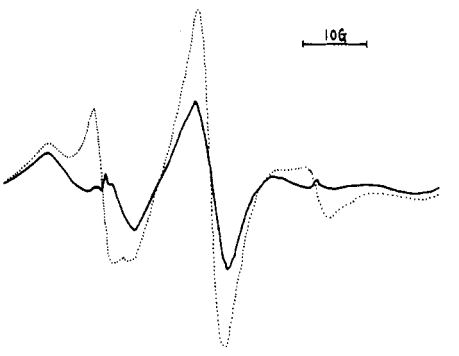

FIGURE 6: Subtraction of the amino-labeled ESR spectrum from the amino- + sulfhydryl-labeled spectrum. (···) Spectrum for both types labeled; (—) the resulting sulfhydryl spectrum. See Discussion for details.

tyrosine residues are also located on the β carbon, it occurred to us that deductions about changes in mobility from spin-label studies might be strengthened by examining the CD band near the absorption of the tyrosines (~277 nm) since this band should also be sensitive to changes in the local dissymmetric molecular conformation.

The results of our CD studies on tropomyosin are given in Figures 4 and 5. Figure 4 displays the effect of Gdn·HCl on the CD spectrum near neutral pH. Figure 4a shows a typical helix backbone double peak which disappears in Gdn·HCl. As can be seen from Figure 4b, native tropomyosin has a strong negative tyrosine band which is greatly diminished in the denaturing solvent.

Figure 4 also shows the effect of pH on the tyrosine band (Figure 4b) and on the much stronger backbone band at 200–230 nm (Figure 4a). As Figure 4a shows, there is a typical helix backbone double peak which decreases steadily in intensity with increasing pH. In the tyrosine region more drastic changes are apparent. As the pH is raised to the region where tyrosines become charged (pH ~11) the negative band near 277 nm disappears and a strong positive band appears in the region of 250 nm. We have also found that addition of Gdn·HCl to the solution at pH 11.3 serves to enhance this positive band (Chao, 1975).

For purposes of comparison, parallel CD studies were carried out on paramyosin, a protein with double α-helical architecture similar to that of tropomysin (Lowey et al., 1963). These data are shown in Figure 5. We find here, too, that charging the tyrosine produces a positive band near 250 nm which is enhanced by addition of Gdn·HCl.

Discussion

Denaturation of Tropomyosin by Gdn·HCl. To follow the denaturation, it is necessary to extract from the spectrum some quantitative measure of the process. It appears from Figure 1a that the leading half of the $M = 1$ band (marked α on the figure) for the loose spins is relatively isolated, whereas the remaining half of this band, marked as Δ on the figure, is strongly overlapped by a contribution from the tight spins (marked γ; we know that Δ is from loose and γ from tight spins rather than the other way round by applying the peak width for mobile spins of Figure 1b to Figure 1a). It thus occurred to us to use α as a measure of the concentration of loose spins.

This isolation of peak α was verified by subtraction of the mobile spin spectrum (Figure 1b) from the spectrum of Figure 1a. Each ordinate of Figure 1b was multiplied by the constant factor required to reduce α to zero when Figure 1b is subtracted from Figure 1a. The resulting subtracted spectrum is shown in Figure 6. The subtracted spectrum indeed resembles closely the spectrum observed from sulfhydryl spins only (see Stone, 1973). Stone reported spectra for heavy meromyosin, which has especially reactive sulfhydryls that can be labeled without appreciable labeling of amino groups.

It is also apparent from Figure 1a that the central peaks for loose and tight spins overlap strongly. Hence we felt we could use β as a measure of the total spin concentration. In the following, then, we use β/α as a monitor of denatura-

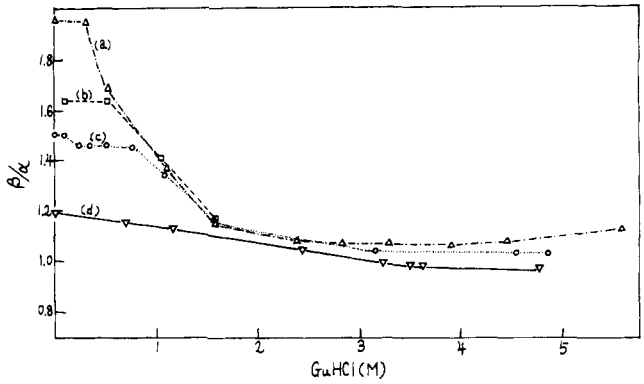

FIGURE 7: The quantity β/α (see Figure 1a) vs. Gdn·HCl concentration. (a) (Δ--) Labeled in $(DTT)_{0.0005}(Gdn{\cdot}HCl)_{5.0}(KCl)_{0.64}(K[PO_4])_{0.064}$ (6.1); (b) (-□-) labeled in $(Gdn{\cdot}HCl)_{5.0}(KCl)_{0.64}K[PO_4])_{0.064}$ (6.1); (c) (O--O) labeled in $(KCl)_{1.0}(K[PO_4])_{0.1}$ (7.3); (d) (∇-∇) all sulfhydryls blocked before labeling.

tion since it measures the ratio of total to mobile spins. This quantity is expected to decrease as denaturation converts immobile to mobile spins. Moreover, rather than reporting on denaturation of the molecule as a whole, this quantity measures the increased mobility of the sulfhydryl spins and therefore monitors conformation at the sulfhydryl positions.

Figure 7 shows β/α as a function of Gdn·HCl concentration for tropomyosin labeled in various ways (see figure legend). The reference for these various curves is curve (d) which is for tropomyosin labeled after all sulfhydryls were blocked. This curve is almost flat, the small negative slope coming from the small decrease of the rotational correlation time of the loose (amino) spins with denaturation. The value of β/α at zero concentration of Gdn·HCl is, as expected, larger the greater the relative number of sulfhydryl spins; thus these points increase in value as we pass from tropomyosin labeled in KCl (curve c) to tropomyosin labeled in Gdn·HCl (curve b) to that labeled in Gdn·HCl + DTT (curve a). With increasing concentration of Gdn·HCl, all curves eventually fall close to the reference curve. The denaturation appears to be over at about 1.5 *M* Gdn·HCl. This is to be compared with other methods of following denaturation that are sensitive to denaturation of the molecule as a whole, e.g., optical rotatory dispersion (ORD). The latter shows that denaturation is not complete until a Gdn·HCl concentration of 3.5 *M* is reached (Noelken and Holtzer, 1964; Noelken, 1962).

The most likely explanation of this is that the region of helix in the vicinity of the sulfhydryl groups is more readily denatured by Gdn·HCl than is the rest of the molecule. The possibility exists, of course, that the presence of the bulky spin-label itself reduces the stability of the local helix. Thus, it is possible that the prior denaturation of the sulfhydryl region might be an artifact of the spin-label technique. This possibility is, of course, an ever present hazard of any labeling technique: that the label is reporting behavior that it itself has induced.

There is still another possible explanation. It could be that there is a side chain order–disorder transition that precedes the backbone transition as the Gdn·HCl concentration is increased. However, we do not think this can occur for the following reason. If we are observing a side-chain transition at 1.5 *M* Gdn·HCl, the main-chain transition, occurring at higher Gdn·HCl, should induce still greater freedom of movement and we should see a second transition. No such is observed. The objection could be raised, however, that the side-chain transition already produces such a great mobility that the main-chain transition cannot produce much more and the second transition is therefore not seen. We do not think this is very likely. We might note also that such a side-chain transition has been implicated in poly(γ-benzyl-L-glutamate) (Yan et al., 1968) where there are large aromatic side chains, but not in any other polypeptide, nor has any such been demonstrated for any protein.

If these alternative explanations are rejected, then our experiments provide further evidence that the tropomyosin denaturation is not of the two-state sort, because the denaturation of the sulfhydryl region does not coincide with that of the molecule as a whole. Prior evidence of this is provided by Pont and Woods (1971), who found (through measurement of molecular weights by sedimentation equilibrium) that separated chains of tropomyosin do not appear until a Gdn·HCl concentration of 2 *M* is reached, although the helix content has already decreased appreciably. We have also found (Chao, 1974) that if the ORD data of Pont and Woods on thermal denaturation are used to plot log K_{app} vs. $1/T$, where K_{app} is the apparent equilibrium constant for the helix to coil transition, the apparent transition enthalpy change derived from the plot shows a maximum when plotted vs. T. This is the classic symptom of a non-two-state transition (Lumry et al., 1966).

ESR Spectra of Oriented Samples. The spectra of Figure 3 can be used in conjunction with the equation $h\nu = \beta g H_{00}$ (Carrington and McLachlan, 1967) to determine values of $g_\parallel$ and $g_\perp$. H_{00} is the magnetic field at the center of the central peak; the other symbols have their usual meaning. In doing so, the values of H_{00} are taken directly from the panel reading. The panel reading for ν, however, is not sufficiently precise; it differs by a constant from the correct ν. We determined the constant by measuring H_{00} for the isotropic spin *tert*-butyl nitroxide and using the known value $g = 2.0059$ (Libertini and Griffith, 1970). In this way, we find $g_\parallel = 2.0046$ and $g_\perp = 2.0059$. The central peak position separation of the two orientations is 2.1 G.

For the amino-labeled spins we found that the hyperfine splittings are independent of the fiber orientation (that splitting is 16.64 ± 0.01 G), and the difference $g_\perp - g_\parallel$ is calculated from the above equation to be less than 0.0004.

If our spectra were only of oriented immobile spins, it would be a relatively straightforward matter to calculate the angle, θ, between the fiber axis and the normal to the plane of the nitroxide groups. We would proceed as follows. The hyperfine splittings (A's) between the centers of, say, the $M = 1$ and $M = 0$ peaks could be obtained for both parallel and perpendicular spectra. These could each be used, in turn, in the relationship (Libertini and Griffith, 1970)

$$A^2 = A_{xx}^2 + (A_{ZZ}^2 - A_{xx}^2)\cos^2\theta \qquad (2)$$

The value of A_{ZZ} could be obtained from the separation of the extreme peaks in the rigid glass spectrum of the spin (Hamilton and McConnell, 1968) and A_{xx} from the relationship (Carrington and McLachlan, 1967)

$$A_{xx} = (1/2)(3a - A_{ZZ}) \qquad (3)$$

with a, the isotropic splitting, obtained from the spectrum of loose spin in solution. The resulting values of θ obtained from $A_\parallel$ and $A_\perp$ could then be averaged to obtain the best value.

Unfortunately, we cannot be this direct because our spectra are contaminated by the spectrum of the loose spins. We therefore adopted the following ruse. Since the loose spin spectrum is independent of orientation, subtraction, at each abscissal value, of the ordinate for the perpendicular spectrum from the ordinate for the parallel spectrum provides curves that are independent of the presence of the mobile spins. The question is, can we determine the hyperfine splitting of the immobile spins from the resulting difference spectra? We think it is indeed possible to determine $A_{\parallel}$ from such graphs because the $M = 1$ and $M = -1$ peaks of the parallel spectrum are relatively isolated. This is not possible for the peaks for perpendicular fibers, which are located closer to the middle of the spectrum and are obscured by the subtraction. In order to make sure that this apparent value of $A_{\parallel}$ is valid we performed a similar subtraction for a lecithin system from the data of Jost et al. (1971), where there are no loose spins, to see if the value known to be correct is found in that case. It checks out very well. In order to be sure that the resulting values of $A_{\parallel}$ are not overly affected by the central peak separation between the two orientations, apparent $A_{\parallel}$ values were calculated for several such separations; the resulting values are insensitive to ΔH_{00}. The subtracted spectra for the lecithin system and for our own system at several chosen ΔH_{00} values are shown in Figure 8. The value of $2A_{\parallel}$ is marked as the double-headed arrow on Figure 8a. As is seen from the figure, after subtraction our spectra bear a marked resemblance to that of the lecithin case (Figure 8a).

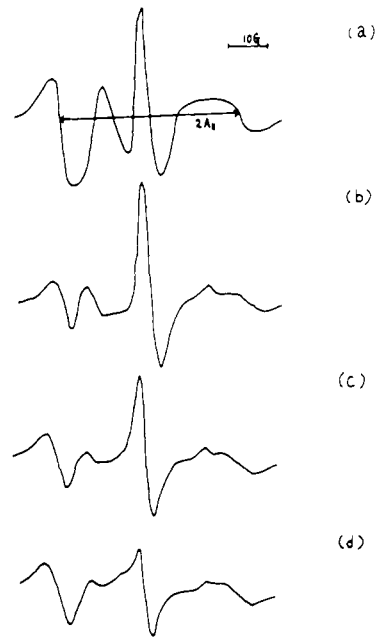

FIGURE 8: Subtracted ESR spectra. Parallel fiber spectra minus perpendicular fiber spectra. (a) The lecithin system, Jost et al. (1971). The quantity $2A_{\parallel}$ is marked on the figure. See text for details. (b) The tropomyosin system, Figure 3, but with a central peak separation of 2.3 G. (c) The tropomyosin system, Figure 3, but with a central peak separation of 1.2 G. (d) The tropomyosin system, Figure 3, but with a central peak separation of 0.8 G.

Using the resulting value of $A_{\parallel}$ (23.4 G) in eq 2 with values of A_{ZZ} determined from a rigid glass spectrum of labeled tropomyosin and A_{xx} from eq 3, with $a = 16.64$ G (as gotten from the spectrum of the loose, i.e. amino, spins) we find a value of $\theta \simeq 50°$.

It is apparent, then, that the sulfhydryl groups have a quite specific orientation with respect to the protein fiber axis. This observation bears on the hypothesis that cysteine's occurrence may lead to local disruption of the regularity of the coiled-coil (Hodges et al., 1972). Our experiments insist that if such disruption occurs, the resulting perturbed structure cannot be disordered but must be relatively rigid. The most conservative interpretation of the results is that the sulfhydryls are on intact α-helical coiled-coil segments; i.e., that no disruption occurs.

CD Spectra. From our ESR studies, it is clear that there is a large change in the mobility of the sulfhydryls in denaturation. Since the phenolic groups of tyrosine residues are also located on β carbons we would expect an equally evident change in mobility. The tyrosine residue CD band (~277 nm) depends very critically on the local dissymmetric molecular conformation. Denaturation should then exert a large effect on this band. Our observations give independent evidence of the correctness of this view and also, we will see, allow us to draw tentative conclusions concerning the nature of the interactions responsible for the aromatic side-chain contribution to the rotational strength.

Figure 4 clearly demonstrates the change in the tyrosine CD band with addition of Gdn·HCl and confirms the idea that motional changes occur at the β carbons. The tropomyosin helix is also sensitive to pH, being successively less stable as pH changes from 2 to 7 to 11 (Noelken, 1962; Noelken and Holtzer, 1964; Lowey, 1965). This is readily apparent from examination of the backbone CD bands (near 215 nm) which show a steady decrease in the characteristic α-helix double peak with increasing pH (Figure 4a). At the same time, there are drastic changes in the tyrosine band (Figure 4b); in particular, the appearance of a strong positive band at pH 11 clearly is due to the charging of the phenolic groups.

We might pose the question whether the negative band at lower pH and the positive band at pH 11 are caused primarily by the interaction of the tyrosine with the dissymmetric constellation of neighboring side chains, with the dissymmetric helix backbone, or with the dissymmetric α carbon of the backbone. As already noted above, addition of Gdn·HCl at pH 11 greatly augments the positive band (Chao, 1975) while it destroys helix. We therefore consider it most likely that this band arises because of interaction of the charged tyrosine with the dissymmetric backbone α carbons.

We can obtain further evidence on this point by an examination of the CD spectra of paramyosin under similar conditions. Paramyosin has the same general double α-helical architecture as tropomyosin, but there is no reason to expect great similarity in their amino acid sequence; indeed, the amino acid compositions differ considerably (Lowey, 1965). Consequently, we expect the array of side chains near a given tyrosine to be quite different in the two proteins. In spite of that, as Figure 5 makes clear, the behavior of the tyrosine CD band in paramyosin is very similar to that in tropomyosin. The strong positive band appears at 250 nm at pH 11 and is augmented by addition of Gdn·HCl. This, again then, indicates that this band does not originate from the influence of the dissymmetric constellation of side chains about the tyrosine, but from interaction of the charged phenolic groups with the backbone α carbons.

Similarly, the disappearance of the negative band with addition of Gdn·HCl at lower pH in both proteins suggests that this band is due to interaction of the uncharged phenolic group with the helical backbone.

There have been many studies of the tyrosine optical activity in polypeptides and proteins (Beychok and Fasman, 1964; Chen and Woody, 1971; Goodman et al., 1968), but there is still no firm theory that explains the results fully. In that connection, these observations implicating, albeit tentatively, the dissymmetric α carbon in one case and the helix backbone in another may be of interest.

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