Assignment and Characterization of the Histidine Resonances in the ^1H Nuclear Magnetic Resonance Spectra of Rabbit Tropomyosins†

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ABSTRACT: Tropomyosin is a major component of the regulatory system of mammalian muscle. In this study ^1H nuclear magnetic resonance (NMR) has been used to investigate the properties of rabbit skeletal and cardiac tropomyosins. The former, being heterogeneous, was prepared as renatured $\alpha\alpha$ and $\beta\beta$ coiled coils; the latter was isolated without denaturation as an $\alpha\alpha$ coiled coil. The complete sequence of 284 residues is known for both types of chains (Stone, D., et al. (1975) *Fed. Eur. Biochem. Soc., Meet., 9th,* 125; Mak, A., & Smillie, L. B., personal communication); combined with the probable coiled-coil arrangement (Crick, F. H. C. (1953) *Acta Crystallogr. 6,* 689), these sequences specify the structure of the tropomyosins. Since each α chain of the coiled coil has one histidine residue near the center (His-153) and one near the C-terminal end (His-276), we have assigned the C-2 resonances using carboxypeptidase digestion. A comparison with the NMR spectrum of the $\beta\beta$ tropomyosin, whose chains lack the histidine in position 276, confirmed the assignment. The pK_a values of the histidine residues of the various tropomyosins were determined in 0.03 M and 1.0 M KCl buffers at 28, 34, and 40 °C. The tripeptide Gly-His-Gly was titrated under the same conditions as a control. The pK_a values varied around neutrality but interesting anomalies were observed during the titrations. The resonances of the pair of central histidines, which superimposed below pH 6 and above pH 8, broadened into a distribution of resonances (more than two) in the middle of the titration. These resonances reflect a series of interconverting conformational forms of differing pK_a, which can be "melted" together to different extents for the different tropomyosins by raising the temperature to 34 °C. A simple Hill analysis gave coefficients approaching 2.0 under certain conditions. The response of the central histidines to variations in ionic strength was also anomalous. They behaved neither like free histidine (Gly-His-Gly) nor like a histidine on a negatively charged rod (His-276).

In mammalian skeletal muscle, contraction is regulated by the interaction of Ca^{2+} ions with the proteins of the I filament: troponin, tropomyosin, and actin (Ebashi & Endo, 1968). A model for this regulation has been developed (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973; Potter & Gergely, 1974; Cohen, 1975; Wakabayashi et al., 1975). In this model tropomyosin, a fibrous protein which binds one troponin molecule, lies along the grooves of the double helix of globular actin molecules (Hanson & Lowy, 1963; Ebashi et al., 1969), sterically blocking their combination with myosin and the development of tension. When calcium binds to troponin, tropomyosin rolls out of its blocking position; it rolls back to restore inhibition when the calcium concentration falls below the calcium binding constant of troponin. Since one tropomyosin molecule interacts with seven actin molecules per strand of the actin double helix (Ebashi et al., 1969), tropomyosin amplifies the calcium initiation. Moreover, in the I filament the tropomyosin molecules overlap one another slightly at their ends and Tawada et al. (1975) have shown this overlap is required for the full cooperativity of activation. Using nuclear magnetic resonance at 270 MHz, we have studied solutions of purified tropomyosin, which retain this ability to polymerize in an end-to-end fashion, with the related goals of studying the intrinsic properties which might explain its function and of investigating the physical aspects of its unusual rod-like structure.

When depolymerized by salt or extreme pH, tropomyosin behaves as a thin rod approximately 20 Å wide and 400 Å long (Caspar et al., 1969) with a molecular weight of 66 000 daltons (Woods, 1967). No x-ray structure is available but for the purposes of this NMR study the structure of rabbit skeletal tropomyosin has been adequately defined: it is a coiled coil of two α-helical polypeptide chains (Crick, 1953); it is almost completely helical by optical rotary dispersion (Cohen & Szent-György, 1957); the chains are parallel and in register (Caspar et al., 1969; Johnson & Smillie, 1975; Stewart, 1975; Lehrer, 1975); the amino acid sequences of the α chains (Stone et al., 1974) and β chains (Mak and Smillie, personal communication) are known. They each have 284 residues and exhibit the expected regularities of the coiled-coil structure (Figure 1A). However, in this paper we consider only the aromatic residues of tropomyosin (Figure 1B).

Experimental Procedures

NMR Procedures. All ^1H NMR spectra displayed in this paper were taken at 270 MHz on a Bruker HXS-270 NMR spectrometer operating in the Fourier mode. Typical instrument settings were acquisition time 0.5 s, sweep width 4000 Hz, spectrum size 4096 data points, and line broadening 1 Hz. The HDO resonance was reduced with homonuclear decoupling. Chemical shifts were measured relative to the major resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)[1] as an internal standard.

Sample Preparation and pH Titrations. The NMR samples were prepared by dissolving a weighed amount of lyophilized tropomyosin directly into the desired D_2O buffer. Protein concentrations were verified by absorbance ($E_{277}^{1\%} = 3.3$; Woods, 1967) and by amino acid analysis after 36 h of acid hydrolysis at 110 °C. The pH titrations were accomplished by

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[1] Abbreviation used: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

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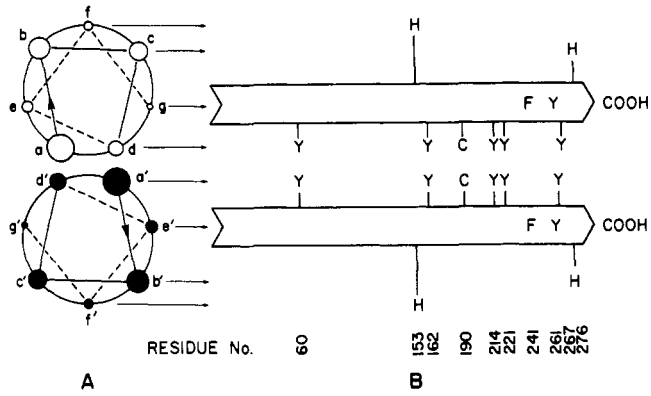

FIGURE 1: The coiled-coil structure of tropomyosin. (A) A cross-sectional view (adapted from McLachlan & Stewart, 1975) showing the paired heptet of residue positions which repeats throughout the length of the molecule. Positions a, a′, d, d′ are usually hydrophobic residues and are thought to pack together in a knobs-into-holes fashion such that the two α helices coil about one another with a pitch of 137 Å (Parry, 1975). Positions e, e′ and g, g′ are predominantly charged residues, acidic and basic, respectively, whose salt links could contribute to the stability of the structure. Positions b, b′, c, c′, f, f′ are also predominantly charged residues in the sequence of α-tropomyosin. (B) A diagrammatic representation of the amino acid sequence of α-tropomyosin showing the aromatic residues—tyrosine (Y), histidine (H), and phenylalanine (F)—and the cysteine residue (C). The cysteines can form an intramolecular disulfide bond (Johnson & Smillie, 1975; Stewart, 1975; Lehrer, 1975).

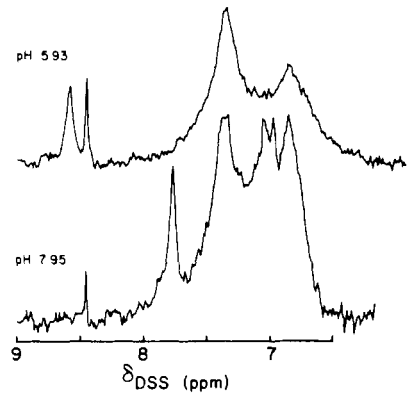

FIGURE 2: A spectrum of the aromatic residues of α-tropomyosin at 27 °C showing the coincidence of the histidine C-2 reasonances in the protonated (top, 8.6 ppm) and unprotonated (bottom, 7.8 ppm) forms. The other resonances are those of formate ion (8.45 ppm), of tyrosine meta protons and phenylalanine protons (7.45 ppm), of histidine C-4 protons (top, 7.35 ppm; bottom, 7.1 and 6.95 ppm), and of tyrosine ortho protons (6.85 ppm). The sample contained 18 mg/mL of protein in 40 mM potassium phosphate, 1 M KCl, 0.5 mM EDTA, 0.25 mM DSS, D_2O.

adding microliter aliquots of NaOD or DCl directly to the sample in the NMR tube, mixing, and then measuring the pH with an Ingold microelectrode (Model 6030-04) attached to either a Beckman Expandomatic SS-2 or Radiometer 26 pH meter. Before each reading, the electrode was standardized in H_2O buffers; between each reading the electrode was cleaned in 0.1 M HCl to ensure reproducible standardization. All pH measurements discussed in this paper are uncorrected for D_2O and are at room temperature, with one exception. The pH measurements for titration 10 were read at 40 °C. Because the temperature range was small and because phosphate buffers have a very small temperature coefficient the pH measurements for titrations at 28 and 34 °C were taken at room temperature.

Tropomyosin Preparation. The various tropomyosins used in this study were obtained as a gift from Dr. L. B. Smillie. Rabbit skeletal tropomyosin, which is heterogeneous, was separated into pure α and β chains by the method of Cummins & Perry (1973) which used 8 M urea to dissociate the coiled coil. The purity of the β-tropomyosin was improved by isolating it from heterogeneous tropomyosin in which the cysteines had been carboxymethylated (Mak & Smillie, personal communication). In this paper α- and β-tropomyosins are renatured dimers of two α or two β chains, respectively. Because renaturation might introduce artifacts we used rabbit cardiac tropomyosin in later experiments. It is at least 90% α chains (Cummins & Perry 1973; Lewis & Smillie, private communication) and can be prepared (Bailey, 1948) without denaturing the protein.

Digestion of Tropomyosin with Carboxypeptidase A. The procedure of Tawada et al. (1975), as modified by Johnson & Smillie (1977), was used to digest α-tropomyosin. Because histidine-276, the ninth residue from the C-terminal end, was removed slowly, the digestion was prolonged for up to 29 h with one or two additional charges of carboxypeptidase A that had been treated with diisopropyl fluorophosphate. The reaction mixture was applied to a column of A-1.5m agarose (Bio-Rad), equilibrated with 0.02 M sodium phosphate, 1 M NaCl, pH 7.2, to remove carboxypeptidase and tropomyosin fragments. A sample of the final reaction mixture was analyzed for free histidine on a Beckman 120C amino acid analyzer.

Analysis of the Titrations. The pH titration data were fitted to a modified form of the Hill equation similar to that used by Markley (1975) but in terms of the protonated species. If one assumes that protonation is in the fast exchange limit, then the observed chemical shift (δ_{obsd}) is the sum of the protonated (δ_{AH}) and unprotonated (δ_A) chemical shifts weighted by their respective fractional populations (f_{AH} and $f_A = 1 - f_{AH}$).

$$\delta_{obsd} = \delta_A(1 - f_{AH}) + \delta_{AH}(f_{AH})$$

For the multiple protonation, $A + nH \rightleftharpoons AH_n$, the Hill model assumes that only A and AH_n exist in significant concentrations. Therefore, f_{AH} is equal to $[AH_n]/([A] + [AH_n])$ and we have

$$\delta_{obsd} = \delta_A + (\delta_{AH} - \delta_A)\frac{[AH_n]}{[A] + [AH_n]}$$

If each protonation step is assumed to have the same acid dissociation constant, K_a, then the overall dissociation constant, which is the product of the individual dissociation constants, K_a^n, is given by $[A][H]^n/[AH_n]$ and the equation can be recast to give the actual equation that was used in this work.

$$\delta_{obsd} = \delta_A + (\delta_{AH} - \delta_A)\frac{[H]^n}{K_a^n + [H]^n}$$

Results

The Assignment of the Histidines. Although they coincide at high and low pH (Figure 2), the resonances of histidines-153, 153′ and -276, 276′ separate clearly at pH 7.4 and 34 °C as seen in spectrum A of Figure 3. Digestion with carboxypeptidase A decreased the downfield histidine C-2 resonance, thereby assigning it to histidine-276, 276′; the spectra of Figure 3 correlate this decrease with the amount of histidine-276, 276′ released. We attribute the rapid broadening of the histidine-276, 276′ resonance to the distribution of partially digested tropomyosins with resulting histidine environments ranging from C terminal to ninth from the end. As evident in Figure 4, the titration curve of the resonance which had survived the digestion followed closely the upfield titration curve of undigested tropomyosin. A pH titration of β-tropomyosin, which has only histidine-153, 153′ (asparagine replaces histidine-276, 276′; Mak & Smillie, private communication), confirms the

TABLE I: Least-Squares Analysis of the NMR Titration Data for the Histidine C-2 Proton Resonances of Tropomyosin.[a]

No.	Sample[b]	Conditions mg/mL	μ[c]	°C (±1 °C)	Histidine-153 pK_{a1}[d]	n_1	pK_{a2}[d]	n_2	Histidine-276 pK_a[d]	n
1	α-TM	20	0.03	27	7.15 (0.02)	1.2 (0.1)	6.89 (0.03)	1.2 (1)	7.39 (0.02)	1.1 (0.1)
2	α-TM	18	1.1	27	7.04 (0.03)	1.5 (0.2)	6.98 (0.02)	2.1 (0.3)	7.1 (0.04)	1.2 (0.2)
3	β-TM	20	1.1	28	7.11 (0.04)	1.0 (0.1)	6.85 (0.04)	1.0 (0.1)		
4	β-TM	20	1.1	34	6.73 (0.02)	0.93 (0.03)				
5	c-TM	15	1.1	28	7.14 (0.04)	1.1 (0.1)	6.93 (0.05)	1.5 (0.2)	7.28 (0.03)	0.9 (0.1)
6	c-TM	15	1.1	34	6.99 (0.03)	1.1 (0.1)	6.6 (0.2)	1.0 (0.02)	7.16 (0.04)	0.9 (0.1)
7	c-TM	30	1.1	28	7.16 (0.04)	1.3 (0.2)	6.95 (0.06)	2.0 (0.5)	7.26 (0.04)	1.0 (0.1)
8	c-TM	30	1.1	34	6.99 (0.05)	1.4 (0.2)	6.89 (0.1)	1.3 (0.3)	7.16 (0.04)	1.1 (0.1)
9	c-TM	30	1.1	40	6.84 (0.03)	1.2 (0.1)	6.66 (0.03)	0.9 (0.1)	7.11 (0.03)	1.1 (0.1)

[a] Numbers in parentheses represent standard deviations from the least-squares fits. [b] Samples 1–2 contained no reducing agent and the cysteines were oxidized to an unknown extent; samples 3 and 4 had been carboxymethylated; samples 5–9 contained 10 mM dithioerythritol. [c] Buffer for μ = 0.03 was 10 mM K_2DPO_4, 0.5 mM EDTA, 0.25 mM DSS, D_2O. Over the titration μ increased to 0.1 M. Buffer for μ = 1.1 was 40 mM K_2DPO_4, 0.5 mM EDTA, 0.25 mM DSS, 1.0 M KCl, D_2O. [d] Uncorrected for D_2O.

TABLE II: Least-Squares Analysis of the NMR Titration Data for the Histidine C-2 Proton Resonance of Gly-His-Gly.[a]

No.	μ[b]	°C	pK_a	n
10	0.03	27	6.62 (0.02)	0.93 (0.04)
11	0.03	34	6.50 (0.02)	0.94 (0.05)
12	1.1	27	7.00 (0.01)	1.04 (0.03)
13	1.1	34	6.87 (0.01)	1.02 (0.03)

[a] All samples had 0.6 mM GHG, 10 mM K_2DPO_4, 0.5 mM EDTA, 0.25 mM DSS. Numbers in parentheses represent standard deviations from the least-squares fits. [b] Ionic strength was increased with 1 M KCl.

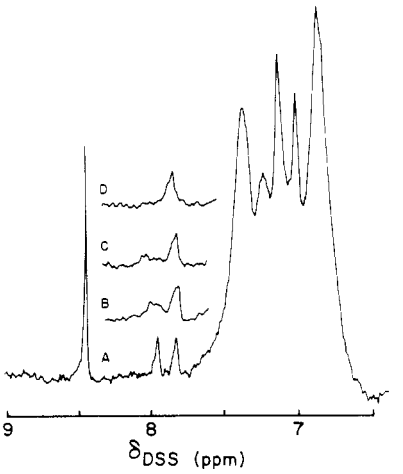

FIGURE 3: Spectra of α-tropomyosin after digestion with carboxypeptidase A. By amino acid analysis, the amounts of free histidine released are 0, 13, 20, and 60% going from A to D. The buffer and protein concentrations were the same as in Figure 2, but the temperature was 34 °C.

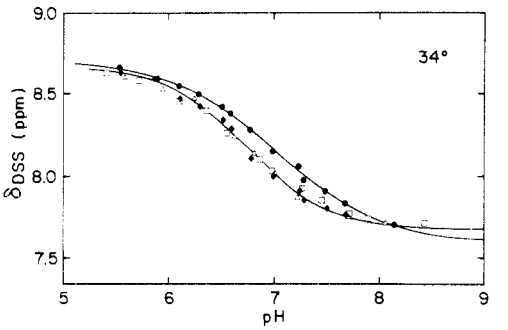

FIGURE 4: pH titrations of α-tropomyosin (His-276 (●); His-153 (♦)), of β-tropomyosin (□), and of α-tropomyosin (△) with 60% of histidine-276 removed by carboxypeptidase A (D in Figure 3) showing the coincidence of the histidine-153 titration data. The solid curves are calculated from least-squares fits to the data from the pH titration of whole α-tropomyosin.

assignment. Its single histidine C-2 resonance also titrates with the upfield resonance of α-tropomyosin in Figure 4.

Titration of the Histidines. The histidine resonances of α-, β-, and cardiac tropomyosin have been titrated under various conditions. Table I lists these conditions along with the pK_a values and Hill coefficients calculated from least-squares fits to the data. The chemical shifts, δ_A and δ_{AH}, calculated for the nine titrations, were similar and varied randomly. For histidine-276, 276′ the mean values (standard deviation, minimum, and maximum value) were respectively 7.67 (0.02, 7.65, 7.70) and 8.62 (0.02, 8.60, 8.64). Similarly, for histidine-153, 153′ the mean value of δ_A was 7.70 (0.03, 7.66, 7.75) and the mean value of δ_{AH} was 8.62 (0.05, 8.53, 8.74). The tripeptide, Gly-His-Gly, was titrated as a model for histidine in a polypeptide chain (Markley, 1975). The conditions and least squares parameters are given in Table II. For δ_A the mean value was 7.69 (0.01, 7.68, 7.70) and for δ_{AH} it was 8.62 (0.01, 8.62, 8.63).

During the histidine titrations of α-, β-, and cardiac tropomyosin, the resonance corresponding to histidine-153, 153′ is further split into a series of closely spaced resonances. As the temperature is raised this splitting can be collapsed. This is most clearly seen for β-tropomyosin (Figure 5) where the histidine region of the spectrum contains only histidines-153, 153′. The collapse occurs at lower temperatures for β-tropomyosin than for α-tropomyosin and cardiac tropomyosin. When a distribution of peaks for histidine-153, 153′ was present during a titration, the values of pK_{a1} and pK_{a2} listed in Table I reflect the pK_a of the most downfield and most upfield peak, respectively, in the distribution.

Effects of Salt. Tropomyosin is monomeric in 1 M KCl (Tsao et al., 1951; Kay and Bailey, 1960); it polymerizes increasingly as the ionic strength is dropped. Complete pH titrations have been done on α-tropomyosin in solutions of ionic strengths 0.03 M and 1.1 M. At the higher ionic strength and 27 °C, the pK_a of histidine-276, 276′ decreased while those used to characterize histidine-153, 153′ approached one another (the broadening decreased) but had the same average. A titration of α-tropomyosin with KCl at a fixed pH of 7.4 showed the same qualitative behavior. The titration was done at 34 °C to resolve more clearly the histidine-153, 153′ and -276, 276′ resonances. In the spectra of Figure 6 the resonance of histidine-276, 276′ shifts upfield (decreasing pK_a) with in-

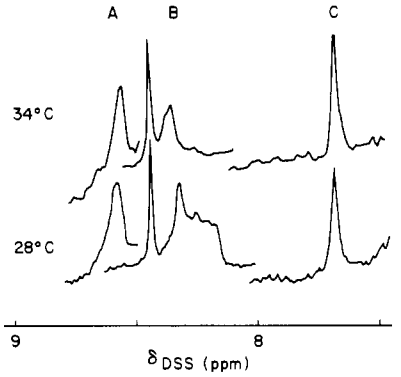

FIGURE 5: Spectra from titrations 3 and 4 of the C-2 proton of histidine-153, 153′ of β-tropomyosin. The relative intensities are not to scale. (A) Spectra at pH 5.7. (B) Spectra showing the greatest broadening observed during each titration; top spectrum is at pH 6.4, and bottom spectrum is at pH 6.8. The former was taken 5 days after the bottom spectrum: deuteration has reduced the histidine C-2 resonances to about half the area—relative to formate—seen in the bottom spectrum. (C) Spectra at pH 8.5. The sample conditions are given in Table I.

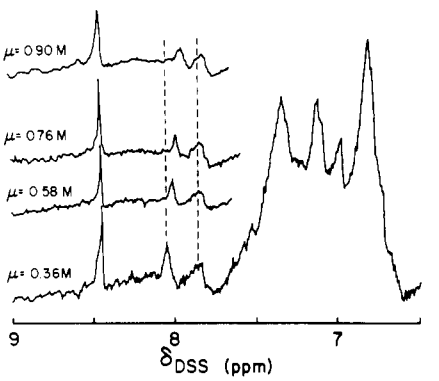

FIGURE 6: Spectra of α-tropomyosin (20 mg/mL) at pH 7.4 and 34 °C showing the upfield shift of the C-2 resonance of histidine-276, 276′ with increasing ionic strength. The buffer was the same as that in titration 1.

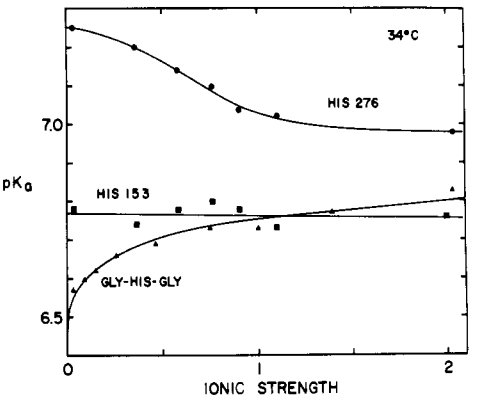

FIGURE 7: The effect of ionic strength on the pK_a values of the histidines of α-tropomyosin and Gly-His-Gly. For α-tropomyosin the conditions were the same as in Figure 6; for Gly-His-Gly the conditions were the same as in titration 11; and in both experiments the ionic strength was varied with KCl.

creasing ionic strength while that of histidine-153, 153′ remains fixed. When the same experiment was done with Gly-His-Gly, the histidine resonance shifted downfield (decreasing pK_a). These data were converted into pK_a values using the inverse of the equation for NMR pH titrations and the chemical-shift parameters from least-squares fits (n fixed at 1.0) to the respective complete titration data. Scant credance can be given to the absolute values of one point pK_a determinations but the relative values of Figure 7 reflect the real effects seen in the spectra.

When the calculated pK_a values for Gly-His-Gly were tested against the Debye–Huckel equation (Edsall & Wyman, 1958) for aqueous solutions at room temperature

$$pK_a^{\mu} = pK_a^{\mu=0} + \frac{0.5\sqrt{\mu}}{1 + 0.33r\sqrt{\mu}}$$

a least-squares fit gave the reasonable values of 6.59 (0.01) for $pK_a^{\mu=0}$, the dissociation constant at zero ionic strength, and 1.8 (0.2) Å for r, the radius of the ion (histidine). Because the α-tropomyosin data in Figure 7 clearly did not follow the above equation, they were fit to a straight line (histidine-153, 153′; intercept = 6.77 (0.02), slope = −0.01 (0.02)) or to a smooth curve (histidine-276, 276′).

Discussion

The NMR spectra of tropomyosin offer several insights into the structure of the coiled coil. The chemical shifts of the histidine and tyrosine residues, which correspond closely with those of free amino acids in aqueous solution, suggest a loose structure, relatively open to solvent. Five of the six tyrosine residues per chain are found in the hydrophobic and most "buried" positions of the coiled coil; yet no significant spread in chemical shifts is observed. By contrast bovine pancreatic trypsin inhibitor, a small protein of only 58 amino acids, has tyrosine resonances distributed over a greater range of chemical shifts (Snyder et al., 1975). Lowey (1965) drew the same conclusion from her optical studies of the tyrosine residues of tropomyosin. The line widths of the tyrosine resonances also indicate a flexible, open structure; for a molecule of 66 000 daltons, even a very rod-like one, the line widths are unusually narrow. Even at 0.03 M ionic strength, where tropomyosin is polymerized into linear units of several hundred thousand daltons average molecular weight (Kay & Bailey, 1960; Ooi et al., 1962), the tyrosine line widths are no broader than those seen in the spectra of Figures 2 and 6, when observed at the same temperature and pH. We have analyzed them by calculations modeled on internal motions of the tyrosine residues (Edwards & Sykes, 1977; Edwards et al., 1977).

The relatively narrow line widths of the histidine resonances are less surprising, because the model of a coiled coil for tropomyosin places them in the f, f′, and c, c′ positions which are expected to be comparatively unhindered and exposed to solvent. For the same reason, the chemical shifts of the four C-2 resonances of each tropomyosin dimer are standard and undifferentiated in both their protonated and deprotonated forms. We will discuss their titration behavior later but we point out here that local differences in electrostatic environment have been observed; the acid dissociation constant of histidine-153, 153′ differs from that of histidine-276, 276′. Also, in Figure 2 the histidine C-4 protons, which lie closer to the main chain, have different chemical shifts when the C-2 protons have identical ones. The paired behavior of the histidine resonances (153 and 153′ coincide, 276 and 276′ coincide) supports the finding that the two polypeptide chains are in register, with equivalent residues in identical environments.

We have investigated the effects of chain type, ionic strength, and temperature upon the pH titration behavior of the histidine resonances of tropomyosin. Table I shows that all the titrations yielded reasonable pK_a values (Markley, 1975) but some titrations gave unusual Hill coefficients. Because the resonances that we observe are the superposition of resonances

TABLE III: Thermodynamic Parameters of the Histidine Titrations.[a]

Titrations[b]	Residue	pK_a[c]	ΔH (kcal/mol)	ΔS (eu)
3, 4	153	6.73	18 (3)	26 (14)
5, 6	153	6.81	17 (9)	22 (32)
	276	7.16	9 (4)	−5 (14)
7, 8, 9	153	6.94	11 (2)	5 (7)
	276	7.16	5 (2)	−15 (6)
10, 11	GHG	6.50	7 (2)	−6 (8)
12, 13	GHG	6.87	8 (1)	−6 (7)

[a] The numbers in parentheses are standard deviations calculated from those in Tables I and II. [b] The numbers are the same as Tables I and II. [c] The value for histidine-153 is an average. The pK_a values are at 34 °C.

from two equivalent histidines (e.g., 276 and 276′), the Hill coefficient can have the range $0 < n \leq 2$ with $n = 1$ being the expected value for independent titrations. Markley (1975) has observed that "a Hill coefficient less than unity may be explained by the presence of one or more groups in the vicinity of the residue being observed that titrate in the histidine pK region". However, in this instance data error could also explain the calculation of Hill coefficients less than unity. In titrations 3, 6, and 7, which had the lowest Hill coefficients, a value of 1.0 could be discounted at no better than the 25% confidence level (i.e., about one chance in four of being 1.0) using Hamilton's ratio test (Hamilton, 1965; Fraser & Suzuki, 1973). It is interesting to note that the same test applied to the Gly-His-Gly titrations at low ionic strength (n = 0.93, 0.94) excludes a Hill coefficient of 1.0 at the 1% confidence level. The "negative cooperativity" is statistically significant and probably reflects the competing and inhibiting protonation of the α-amino group which has a slightly higher microscopic acid dissociation constant (Rabenstein et al., 1977). In 1 M KCl the imidazole ring, which is isolated from the positive charge of the protonated α-amino group, titrates independently.

By the Hamilton ratio test the positive cooperativity which appears in several titrations of histidine-153, 153′ is statistically significant. In titration 2, which gave the highest Hill coefficients, a value of unity is excluded at better than the 1% confidence level. However, because these analyses are based on the extrema of the distributions, not on the chemical shifts of individual species, we cannot yet attribute biological relevance to this cooperativity.

The Hill analysis is an unsophisticated model which assumes that only the initial and final states (both histidines deprotonated or protonated) exist in significant concentrations. A glance at Figure 5 shows that more than two resonances appear during the titration of histidine-153, 153′ at 28 °C. The pH titration at 34 °C suggests that these resonances are derived from interconverting conformational states of differing pK_a since they can be "melted" together. The effect is completely reversible. In fact titrations 3 and 4 used the same sample, which was subjected to the two temperatures in turn, and to room temperature for pH measurements. This complexity is reflected in the thermodynamic parameters in Table III. The enthalpy and entropy changes for the ionization of histidine-153, 153′ of β and cardiac tropomyosin are positive, whereas the values for histidine-276, 276′ are closer to those found for free histidine (Edsall & Wyman, 1958; $pK_a^{H_2O}$ = 6.00; ΔH = 6.9 kcal/mol, ΔS = −4 eu) and for the six "normal" histidine residues of myoglobin (Hermans & Rialdi, 1965; $pK_a^{H_2O}$ = 6.62; ΔH = 7.1 kcal/mol, ΔS = −6.5 eu). Although the differences in temperature are small, the differences in pK_a values are small, and the possible errors in both are significant, the data do yield reasonable thermodynamic values for our "control", the Gly-His-Gly proton dissociation.

Because tropomyosin probably moves by means of a conformational change between its repressing and derepressing positions on the actin helix (Potter & Gergely, 1974), its intrinsic, conformational equilibria have great potential significance in the regulation of muscle contraction. A detailed analysis of the distribution of states of histidine-153, 153′ of tropomyosin will be the subject of another paper; here we limit the discussion to a few observations. If the widest splitting seen in the spectra of Figure 5, about 0.14 ppm, is assumed to occur between two interconverting states of equal populations and to be in the slow exchange limit at 28 °C, we can calculate that the rate of interconversion is less than 84 s^{-1} (Carrington & McLachlan, 1967). The maximum rate will be slower if closer resonances within the envelope are derived from directly interconverting states. The assumption of slow exchange is supported by the similar splitting of the histidine-153, 153′ resonance in cardiac tropomyosin which is unchanged when the temperature is raised from 28 to 40 °C. Of course this experiment also says that the rate of interconversion at a given temperature must be lower for cardiac tropomyosin than for β-tropomyosin since the latter passes out of the slow exchange region at a lower temperature. This difference between the two types of chain cannot be explained by the fact that the β-tropomyosin has been carboxymethylated or previously denatured; the corresponding α-tropomyosin, isolated from the same preparation of carboxymethylated skeletal tropomyosin, behaved essentially the same as cardiac tropomyosin.

Another interesting observation, which comes from preliminary experiments with cardiac tropomyosin, is that the distribution of histidine-153, 153′ resonances is narrowed by cross-linking the two polypeptide chains with a disulfide bond at cysteine-190 and 190′. McLachlan & Stewart (1976) have assigned the troponin binding site to a region near cysteine 190, 190′ which is some 50 Å along the coiled coil from histidine-153, 153′. By contrast, the distribution of states appears to be unaffected by magnesium ions (5 mM) at physiological ionic strengths.

Several other experimental techniques have detected at least one conformational change in tropomyosin. Fluorescent polarization (Mihashi, 1972; Satoh & Mihashi, 1972; Ohyashiki et al., 1976), electrical birefringence (Asai, 1961), electron spin resonance (Chao & Holtzer, 1975) and denaturation by guanidine, urea, or heat (Noelken & Holtzer, 1964; Bodwell et al., 1965; Mani et al., 1975; Woods, 1976) exemplify but do not exhaust the variety of such studies. The accumulated evidence indicates that a conformational change in the structure of tropomyosin, which is probably a local disruption of the coiled coil, occurs at or above 30 °C. It has been variously reported to be independent of the degree of polymerization (Satoh & Mihashi, 1972; Asai, 1961) or to be dependent (Ohyashiki et al., 1976), although the same conformational change is not necessarily detected by all the studies. The conformational equilibria which we have detected seem to be independent of the degree of polymerization. Neither dilution at pH 7.4 nor partial digestion with carboxypeptidase A, which destroys the ability to polymerize (Tawada et al., 1975; Johnson & Smillie, 1977), nor the addition of salt prevents the broadening of the C-2 resonance of histidine-153, 153′.

Changes in ionic strength do affect the pH titration of the histidine residues of tropomyosin, but the effects of depolymerization by salt are entangled with those of ionic strength and electrostatic charge. McLachlan & Stewart (1975) have proposed that histidine-276, 276′ is salt-linked to aspartate-2,

2′ of the next monomer in the polymerized form of tropomyosin. Because any histidine involved in a salt bridge should have a higher observed pK_a, the effect of increasing ionic strength, which depolymerizes tropomyosin, would be to lower the observed pK_a. On the other hand, increasing the ionic strength should raise the pK_a of an isolated histidine ring and Gly-His-Gly follows this pattern. Its data in Figure 7 fit the Debye-Huckel equation (Edsall & Wyman, 1958) quite well up to an ionic strength of 2.0 M, even though the equation has little theoretical validity beyond about 0.2 M. But the histidine rings of tropomyosin are not isolated. Although we expect them to be exposed to solvent, they are attached to a long rod which bears an electrostatic charge of approximately −40 esu at pH 7.4 (Iida & Imai, 1969). The Linderstrøm-Lang equation is commonly used to explain the effect of ionic strength on the acid dissociation constant of a titrating group attached to a polyelectrolyte of average charge, Z (Martin, 1964). If pK_a^{intr} is the intrinsic acid dissociation constant when the group is isolated (i.e., charge independent) and pK_a^{obsd} is the observed acid dissociation constant when the group is attached to the polyelectrolyte, then for aqueous solutions at room temperature

$$pK_a^{obsd} = pK_a^{intr} - 0.868wZ$$

where w is a function of the ionic strength and of the shape of the polyelectrolyte. Iida & Imai (1969) have determined experimental values for w of 0.04 and 0.008 for tropomyosin in 0.01 M and 1 M KCl, respectively. These values predict that the observed pK_a of the histidine residues should fall by approximately 1.1 units when the ionic strength is raised to 1 M.

Acknowledgment

In this work we have benefited greatly from the interest and advice of Dr. C. M. Kay and of Dr. L. B. Smillie and members of his research group, especially Dr. P. Johnson and M. D. Pato. We thank Dr. L. B. Smillie and his group for the generous gift of the various tropomyosin preparations used in this study.

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