# Nuclear Magnetic Resonance Evidence for the Coexistence of Several Conformational States of Rabbit Cardiac and Skeletal Tropomyosins†

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ABSTRACT: Tropomyosin, a rodlike molecule (420 × 20 Å) containing two helical chains in a parallel, coiled-coil structure, plays a pivotal role in the regulation of muscle contraction in mammals. The NMR resonances at 270 MHz from the two histidine residues of rabbit skeletal and cardiac tropomyosins have been analyzed in detail to explain the anomalous splitting of the histidine-153 resonance which was observed during the pH titrations reported in a previous paper [Edwards, B. F. P., & Sykes, B. D. (1978) *Biochemistry 17*, 684]. The splitting derives from conformational differences in the local structure about the histidine residues which produce different p$K_a$ values. These conformations are in slow exchange with one another. The temperatures at which the splitting can be observed correlate with the relative thermal stability of the various tropomyosins studied: reduced, cross-linked, carboxymethylated, or carboxyamidomethylated $\alpha,\alpha'$-tropomyosin and reduced or carboxymethylated $\beta,\beta'$-tropomyosin. The circular dichroism at 222 nm was used to monitor the thermal unfolding of the coiled coil. In general, reduced $\beta,\beta'$-tropomyosin is less stable than reduced $\alpha,\alpha'$-tropomyosin. In particular, carboxymethylation or cystine formation at cysteine-190 lowers the stability of the coiled coil and the temperature at which the histidine-153 resonance splits. Since histidine-276, which is 183 Å from histidine-153, also exhibits several coexisting p$K_a$ values, these local conformational differences probably extend over most of the molecule and are intermediates in the thermal unfolding of tropomysosin.

The contraction of mammalian skeletal and cardiac muscle is regulated by the interaction of calcium ions with the proteins of the thin ("I") filament: troponin, tropomyosin, and actin. The tropomyosin molecules have a pivotal role in this system. They are thin rods ~20 Å wide and ~420 Å long (Caspar et al., 1969) which overlap one another to form a continuous filament (Wakabayashi et al., 1975) along each groove of the actin double helix. Each tropomyosin molecule binds one troponin molecule and spans seven actin molecules in such a way as to inhibit their tension-generating interaction with the myosin heads. When calcium ions saturate the troponin molecules, a perturbation is generated that causes the associated tropomyosin molecules to release this inhibition. For reviews of the pertinent structures, mechanisms, and references, consult Cohen (1975) and Smillie (1976).

Using nuclear magnetic resonance at 270 MHz, we have studied solutions of purified tropomyosin with the goal of elucidating the intrinsic properties of its unusual rodlike structure which might relate to its function. When depolymerized by salt (1 M KCl) or by extreme pH, tropomyosin behaves as a thin rod of 66 000 molecular weight (Woods, 1967). In solutions of low ionic strength and neutral pH, the protein polymerizes indefinitely by end to end overlap in a fully reversible manner. The recently published X-ray crystallographic structure (Phillips et al., 1979) was limited to a resolution of 20 Å by the crystal form which contained only 5% protein. Fortunately, the structure of tropomyosin has been adequately detailed by physicochemical studies. It is a coiled coil of two helical polypeptide chains (Crick, 1953), it is almost completely helical, as measured by optical rotary dipersion (Cohen & Szent-Gyorgyi, 1957), the two chains are parallel (Caspar et al., 1969) and in register (Stewart, 1975; Johnson & Smillie, 1975; Lehrer, 1975), and, finally, the amino acid sequences of the two chain isomers found in rabbit tropomyosin, designated $\alpha$ and $\beta$, are both known (Stone & Smillie, 1978; Mak et al., 1979). Both chains have 284 residues and exhibit the expected regularities of the coiled-coil structure which assigns each residue to a specific position of the repeating heptet [see Figure 1 of Edwards & Sykes (1978)].

In previous publications we have discussed the NMR spectra of the tyrosine residues (Edwards & Sykes, 1977; Edwards et al., 1977) and of the histidine residues (Edwards & Sykes, 1978). In the latter paper we assigned the resonances of histidines-153 and -276 and analyzed their titration data. At that time we reported two unusual features of the resonances from histidine-153, their pH titration curve was cooperative and they broadened into a plateau of resonances near the midpoint, but we did not explain these anomalies. This paper analyzes the broadening in detail; the cooperativity will be discussed in a later publication (B.F.P. Edwards and B. D. Sykes, unpublished experiments).

## Experimental Methods

*NMR Procedures.* The $^1$H NMR spectra displayed in this paper were taken in the Fourier mode at 270 MHz on a Bruker HXS 270 spectrometer equipped with quadrature detection. Typical instrument settings were the following: pulse length, 8 $\mu$s (76°); acquisition time, 0.5 s; sweep width, ±2000 Hz; spectrum size, 4096 data points; line broadening, 1 Hz. The Bessel filters were set to 4000 Hz. The free induction decays were collected in blocks of 250 and then were summed in double precision on a magnetic disk using Nicolet software. An adequate spectrum of the histidine resonances required 3000–6000 transients. The HDO resonance was suppressed with homonuclear decoupling. Chemical shifts were measured relative to the major resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

*Sample Preparation.* The NMR samples were prepared and analyzed as reported previously (Edwards & Sykes, 1978). All pH values reported are uncorrected for $D_2O$ and were measured at room temperature, 24 °C. The samples for the spectra in this paper contained ~20 mg/mL protein in a

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standard $D_2O$ buffer of 40 mM $K_2HPO_4$, 1 mM EDTA, 1 M KCl, and 10 mM DTE. The air above the sample solutions was routinely replaced with argon. For some samples the reducing agent was varied or omitted; these cases are specified in the text.

*Nomenclature.* Although tropomyosin has two chains, we have assumed the diad in this paper and refer only to one residue of a pair. When required, a prime denotes the second residue or chain. For clarity, we have abbreviated the various tropomyosins, whose preparations are described below, as follows: sTM, skeletal tropomyosin, a mixture of $\alpha\alpha'$ and $\alpha\beta'$ species; $\alpha$TM-SH, reduced $\alpha,\alpha'$-tropomyosin; $\alpha$TM-CM, carboxymethylated $\alpha,\alpha'$-tropomyosin; $\alpha$TM-CA, carboxyamidomethylated $\alpha,\alpha'$-tropomyosin; $\alpha$TM-NB, $\alpha,\alpha'$-tropomyosin cross-linked with 5,5′-dithiobis(2-nitrobenzoate); $\alpha$TM-RD, $\alpha$TM-NB reduced with DTE; $\alpha$TM-OX, $\alpha,\alpha'$-tropomyosin cross-linked as an NMR sample by air oxidation at 34 °C; $\beta$TM-SH, reduced $\beta,\beta'$-tropomyosin; $\beta$TM-CM, carboxymethylated $\beta,\beta'$-tropomyosin.

*Tropomyosin Preparations.* The tropomyosin in rabbit skeletal muscle is heterogeneous; its coiled coils contain either $\alpha\alpha$ or $\alpha\beta$ chains. Because the two isomers cannot be separated without denaturing the coiled-coil structure, we prepared pure $\alpha,\alpha'$-tropomyosin from rabbit cardiac muscle which is known to be homogeneous (Cummins & Perry, 1973; Mak et al., 1979).

Our procedure, which is a modification of the original method (Bailey, 1948), was adopted from Dr. Smillie's laboratory. One kilogram of thawed rabbit hearts (mature New Zealand White, type I, from Pel-Freeze) was shredded in a meat grinder whose metal parts were stainless steel. An acetone powder was prepared from this mince by the following nine extractions which, except for the first one, were of ~3-min duration: 1 L of water for 20 min, 1 L of 95% ethanol, 4 L of 50% ethanol (thrice), 4 L of 95% ethanol (twice), and 4 L of acetone (twice). After each extraction the muscle fiber was recollected by pouring the suspension onto a large square of triply layered cheesecloth which covered a Buchner funnel (~26 cm across). When the free liquid had passed through it, the cheesecloth was gathered up into a ball and the remaining liquid was squeezed out by hand using rubber gloves. The solutions were prechilled to 4 °C, and all steps except the last one were executed in the cold room; the acetone extractions were done quickly with chilled solvent in a fume hood. The acetone powder, ~130 g, was air-dried on filter paper and stored in a refrigerator.

The preparation was continued by extracting 120 to 140 g of muscle powder in 1 L of solution A (0.5 mM DTE and 1 M KCl) for 16 h at room temperature. The suspension was stirred with a glass rod, and 1 M NaOH was added until the pH stabilized at 7.0. The next day the supernatant was extracted by using cheesecloth and 600 mL of fresh solution A was added. After being allowed to stand for 1 h, this solution was squeezed out also and pooled to give ~200 mL of extracted protein. From this point the steps were done at 4 °C. The protein was precipitated at pH 4.6 with HCl. After being stirred for 30 min, it was collected by centrifugation at 6000 rpm for 20 min. The precipitate was redissolved with stirring in 1 L of solution A for 30 min and then cleared by centrifugation at 8000 rpm for 30 min. This isoelectric precipitation of the tropomyosin was repeated 2 more times. In the third precipitation the pH was adjusted to 4.3 and the centrifuged precipitate was redissolved in 1 L of solution B (0.5 mM DTE, pH 7.0). In the later preparations 1 mM EDTA was included in solutions A and B to improve the separation of tropomyosin from troponin (Nagy, 1977). Solid ammonium sulfate was added slowly to achieve 53% saturation at 4 °C. When all the salt had dissolved, the precipitate was collected at 8000 rpm for 30 min and discarded. The ammonium sulfate concentration was then raised to 65% saturation, and the precipitated tropomyosin was collected. It was redissolved in ~200 mL of solution B and dialyzed exhaustively against a mercaptoethanol solution (~6 mM) before being lyophilized. The yield was greater than 800 mg.

The $\beta,\beta'$-tropomyosin was a gift from Dr. Mak in Dr. Smillie's laboratory. Heterogeneous tropomyosin was prepared from rabbit skeletal muscle as above, and the $\beta$ chains were purified by the procedure of Cummins & Perry (1973).

*Carboxymethylation.* Some cardiac $\alpha,\alpha'$-tropomyosin was carboxymethylated or carboxyamidomethylated by the method of Crestfield et al. (1963) using the appropiate reagent which had been recrystallized from carbon tetrachloride. This procedure, which uses an equimolar ratio of reagent to free sulfhydryl in 6 M urea at room temperature for 15 min, was chosen for its mildness and specificity. We also received a large gift of $\alpha$TM-CM and $\beta$TM-CM from Dr. Mak. These samples had been carboxymethylated together as sTM before they were separated. Since Dr. Mak had treated them with a 10-fold excess of iodoacetic acid in 6 M urea at room temperature for 30 min, there was a possibility of methionine modification (Wong et al., 1979). However, the assay of Neuman et al. (1962) indicated that 5% or less of the total methionine had been modified. The two preparations of $\alpha$TM-CM were indistinguishable in the experiments reported here.

We often used carboxymethylation to assay the cysteines of NMR samples of tropomyosin. In these cases 1 mL of 0.5 M Tris, 4 mM EDTA, and 5 M guanidine hydrochloride, pH 8.1, in a disposable plastic culture tube was degassed with argon, solid iodoacetic acid was added in an amount that ensured a 2 times molar excess over all sulfhydryls in the aliquot of the sample, the aliquot itself, containing 2–4 mg of protein, was added quickly afterwards, argon was blown on top, and the tube was sealed. After the reaction had proceeded (in the dark) for at least 30 min, an excess of mercaptoethanol was added and the sample was dialyzed against distilled water in the cold room before being lyophilized and subsequently hydrolyzed for amino acid analysis.

*Cross-Linked Tropomyosin.* Reduced $\alpha,\alpha'$-tropomyosin was cross-linked at cysteine-190 and -190′ by using Ellman's reagent. We followed the procedure of Lehrer (1978) but used a Sephadex G-25 column to remove excess reagent and by-products.

*Amino Acid Analysis.* The acid hydrolyses were done at 110 °C for 24–36 h with constant boiling HCl containing 0.1% phenol. When a tropomyosin concentration was being determined, a known amount of norleucine was added to the original sample before lyophilization, hydrolysis, and analysis on either a Durrum D500 or a Beckman 120C amino acid analyzer. The values for aspartic acid, alanine, and leucine were used to calculate the protein concentration. Corrections were made for sample losses and color yields by using the norleucine value and analyses of standard solutions which were run concomitantly. The color production (area) for norleucine was taken to be the same as or 97.1% of that of leucine on the Beckman and Durrum instruments, respectively.

(Carboxymethyl)cysteine was determined on the Beckman 120c analyzer by running both a concentrated and a 10 times diluted sample. Its color value was assumed to be 92% that of aspartic acid.

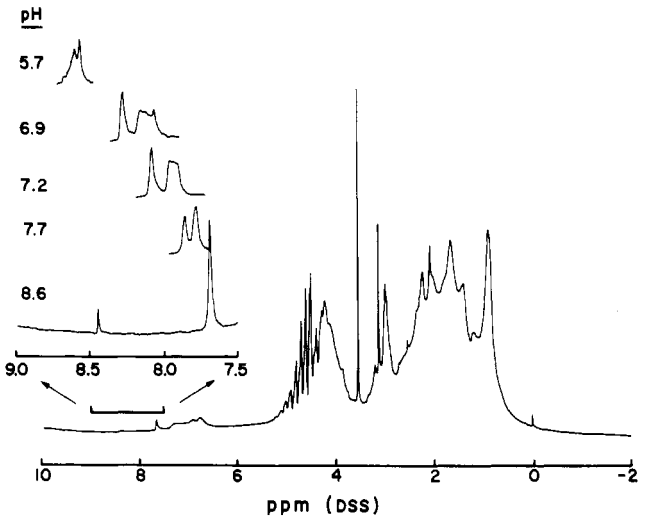


FIGURE 1: Complete spectrum of αTM-CM in the standard $D_2O$ buffer at 34 °C and pH 8.6 is shown along the bottom. The two sharp resonances at 3.12 and 3.54 ppm are from extra EDTA which was carried over from the preparation of the protein. Insert: the region of the histidine C2 resonances has been expanded to show their behavior during a pH titration. The upfield (toward lesser ppm) resonance which is square shaped at pH 6.9 and at pH 7.2 has been assigned to histidine-153. The adjacent downfield (toward greater ppm) resonance which remains sharp has been assigned to histidine-276. The resonance at 8.45 ppm comes from formate ion.

*Analysis for Methionine Sulfoxide.* The procedure outlined by Glazer et al. (1975) was followed without modification.

*Polyacrylamide Gel Electrophoresis.* Tropomyosin samples were analyzed on 8% acrylamide slab gels (Bio-Rad 220) in 1% sodium dodecyl sulfate, 6 M urea, and 0.2 M sodium phosphate, pH 7.0. The samples were normally preincubated for 30 min at 60 °C with a large excess of mercaptoethanol before they were applied to the gel. However, when we assayed for cystine cross-links, the reducing agent was omitted or replaced by an excess of iodoacetic acid or *N*-ethylmaleimide.

Strips from the gels were scanned at 590 nm by using a Gilford spectrophotometer equipped with a gel scanning accessory. The relative areas of the cross-linked (66 000 daltons) and single-chain species (33 000 daltons) were measured with a Keuffel & Esser planimeter.

*Circular Dichroism Measurements.* The circular dichroism of the various tropomyosins was measured on a Cary 60 spectropolarimeter with a Cary 6001 attachment. The samples were dialyzed overnight against a buffer of 50 mM potassium phosphate, 1 mM EDTA, and 10 mM DTE, pH 7.0, and were centrifuged before use. The protein concentrations, measured by amino acid analysis, ranged from 0.4 to 1.5 mg/mL. The DTE was omitted from the cross-linked samples to prevent the reduction of their cystine. Because the ellipticities, $[\theta]_{222}$, of a random coil and of a helix are +1580 and −39 500 deg cm$^2$ dmol$^{-1}$, respectively (Chen et al., 1974), the fractional ellipticities [($[\theta]_{222}$ at $x$ °C)/($[\theta]_{222}$ at 10 °C)] have been converted to approximate percentages of helix by adding 0.04, dividing by 1.04, and converting to a percentage.

*Thermal Denaturations.* The relative thermal stabilities of the tropomyosins were determined by heating samples slowly in the spectropolarimeter and following their loss of negative ellipticity at 222 nm. A complete scan from 255 to 190 nm was taken at 10 °C; then a measurement at 222 nm was taken for increments of 2 °C with a 10-min wait between points; when the ellipticity no longer changed, a second complete scan was recorded; a final scan was taken at 27 °C after a wait of 30 min to verify reversibility. The final temperature, which depended on the stability of the particular tropomyosin, ranged from 61 to 75 °C.

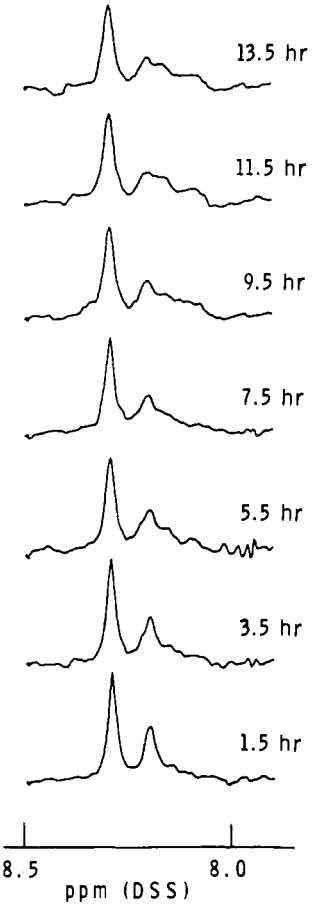


FIGURE 2: These spectra show the effect on the histidine C2 resonances of air oxidation at 34 °C of a sample of αTM-SH in the standard $D_2O$ buffer without DTE and at pH 6.9. The first spectrum was begun 1.5 h after the buffer had been added to the lyophilized sample at room temperature. Each spectrum was collected for 50 min followed by a 70-min wait.

## Results

The expanded spectra of the histidine resonances in Figure 1 illustrate the observation that we seek to explain. In the earlier work we had reported that the resonances of histidine-153 in α,α′-tropomyosin (reduced, partially oxidized, or carboxymethylated) and β,β′-tropomyosin (carboxymethylated) split into a broad envelope of resonances near the midpoint of the titration but were narrow and superimposed at both ends. In 1 M KCl, this broadening was unaffected by varying the concentration of tropomyosin from 10 to 30 mg/mL.

Because the carboxymethylated protein and the native protein, with what we thought to be sufficient DTE, gave a similar broadening of the histidine-153 resonances, we initially discounted the state of cysteine-190 as a possible cause of this phenomenon. However, the spectra of Figure 2 show that cross-linking the cysteines broadens the resonances of histidine-153. The sample was prepared from lyophilized tropomyosin which was 73% reduced by carboxymethylation analysis. After the final spectrum, it was 16% reduced by the same assay and had less than 5% methionine sulfoxide by alkaline hydrolysis. A spectrum of αTM-NB (not shown) under the same conditions resembled the final spectrum of Figure 2.

This experiment was repeated and extended with an identical sample; spectra 1a and 1b of Figure 3 show the histidine

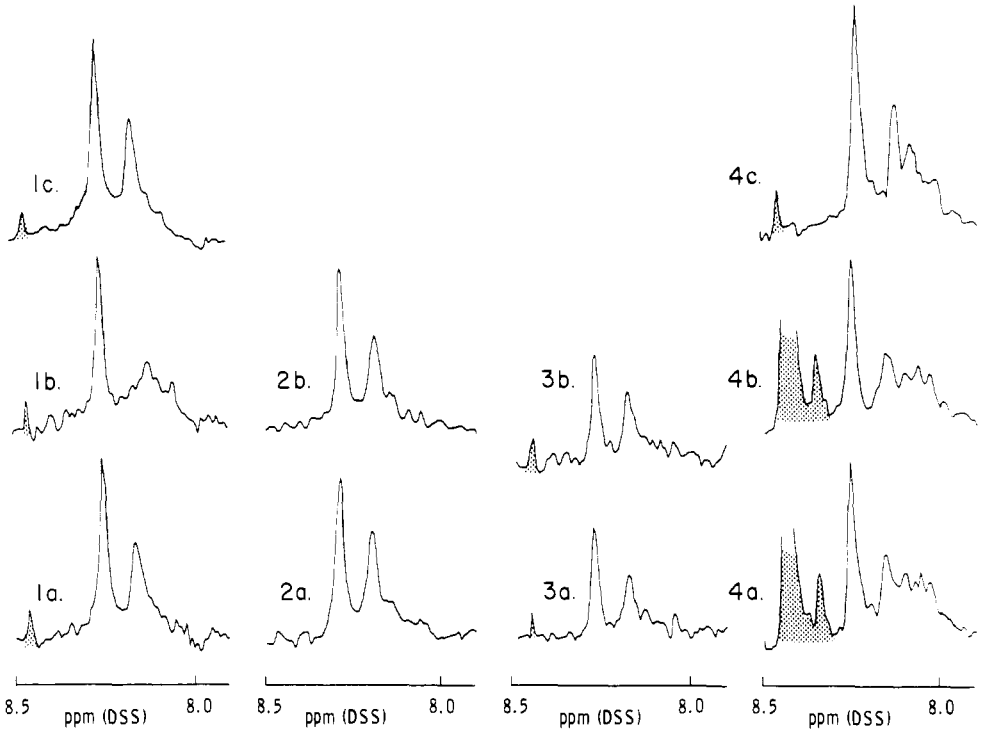


FIGURE 3: Histidine C2 resonances of various tropomyosins are compared at 34 °C and pH 6.9. In each spectrum the resonances, in order of increasing ppm, are from histidine-153, histidine-276, and formate ion. The resonance and spinning sideband, near 8.45 ppm, from formate ion are shaded out. The initial samples were in the standard $D_2O$ buffer with the following qualifications: (1) αTM-SH with no DTE and air above the sample; (2) αTM-SH with 10 mM DTE and argon above the sample; (3) αTM-CA with no DTE and air above the sample; (4) αTM-CM with no DTE and air above the sample. For each sample, (a) is the initial spectrum, (b) is the spectrum after at least 12 h at 34 °C, and (c) is the spectrum after the addition of solid DTE to 20 mM and a wait of at least 16 h at 34 °C under argon. Spectrum 4c is from a second sample of αTM-CM with less formate.

resonances at the beginning and end of 15 h of air oxidation in the spectrophotometer. After spectrum 1b was taken, part of the sample was assayed for free cysteine by carboxymethylation and for un-cross-linked protein by polyacrylamide gel electrophoresis. The assays gave 17 and 30%, respectively. We then made the sample 20 mM in dithioerythritol, covered it with argon, and let it reduce at 34 °C for 4 h. Spectrum 1c shows that reduction of the cystine reversed the broadening of the histidine resonances. By the same two assays the protein was 100% reduced and un-cross-linked. Spectra 2a and 2b of Figure 3 show that reducing conditions will keep the resonances narrow over 12 h at 34 °C.

It is a common procedure to preserve the "reduced" form of a protein by carboxymethylating its cysteines. However, here we have observed that carboxymethylated α,α′-tropomyosin resembles the *oxidized* protein. The last two sets of spectra in Figure 3 address this problem. Spectra 3a and 3b show that the resonances of histidine-153 of αTM-CA begin narrow and remain so even after 12 h under air at 34 °C. By contrast, the histidine resonances in spectra 4a and 4b of αTM-CM begin broad and remain so. Spectrum 4c, taken from a different sample of αTM-CM, shows that 16 h of attempted reduction with 20 mM dithioerythritol at 34 °C does not narrow the histidine resonances. The tropomyosins for samples 3 and 4 were carboxymethylated by the procedure of Crestfield et al. (1963) under identical conditions.

If we assume tropomyosin to be an extended coiled coil with the residues separated by 1.49 Å along the rod axis (Perutz, 1951), then histidine-153 is 55 Å away from the cysteines. Reasoning that a perturbation which extends over 55 Å might affect the grosser characteristics of the structure, we measured the temperature denaturation curves of α,α′- and β,β′-tropomyosins, both native and modified forms, by using circular dichroism at 222 nm. At this wavelength it is an approximate measure of the extant helical structure.

Table I: Comparison of the Thermal Stability of Native and Modified Tropomyosins[a]

| protein | runs | % helix at 34 °C | % helix final | $T_{1/2}$ (°C) |
|---|---|---|---|---|
| αTM-SH | 3[b] | 86.7 ± 2.0 | 14.8 ± 0.7 | 47.5 ± 1.0 |
| αTM-NB | 2[c] | 79.1 ± 0.4 | 16.2 ± 1.3 | 50.4 ± 0.6 |
| αTM-RD | 2 | 82.8 ± 0.6 | 16.5 ± 0.1 | 47.2 ± 0.1 |
| αTM-CA | 2 | 82.6 ± 1.1 | 16.8 ± 0.6 | 44.1 ± 0.1 |
| αTM-CM | 3[d] | 82.1 ± 2.8 | 14.1 ± 0.5 | 42.0 ± 0.5 |
| βTM-SH | 2 | 78.1 ± 3.4 | 14.9 ± 0.7 | 41.9 ± 1.8 |
| βTM-CM | 3[b] | 51.3 ± 5.1 | 16.3 ± 3.0 | 32.4 ± 0.4 |

[a] All samples were dialyzed against 50 mM potassium phosphate, 1 M KCl, 1 mM EDTA, 10 mM DTE, and $H_2O$, pH 7.0, except as noted. [b] One determination was done in the standard $D_2O$ buffer at pH 7.0. [c] As in footnote *a* but no DTE. Equivalent to αTM-OX. [d] As in footnote *b* but no DTE.

Within the experimental error, all the tropomyosins were completely helical at 10 °C ($[\Theta]_{222} = -39\,500$ deg cm$^2$ dmol$^{-1}$; Chen et al., 1974). Therefore, we chose to analyze the data as a fraction of the ellipticity at 10 °C, a normalization which cancels out the concentration terms with their attendant errors. Table I lists averages and standard errors of the percent helix at 34 °C and at the end of the unfolding and the temperature at the midpoint of the thermal unfolding. Three of the samples were in $D_2O$; their data were indistinguishable from those of the corresponding runs in $H_2O$, and their parameters were averaged with the latter. The complete denaturation profiles will be published later.

In Figure 4, spectra of αTM-SH and βTM-CM have been matched approximately by the shape of their histidine-153 resonances. The corresponding spectra are separated by 15 to 16 °C, which is also the difference in the $T_{1/2}$ values of these

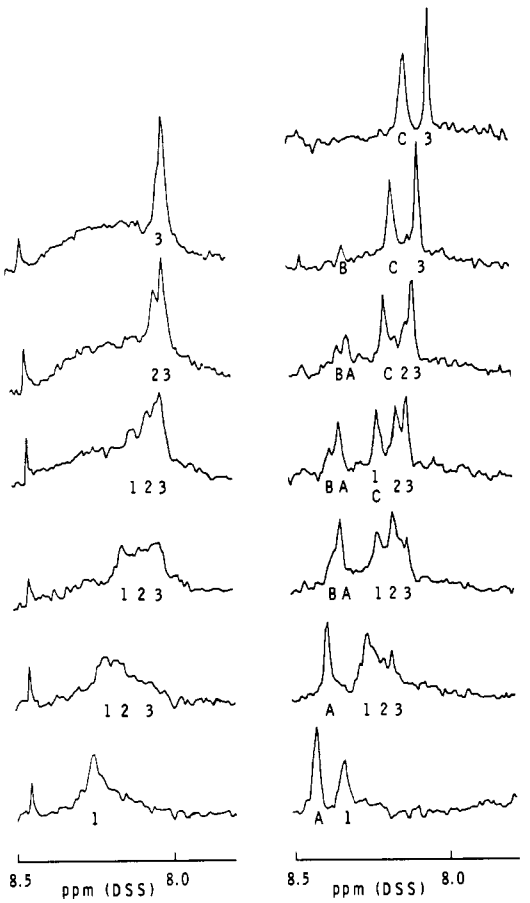


FIGURE 4: Histidine C2 resonances of βTM-CM and αTM-SH are correlated by the shape of histidine-153 resonances as a function of temperature. Both samples were in the standard $D_2O$ buffer with 10 mM DTE and argon above the solution. Left: βTM-CM at pH 7.0; the temperatures of the spectra from bottom to top are 20, 24, 28, 30, 32, and 34 °C. Right: αTM-SH at pH 6.6; the temperatures of the spectra from bottom to top are 37, 40, 43, 45, 47, 50, and 55 °C. The assignment of the resonances to the various conformational forms mentioned in the text is given by numbers (1, 2, and 3) for histidine-153 and by letters (A, B, and C) for histidine-276.

two proteins given in Table I. The spectra shift upfield with increasing temperature because the acid–base equilibria have significant enthalpies. For clarity, the spectra of Figure 4 were taken on samples at a constant pH. Because the temperature affects the $pK_a$ of the histidines, the pH value for each sample was a compromise chosen to keep the resonances within our "window" (see Discussion) over the temperature range that was used.

## Discussion

In the spectra of Figures 1 and 3, acquired at 34 °C, the resonance of histidine-153 is a square-shaped envelope of resonances for αTM-OX and for αTM-CM but it is a narrow, single resonance for αTM-SH and αTM-CA. This unexpected observation that αTM-CM resembles the S–S cross-linked form and not the reduced form can be explained by correlating the thermal stabilities of the different forms with the effects of unfolding the coiled coil on the $pK_a$ value of the histidine.

Under all conditions that we have investigated the histidines of rabbit tropomyosin have constant chemical shifts, within experimental error, for their protonated (8.62 ppm) and unprotonated (7.69 ppm) forms. The observed chemical shift is the mean of these values weighted respectively by the mole fractions of the protonated and unprotonated forms (Markley, 1975). Therefore, a difference in observed chemical shifts, at the same pH, indicates a difference in $pK_a$ value. If we attribute the upfield shoulder which appears over time in Figure 3 to histidine-153 in an exposed or unfolded section of the coiled coil, then the perplexing similarity between αTM-OX (or αTM-NB) and αTM-CM can be explained as follows.

The thermal stability data of Table I show that both of these modified tropomyosins are less stable than αTM-SH at 34 °C, although the $T_{1/2}$ value of αTM-NB is higher than that of αTM-SH. Since the histidine-153 resonance of the former splits at a lower temperature than that of αTM-SH, it must be associated with the "pretransition" between 30 and 40 °C in the unfolding of cross-linked tropomyosin which has been reported by Satoh & Mihashi (1972) and Lehrer (1978). Evidently, modifying the uncharged (at pH 7.0) cysteine-190 with the charged carboxymethyl group destabilizes the region about the cysteine, a destabilization which is felt at histidine-153, some 55 Å away. Moreover, the uncharged carboxyamidomethyl group, which does not split the histidine-153 resonance at 34 °C, destabilizes the coiled-coil structure less than the carboxymethyl group does. At 34 °C, αTM-CA has a marginally higher helical content than αTM-CM but a significantly higher $T_{1/2}$ value. These correlations between the data of Table I and the state of the histidine-153 resonance are acceptable considering that the former measure a global property of the tropomyosin structure in dilute solutions while the latter reflects a local effect in concentrated solutions. The NMR samples are comparable despite the higher concentration of protein becaue 1 M salt depolymerizes tropomyosin (Tsao et al., 1951). For example, a "titration" of a NMR sample (20 mg/mL αTM-SH, pH 7.4, 34 °C) with KCl showed no evidence of further depolymerization, viz., increasing height of the methyl resonance or decreasing line width of the histidine-276 resonance, above 0.8 M KCl.

The coiled-coil structure appears to be very sensitive to charged residues in its core positions [a and d of Figure 1 in Edwards & Sykes (1978)]. Witness the initial cleavage by trypsin near aspartate-137, the only charged residue in a core position of αTM-SH (Pato & Smillie, 1978), and our observation that βTM-CM, which has an additional (carboxymethyl)cysteine in its core at position 36, is the least stable tropomyosin in Table I.

The above comparisons among the various tropomyosins have been made at 34 °C. However, our conclusion that the downfield shoulder of the square envelope of resonances reflects the $pK_a$ of histidine-153 in the intact coiled coil while the upfield shoulder reflects its $pK_a$, ~0.2 unit lower, in an unfolded state implies that every tropomyosin that we have discussed should exhibit first a narrow resonance, then a square one, and then a narrow one as the temperature is raised and its structure passes from being wholly helical, to partially unfolded, to fully unfolded in the neighborhood of histidine-153.

Two examples of this progression are given in Figure 4. The spectra of βTM-CM reveal clearly the progression of histidine-153 from a single species of higher $pK_a$ at 20 °C, through a mixture of species at 28 °C, to a single species of lower $pK_a$ at 34 °C. It is important to remember that since we are actually seeing differences in $pK_a$ values, not differences in chemical shifts, we can only observe the distribution of conformations at pH values where the various histidines are partially protonated, although the distribution very probably exists at other pH values. Consequently, their observed chemical shifts must be in a window centered at ~8.15 ppm. When the histidines are mostly protonated or unprotonated,

their resonances will coincide no matter how different their $pK_a$ values might be.

Complete pH titrations at selected temperatures have defined the window and confirmed the patterns seen in Figure 4. Titrations of βTM-CM at 28 °C and of αTM-SH at 40 °C show a broadened resonance for histidine-153 between 7.9 and 8.4 ppm. Other pH titrations have confirmed that at 34 °C histidine-153 in both βTM-CM and αTM-SH has a single resonance and therefore a single $pK_a$. In other words, at 34 °C βTM-CM has finished the progression of conformational changes while αTM-SH has not yet begun it.

We have interpreted the multiple resonances for histidine-153 as evidence for the existence of intermediates in the thermal unfolding of the tropomyosin coiled coil. Other investigators have also inferred the existence of such an intermediate during the unfolding of tropomyosin by heat or Gdn·HCl. Pont & Woods (1971) found that free chain did not appear until sTM had lost ~25–30% of its helical structure at 2 M Gdn·HCl. Woods (1976) later presented a thermal unfolding profile for αTM-SH (his single-banded tropomyosin) that was clearly biphasic. His analysis gave half-transition values of 30 and 54 °C for the two steps. Satoh & Mihashi (1972) used the depolarization of the tyrosine fluorescence to follow the unfolding of sTM. They observed a sudden depolarization near 34 °C which was independent of polymerization (0.01–1 M KCl) or pH (6–8). They attributed it to a partial unfolding which released 15–20% of the tyrosines from rotational restraints. Chao & Holtzer (1975) located this region of lesser stability around cysteine-190. A spin-label attached to this residue was fully mobile at 1.5 M Gdn·HCl while the protein was not fully denatured until 3.5 M.

Recently, Lehrer and co-workers have used circular dichroism, fluorescence (Lehrer, 1978), and calorimetry (Krishnan et al., 1978) to analyze the effects of cross-linking sTM at cysteine-190 and -190′. They determined that the cystine cross-link induced a transition near 35 °C followed by a main transition that was shifted upward to 52 °C, an increase of 7 °C from the single transition temperature evinced by reduced or carboxyamidomethylated sTM. Lehrer further showed (Betcher-Lange & Lehrer, 1978) that cysteine-190 and -190′ separated locally at a lower concentration of Gdn·HCl than that required to fully denature their chains.

All investigators concur that reduced skeletal and cardiac α,α′-tropomyosins have a region of local instability about cysteine-190. Our NMR experiments support this observation and extend it in two important details. First, the two spectra in Figure 4 at 28 °C and at 43 °C show unequivocaly that there are also three different conformations possible for histidine-153 some 55 Å from the cysteine-190. We assign the resonances to a fully folded state (1; downfield resonance; highest $pK_a$), a partially unfolded state (2; middle resonance; intermediate $pK_a$), and an effectively unfolded state (3; upfield resonance; lowest $pK_a$). The last assignment is qualified because at 34 °C for βTM-CM or at 50 °C for αTM-SH the samples retain ~45% of their helicity and, if heat produces the same unfolding intermediates as Gdn·HCl, coiled coils still exist in equilibrium with free chains (Pont & Woods, 1971). That is, the peptide chain about histidine-153 is locally unfolded in the coiled coil and is indistinguishable from the free, unfolded chains at these or higher temperatures.

Second, the spectra in Figure 4 also report on the carboxy-terminal part of the coiled coil. If we assume that histidine-153 in αTM-SH has the same spectrum as it does in βTM-CM, allowing for narrower resonances due to the higher temperatures, then the behavior of the histidine-276 resonances can be disentangled. At 37 °C they are a single resonance well downfield (higher $pK_a$) of the histidine-153 resonances. With increased temperature an intermediate species of very slightly higher $pK_a$ appears as well as the "effectively unfolded" species whose $pK_a$ and resonance coincide with those of histidine-153 in its low-temperature conformation. Having this assignment, we can then deduce that the chain about histidine-276 unfolds later than that about histidine-153, as shown by the αTM-SH spectrum at 50 °C. Histidine-153 is entirely in the "denatured" conformation (3) while some histidine-276 remains in the intermediate conformation (B). If the three states of each histidine are independent, there are at least nine conformations possible for the tropomyosin coiled coil. The spectra in Figure 4 argue against three of them existing (1B, 1C, and 2C), but to go farther requires a detailed analysis of the relative intensities of the individual resonances.

Parenthetically, we note that such an analysis also offers the possibility of calculating the equilibrium constants among the various species. Because the species are in slow exchange with one another [see, e.g., Carrington & McLachlan (1967)], the relative areas of their respective histidine resonances are potentially proportional to their relative concentrations. However, an accurate analysis requires spectra of greater digital resolution and longer recovery times.

In summary, we have shown that the broadened resonance of histidine-153 derives from the coexistence of at least three conformational states of tropomyosin. They have different $pK_a$ values for the histidine residue, they are in slow exchange with one another, and they correlate with the temperature stability of the various tropomyosins. The last fact gives possible significance to the difference in thermal stability between βTM-SH and αTM-SH. In particular, cross-linking the coiled coil at cysteine-190 perturbs its structure in the vicinity of histidine-153 which is 55 Å away. In general, these conformational states seem to extend over most of the coiled coil since histidine-276 also exhibits multiple $pK_a$ values.

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# Proton Magnetic Resonance Study of the Histidines in Hemerythrin and Chemical Identification of the Nonligand Histidines†

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ABSTRACT: Three non-iron-liganded histidines have been studied in methemerythrin azide monomers from *Phascolopsis gouldii* by 250-MHz proton correlation nuclear magnetic resonance (NMR) spectroscopy. Four of the seven histidines in the protein are not observed because of paramagnetic broadening by the coordinated iron; neither are they observed as contact or pseudocontact shifted resonances. The NMR titration of the three free histidines establishes them as normal histidines with p$K'$ values of 7.00 ± 0.03 and Hill coefficients of 0.90, 0.81, and 0.81 ± 0.03. The chemical shift of the protonated and neutral histidines is normal, and the bandwidth of the resonance absorption is 5 Hz. A pH-dependent reversible transition in the chemical shift of the histidine C(2)H occurs at pH 6.5; above this pH the three protons occur as a singlet but break into three singlets of different chemical shift at acid pH values. Two of the three "free" histidines have been identified by their susceptibility to photooxidation as His-82 and His-34.

Hemerythrin is a non-heme iron protein which reversibly binds oxygen (Klotz et al., 1957; Boeri & Ghiretti-Magaldi, 1957). A number of chemical and biophysical techniques, predating recent X-ray studies, have been used to elucidate the nature of the iron binding site. Mössbauer spectroscopy has shown that two antiferromagnetically coupled iron atoms (per monomeric unit of 13 000 daltons) comprise the oxygen binding site (Okamura et al., 1969; York & Bearden, 1970; Moss et al., 1971). The chemical studies have suggested that four histidyl (Fan & York, 1969; York & Roberts, 1976) and two or three tyrosyl (Rill & Klotz, 1970, 1971; York & Fan, 1971; Fan & York, 1972) residues are iron ligands. There are a total of seven histidines and five tyrosines per monomer. X-ray analysis of the myohemerythrin from *Themiste pyroides* (Hendrickson et al., 1975) suggested that histidine-25, -54, -73, and -106 and tyrosine-67 and -109 were iron ligands. Similar studies on the methemerythrin octamer from *Themiste dyscritum* (Stenkamp et al., 1978) suggested that a fifth histidine (His-77) and only one tyrosine (Tyr-114) are ligands to the two iron atoms at the active site. It is now agreed that the latter interpretation is correct (Hendrickson, 1978). In an attempt to resolve the apparent conflict between the crystallographic and chemical data relating to the histidine composition of the iron binding site, we have investigated hemerythrin by NMR spectroscopy and in addition have identified two of the three histidines which are readily photooxidized.

## Materials and Methods

Hemerythrin azide monomers were prepared from the crystalline hemerythrin azide octamer obtained from *Phascolopsis gouldii* by disulfide interchange with cystine as previously described (York & Roberts, 1976), but with all reagents prepared in 92% $D_2O$. The preparation was concentrated to 2 mM under vacuum in a S and S collodion bag at 25 °C. These procedures for preparing $D_2O$ solutions of hemerythrin were necessitated by the susceptibility of heme-

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