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Nuclear Magnetic Resonance Studies of Trypsin Inhibitors. Histidines of Virgin and Modified Soybean Trypsin Inhibitor (Kunitz)†

John L. Markley

ABSTRACT: Titration studies of the two histidine residues of virgin and modified soybean trypsin inhibitor (Kunitz) have been carried out. The pK values are 7.00 and 5.27 in the virgin inhibitor and 6.82 and 5.91 in the modified inhibitor. The histidine residue of soybean trypsin inhibitor having the lower pK exists in an abnormal environment (shielded chemical shift) in its positively charged form in virgin, but not in modified, inhibitor. The line width of the histidines (particularly the histidine with the higher pK values) undergoes a maximum at pH* 6.75 in modified but not virgin soybean trypsin inhibitor suggesting the existence of a pH-dependent conformational equilibrium in modified inhibitor. There is a large difference in the rate of deuterium exchange of the C-2 protons of the two histidine residues of virgin soybean trypsin inhibitor at pH* 5.0 but not at pH* 6.0.

Protein proteinase inhibitors make up an important class of biological macromolecules whose role appears to be the regulation of activity of proteolytic enzymes. Since proteolytic enzymes are potentially dangerous molecules occurring in the external or internal environments of cells, it is not surprising that protein proteinase inhibitors have been discovered in a wide range of organisms from bacteria to man (for a recent review see Laskowski and Sealock, 1971). Like other macromolecules having a regulatory function, protein proteinase inhibitors have a diversity of specificities. Physiological target proteinases are still unknown for many of the protein proteinase inhibitors that have been discovered. The usual method of classifying inhibitors is by their specificity toward known classes of proteinases (*e.g.*, trypsins, chymotrypsins, elastases, etc.).

Soybean trypsin inhibitor (STI)[1] (Kunitz, 1947) is a protein of mol wt 21,500. The sequence of its 181 amino acid residues and the pairing of its two disulfide bridges are known (Koide *et al.*, 1972). The inhibitor has been the subject of numerous physical chemical studies (Laskowski and Sealock, 1971). Furthermore, the kinetics of its interaction with trypsin have been studied in great detail making it one of the best understood examples of protein–protein interaction (Laskowski, 1970).

Finkenstadt and Laskowski (1965) discovered that a peptide bond is split when soybean trypsin inhibitor is incubated with a catalytic amount of trypsin. They named the intact and nicked forms of the inhibitor the *virgin* (S) and *modified* (S*) forms, respectively. The point of trypsin cleavage between Arg^{63} and Ile^{64} (Ozawa and Laskowski, 1966) identifies the *reactive-site* region of the inhibitor (see Figure 1 in the preceding article, Mattis and Laskowski, 1973). In an elegant series of experiments, Laskowski's group has investigated the role of various amino acids in the active-site region. They have shown conclusively that Arg^{63} determines the specificity of STI toward trypsin (Leary and Laskowski, 1973). Until the present there has been no indication of a conformational change in STI on modification (Niekamp, 1971). However, an anomalous pH dependence of the trypsin-catalyzed equilibrium between S and S* has been reported (Hixson, 1970, quoted in Laskowski and Sealock, 1971). This small perturbation suggests the existence of one or more groups in STI whose pK values are shifted upward in the region 5.3–5.9 upon modification (Hixson, 1970; Mattis and Laskowski,

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[1] Abbreviations and symbols used: STI, soybean trypsin inhibitor in all its forms; S, virgin soybean trypsin inhibitor; S*, modified soybean trypsin inhibitor; His^{H1} and His^{H2}, the two histidines of soybean trypsin inhibitor (the identification, H1 and H2, is by nmr peak; see Figure 1); His^{H1^+}, histidine residue H1 in its positively charged form; δ, chemical shift (parts per million).

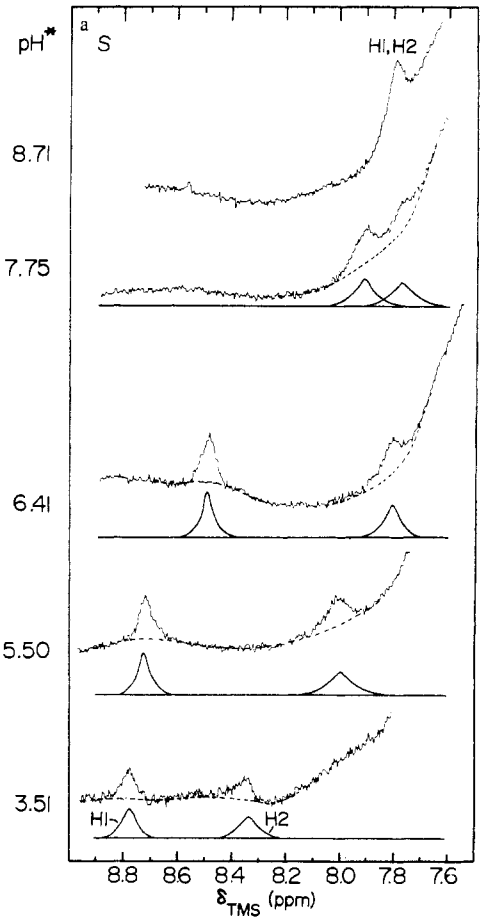
1973). The author wishes to report here nuclear magnetic resonance titration data (Meadows, 1972) on the two histidine residues of virgin and modified STI which indicate that a conformational change upon modification raises the pK_a of one histidine residue from 5.27 to 5.86 and causes a slight change in the environment and pK_a of the second histidine. As is discussed in the preceding paper (Mattis and Laskowski, 1973), these pK changes explain the anomaly in the pH dependence of the S–S* equilibrium.

Experimental Procedures

Materials. S and S* were prepared as described in the preceding paper (Mattis and Laskowski, 1973). The S* was shown by disc gel electrophoresis to consist of a 90–10% mixture of S* and S. Protein samples were lyophilized three times from 99.8% ^2H_2O (Bio-Rad) and then dissolved in ^2H_2O containing 0.50 M KCl and 0.050 M $CaCl_2$. With the exception of ^2H_2O, this is the standard solvent used in Laskowski's laboratory in their trypsin inhibitor studies. STI concentrations were measured using an optical factor of 1.1 mg/ml per absorbance unit at 280 nm (Kunitz, 1947). The STI concentrations were 1.4 mM at high pH and approximately 0.4 mM below pH 4.8 for S and below pH 4.6 for S* where the inhibitors become abruptly less soluble. Spectra of S at pH 5.0 showed no concentration dependence between 0.27 and 2.0 mM. The tripeptide Gly-His-Gly (Sigma) was lyophilized from ^2H_2O and dissolved in ^2H_2O containing 0.3 M NaCl. The Gly-His-Gly concentration was 50 mM.

Methods. pH measurements of solutions in ^2H_2O were made at 25° using a Corning digital 112 pH meter with a small Fisher combination electrode. The meter was calibrated with two standard pH buffers in ^1H_2O (Sargent-Welch) bracketing the pH reading. The pH values given are the actual meter readings and have not been corrected for the deuterium isotope effect at the glass electrode. The notation pH* is used to designate uncorrected readings obtained in this manner. It has been found (Meadows, 1972) that histidine pK values determined directly from uncorrected glass electrode readings in ^2H_2O agree well with those determined in ^1H_2O. Apparently the isotope effect on pK values is equal and opposite to the glass electrode effect. Adjustment of pH was made by adding 1 M NaO^2H or 1 M ^2HCl by means of a micrometer syringe (Gilmont) and a fine Teflon needle (Hamilton). No significant denaturation of STI was detected as long as the acid or base was added slowly with rapid mixing. The pH of each solution was measured before and after the nmr spectrum was taken. In the His titration region, spectra were used only if the "before" and "after" pH readings agreed by ±0.02 pH unit.

Nmr spectra were taken with a Varian XL 100-15 spectrometer using an internal deuterium lock. The spectrometer settings were as follows: rf field, 85 dB; filter, 4 Hz; spin rate, 30 rps; sweep width, 250 Hz; sweep rate, 2.5 Hz/sec. Time-



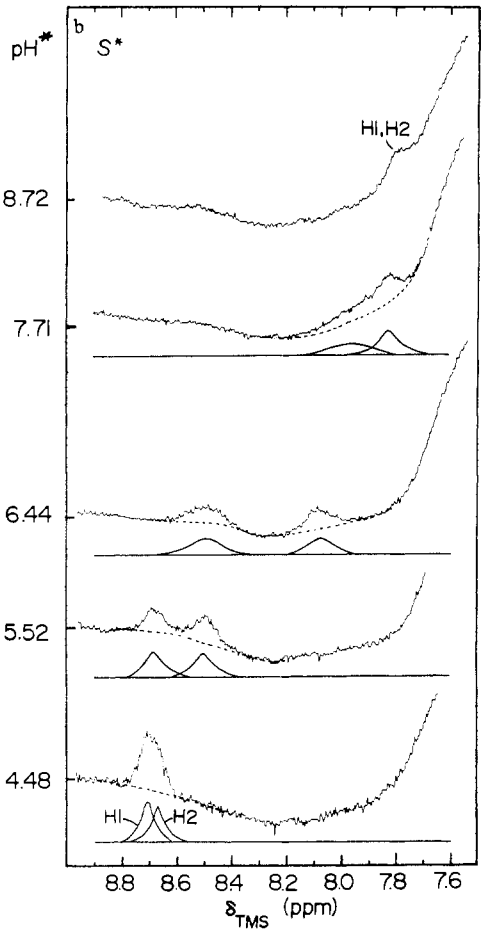


FIGURE 1: Representative proton nmr spectra at 100 MHz of the histidine region of soybean trypsin inhibitor at various pH* values. The spectra have been normalized to equal intensities. Two Lorentzian peaks of intensity equal to one proton have been fit to each experimental spectrum. The assumed base line is shown as a dashed line. Because the base line was ill defined, spectra of samples having pH >8 could not be fit unambiguously: (a) 0.4–1.4 mM virgin soybean trypsin inhibitor in ^2H_2O containing 0.3 M NaCl; (b) 0.4–1.4 mM modified soybean trypsin inhibitor in ^2H_2O containing 0.3 M NaCl.

TABLE I: Line Widths at 32° of the Two Histidine C-2-H Nuclear Magnetic Resonance Peaks of Virgin (S) and Modified (S*) Soybean Trypsin Inhibitor at Selected pH Values.

	$\Delta\nu_{1/2}$ (Hz)			
	S		S*	
pH	H1	H2	H1	H2
3.5	7.0	8.0		
4.5			5.0	5.0
5.5	5.5	9.0	7.5	8.0
6.4	5.0	6.0	12	10
6.75	9.0	~11.0	>25	~15
7.7	9.0	9.0	16	7.5

averaged spectra were accumulated in the continuous-wave mode using a Nicolet 1080 computer. The spectra represent the average of 128–256 scans. Samples (2.0 ml) were placed in 12-mm o.d. sample tubes (Wilmad). Teflon inserts (Thompson Packard) were used in the nmr tubes to prevent vortexing. Chemical shifts are reported in parts per million from external 5% Me_4Si (by volume) in CCl_4 contained in a coaxial capillary in the nmr tube.

Time-averaged spectra were stored on paper tape. For comparison purposes, spectra were adjusted to the same amplitude by equalizing the integrated intensity of the aromatic envelope. It was assumed that the total number of protons in this region does not change with pH. Peak positions and line widths (width at half-height) of His C-2-H resonances were determined by fitting Lorentzian peaks of intensity equal to a single proton to the spectra using a DuPont 310 curve resolver.

The observed histidine chemical shifts (δ_{obsd}) are weighted averages of the chemical shifts of the protonated (δ_{H^+}) and unprotonated (δ_{H^0}) forms (eq 1). Thus, the fraction of protein

$$\delta_{obsd} = \frac{\delta_{H^+}[His^+] + \delta_{H^0}[His^0]}{[His^+] + [His^0]} \quad (1)$$

with a given histidine protonated is given by $(\delta_{H^+} - \delta_{obsd})/(\delta_{H^+} - \delta_{H^0})$, where δ_{obsd} is the observed chemical shift of the ring proton of that histidine. The nmr titration data were fitted by a nonlinear least-squares program to the Hill equation

$$\frac{\delta_{H^+} - \delta_{obsd}}{\delta_{H^+} - \delta_{H^0}} = \frac{K_a{}^n}{K_a{}^n + [^2H^+]^n} \quad (2)$$

where K_a is the dissociation constant of the histidine, $[^2H^+]$ is the hydronium ion concentration calculated from the pH* measurements, and n is the Hill coefficient. Both four-parameter fits (K_a, δ_{H^+}, δ_{H^0}, and n free) and three-parameter fits (n fixed equal to 1) were tried.

Deuterium exchange of STI samples at 40° was carried out in the nmr tubes. Periodic spectra of the exchanging samples were taken at 32°. The period of time at 32° required for obtaining spectra was negligible compared to the half-time for exchange as determined by the decrease in intensity of the histidine C-2-H peak areas.

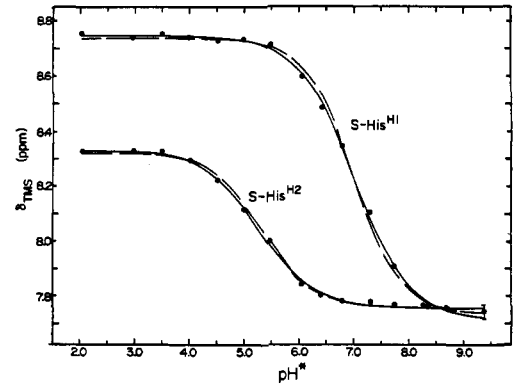


FIGURE 2: Least-squares fit of histidine chemical-shift data of virgin soybean trypsin inhibitor, S, to the Hill equation: solid line, fitted Hill coefficient, n (n = 0.85 for His^{H1}; n = 0.88 for His^{H2}); dashed line, Hill coefficient constrained equal to 1.

Results

Representative proton magnetic resonance spectra of the His C-2-H region of virgin (S) and modified (S*) soybean trypsin inhibitor are shown in Figure 1. The fitted His C-2-H peaks and the assumed base line are given in the figure. The peaks were identified as His C-2-H resonances on the basis of their areas (one proton), the pH dependence of their chemical shifts (Figure 2), and their extremely slow and pH-dependent deuterium exchange rates (Table III). The line widths of the His C-2-H peaks of S and S* at selected pH values are given in Table I. Histidine titration data on S, S*, and Gly-His-Gly are presented in Table II. For S and S*, the four-parameter least-squares fit with n free gave a better approximation to the data than the fit with n = 1. Calculated titration curves with n = 1 and n variable are compared with the experimental data for S in Figure 2 and for S* in Figure 3.

The best-fit titration curves of the histidines of S and S* are compared in Figure 4. The titration curve expected from a "normal" histidine (obtained from the tripeptide Gly-His-Gly) is shown as a dotted line for reference.

The effect of prolonged exposure of S to ^2H_2O at 40° on the intensities of the histidine peaks is shown in Figure 5. Deuterium exchange is much faster at pH* 6.0 than at pH* 5.0, and at pH* 5.0 peak H2 clearly exchanges more rapidly

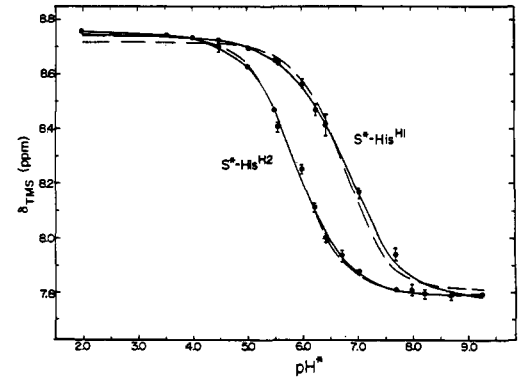


FIGURE 3: Least-squares fit of histidine chemical-shift data of modified soybean trypsin inhibitor, S*, to the Hill equation: solid line, fitted Hill coefficient, n (n = 0.75 for His^{H1}, n = 0.93 for His^{H2}); dashed line, Hill coefficient constrained equal to 1.

TABLE II: Least-Squares Analysis of Nuclear Magnetic Resonance Titration Data for the Histidine Residues of Virgin (S) and Modified (S*) Soybean Trypsin Inhibitor.[a]

	pK			Chemical Shift, δ (ppm)[b]		
	Hill Coeff Fixed at 1	Variable Hill Coeff	Fitted Value of Hill Coeff	δ_{H^+}	δ_{H^0}	$\Delta\delta_{H^+,H^0}$
S-H1	6.98 ± 0.03	7.00 ± 0.03	0.85 ± 0.04	8.75 ± 0.01	7.71 ± 0.01	1.03 ± 0.01
S-H2	5.28 ± 0.03	5.27 ± 0.03	0.88 ± 0.04	8.32 ± 0.01	7.75 ± 0.01	0.57 ± 0.01
S*-H1	6.76 ± 0.05	6.82 ± 0.03	0.75 ± 0.03	8.74 ± 0.01	7.77 ± 0.02	0.99 ± 0.02
S*-H2	5.91 ± 0.03	5.91 ± 0.03	0.93 ± 0.02	8.75 ± 0.04	7.79 ± 0.02	0.96 ± 0.01
Gly-His-Gly	6.81 ± 0.02	6.81 ± 0.02	1.03 ± 0.04	8.65 ± 0.01	7.70 ± 0.02	0.95 ± 0.01

[a] Data for the tripeptide Gly-His-Gly are given for reference. [b] Reference, 5% Me_4Si in CCl_4.

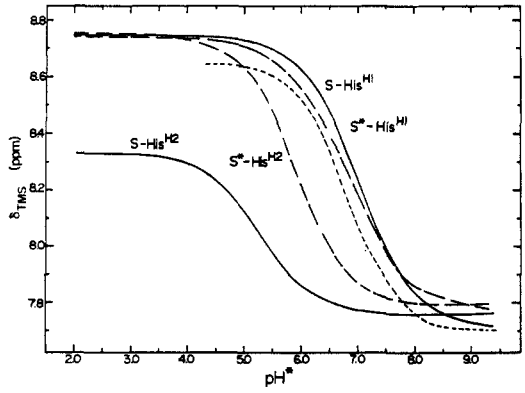


FIGURE 4: Comparison of the best fit nmr titration curves from Figures 2 and 3 for the two histidine residues of virgin (S, solid line) and modified (S*, dashed line) soybean trypsin inhibitor. The nmr titration curve for a "normal" histidine (from data on 50 mM Gly-His-Gly in ^2H_2O containing 0.3 M NaCl) is given as a dotted line for reference.

than H1. Approximate half-times for the exchange (Table III) were calculated assuming first-order kinetics.

Discussion

The two striking features of the nmr histidine titration curves of virgin and modified soybean trypsin inhibitor (Figure 4) are, first, the abnormal chemical shift of S-His^{H2+} resulting in an extremely shallow titration curve, and second, the increase in the pK of this histidine from a low to a more normal value on trypsin modification. The pK value of S-His^{H2} (5.27 ± 0.03) is the lowest of any titratable histidine residue reported thus far in the literature.[2] The present data are insufficient to reveal either the origin of the low pK or of the shallow titration curve of S-His^{H2}. However, some indication of the structural possibilities may be gained from studies of other proteins having similar titration curves.

The closest known histidine pK value is 5.55 for the peak assigned to His^{121} of staphylococcal nuclease (Meadows *et al.*, 1967; Markley, 1969; Jardetzky *et al.*, 1972). His^{121} of staphylococcal nuclease has δ_{H^+} and δ_{H^0} values that are both 0.25 ppm upfield of normal ($\Delta\delta_{H^+,H^0} \approx 1$). The X-ray data on staphylococcal nuclease (Cotton *et al.*, 1972) were obtained from crystals grown at pH 6.7–6.8 where the histidine is almost completely uncharged. The X-ray structure shows a hydrophobic interaction between His^{121} and Leu^7 and the N-2–H of His^{121} hydrogen bonded to the carboxyl group of Glu^{75}. One could argue that the high-field δ_{H^+} and δ_{H^0} values result from the hydrogen bond and the low pK results from placing a charge in a hydrophobic environment.

The peak assigned to His^{48} of ribonuclease (Meadows *et al.*, 1968) gives a shallow nmr titration curve (Meadows *et al.*, 1967) similar to that of S-His^{H2} but with a pK of 6.4. The X-ray structure of ribonuclease S crystallized at pH 6 (Wyckoff *et al.*, 1970; Richards and Wyckoff, 1971) shows the imidazole ring of His^{48} perpendicular to the ring of Tyr^{25} and hydrogen bonded to the hydroxyl of Thr^{82}. The C-2–H of His^{48} points toward the hydroxyl oxygen of Tyr^{25} which is hydrogen bonded to Asp^{14}.

In modified STI the environment of His^{H2+} is normalized, and the pK of the histidine is raised to 5.91 ± 0.03. The pK is still well below that for a "normal" histidine (the pK value of Gly-His-Gly at the same temperature and at somewhat lower ionic strength is 6.81). It is clear that modification must perturb the environment of histidine H2 in such a way as to make protonation of the ring more favorable. This could be accomplished either by removing positive charge from the vicinity of the histidine or by "unburying" the histidine.

The other histidine residue of STI, His^{H1}, has a normal pK and a normal chemical-shift environment in both the protonated and unprotonated forms. On modification it shows a slight but significant pK change from 7.00 ± 0.03 to 6.82 ± 0.03. This pK change is quite small in comparison to that of His^{H2}. On the other hand, there is a profound change in the line width of S-His^{H1} around pH 6–8 on modification (Table I). At

TABLE III: Half-Times for Deuterium Exchange at 40° of the Histidine C-2 Protons of Virgin Soybean Trypsin Inhibitor.

pH	Histidine	$t_{1/2}$ (Days)
5.0	S-H1	61 ± 2
	S-H2	24 ± 2
6.0	S-H1	7.1 ± 2
	S-H2	11 ± 2

[2] Matsuo *et al.* (1972) have reported a pK value for the single histidine of hen egg-white lysozyme of 5.2 based on kinetics of tritium exchange of the C-2–H. This is much lower than the value of 5.8 obtained directly by nmr titration (Meadows *et al.*, 1967).

pH* 6.4 the line width of S-His^{H1} is 5.0 Hz whereas that of S*-His^{H1} is 12 Hz. The width of S*-His^{H1} is maximal around pH* 6.75. At this pH the peak is so broad that it could not be detected at 100 MHz. A smaller increase in line width on modification, also with a maximum at pH* 6.75, is seen in His^{H2} (Table I).

There are two possible explanations for the broadening centered around pH* 6.75 in S*: (1) *dipolar broadening* caused by immobilization of the His C-2 protons in the vicinity of other magnetic nuclei (protons) at the critical pH, or (2) *exchange* broadening caused by a pH-dependent, dynamic conformational equilibrium which modulates the chemical shift of the His C-2 protons by $\Delta\nu$ Hz such that

$$\tau \approx \frac{1}{\pi\Delta\nu} \qquad (3)$$

where τ is the lifetime of one conformational state. The first explanation has been given for failure to detect His C-2-H resonances of two proteins having molecular weights close to that of STI, trypsin (mol wt 23,800), and chymotrypsin (mol wt 23,000) (Bradbury *et al.*, 1971).[3] The other possibility, exchange broadening, has been invoked to explain the broadening of histidine peak 4 (His^{48}) of ribonuclease in 0.2 M NaCl but not in 0.2 M acetate (Roberts *et al.*, 1969) and the behavior of histidine peak 2 (His^{46}) of staphylococcal nuclease under certain conditions (Markley *et al.*, 1970). Exchange broadening can result either from the slowing of a process already in fast exchange (for example, slowing the on-off rate for protonation and deprotonation of a histidine) or from the partial loosening of a previously rigid structure (such as cis–trans isomerization of a peptide bond).

Although dipolar broadening cannot be ruled out completely, exchange broadening is preferred in the present case. Nmr difference spectra of the aromatic region of S and S* (Markley, 1973[4]) reveal a transition affecting an aromatic residue at pH 6.75. It is possible that this transition may occur at the proper frequency and provide the proper chemical-shift perturbation of the histidines to cause the observed broadening.

The pK values for the newly formed Arg^{68} carboxyl terminal and Ile^{64} amino terminal of S* are known from the pH dependence of the trypsin-catalyzed equilibrium between S and S* to be 3.56 and 7.89, respectively (Mattis and Laskowski, 1973). Since the His C-2-H chemical shifts of S* are not perturbed in these pH regions, it seems safe to conclude that neither histidine is close to the active site in S*.

The fitted Hill coefficients for all histidine titrations of S and S* are significantly less than unity. This result is not surprising for groups on a protein since the net positive charge on the protein increases as the pH is lowered, making it increasingly more difficult (energetically) to add protons. The decrease in the Hill coefficient of His^{H1} of STI from 0.85 ± 0.04 to 0.75 ± 0.03 appears to be the only significant change in a Hill coefficient on modification. This change may be linked to the interaction causing the broadening of S*-His^{H1}.

In the preceding paper Mattis and Laskowski (1973) predict the existence of one or more groups whose net pK changes on modification from 5.30 to 5.86. There is a remarkable agreement between this prediction and the pK values of His^{H2} in S and S* (5.27 ± 0.03 and 5.91 ± 0.03). The only complication is the slight change of −0.18 in the pK of His^{H1} on modification which does not show up in Mattis and Laskowski's equilibrium data. This slight inconsistency may be attributable to differences in experimental procedures (deuterium isotope effect or concentration effect) or to the existence of another group having an equal but opposite pK shift. Mattis and Laskowski's (1973) data are based on deviations of the pH dependence of K_{hyd}, the equilibrium constant for the modification reaction, from the theoretical curve. It is interesting in this connection that the pK values of the four histidine residues of virgin ovomucoid, whose K_{hyd} follows the theoretical curve exactly (Schrode and Laskowski, 1971), are not perturbed significantly on modification (Markley, 1973).

Further experiments, designed to assign the two His C-2-H resonances of STI to His^{71} and His^{157} in the linear sequence and designed to investigate the pK values and environments of the histidines in the trypsin–STI complex, are under way.

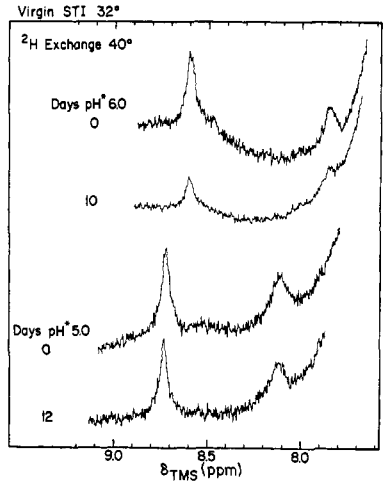


FIGURE 5: Effect of prolonged exposure of virgin soybean trypsin inhibitor to ^2H_2O on the intensities of the two His C-2-H nmr peaks. Deuterium exchange was carried out at 40° on 1.4 mM solutions of virgin soybean trypsin inhibitor in ^2H_2O containing 0.3 M NaCl. Half-times for exchange based on first-order kinetics are given in Table III.

Acknowledgments

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Segmental Flexibility of the S-1 Moiety of Myosin†

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ABSTRACT: The flexibilities of rabbit myosin and heavy meromyosin—labeled on the S-1 moiety with the fluorophore *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl)ethylenediamine—have been examined by the nanosecond fluorescence depolarization technique. It was found that there is considerable flexibility within heavy meromyosin which is localized near the (S-1)–(S-2) connecting joint. The S-1 moiety of myosin depolarized more slowly than free S-1 but with a relaxation time in agreement with a model in which S-1 is free to pivot at the (S-1)–(S-2) connection. S-1 was found to be highly elongate with an axial ratio of greater than 3.5 if considered a hydrated prolate ellipsoid. These results are shown to harmonize with recent suggestions that S-1 rolls on actin to produce muscle contraction.

Current speculations concerning the manner in which a myosin "cross bridge" attaches to the thin filament and imparts a mechanical thrust invariably assume that in the myosin molecule there is at least one point of flexibility between two segments. The existence of such a point has been inferred from enzyme susceptibilities and from morphological features of the filament assemblies. It occurred to us that the more direct approach which Stryer (1968), Tao (1969), and Yguerabide *et al.* (1970) used to examine for "segmental flexibility" in immunoglobulin might also be applicable to myosin.[1] Myosin too is Y-shaped, but its "stem" (light meromyosin (LMM) and S-2 portions) is very long. Here we have used the technique of fluorescence polarization decay to characterize the motion of the two "arms" (S-1 moieties), both when they are integral parts of the Y (as in myosin or heavy meromyosin (HMM)) and when they have been cut free of the stem (as in S-1), and then have argued that the observed relationship between these motions could only be obtained if the arms are *flexibly* attached to the stem. The fluorescence polarization decay technique consists of attaching a fluorophore to the single special (fast-reacting) SH group on each S-1 moiety; then we excite these fluorophores by a brief flash of plane-polarized (‖) light and measure the intensity of the two components ($I_{\parallel}$ and $I_{\perp}$) of the ensuing fluorescence as a function of time. From these intensities is constructed the "polarization anisotropy," $r(t) \equiv (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$; this function decays with time because the directional preference imposed by the polarized flash is progressively randomized by the rotational Brownian movement of the molecule bearing

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[1] In a pioneering investigation using steady-state fluorescence, Young *et al.* (1965) concluded what we shall conclude here, *viz.*, that there is segmental flexibility in myosin; however, the agreement between studies is only apparent. We find the rotational correlation time of the moving segments to be much greater than do they. Also, we find that the moving segments move more slowly when incorporated into the molecule than when free, but that the difference is just what mechanics predicts for attachment by a "swivel joint"; Young *et al.* find the motion to be the same whether the segments are incorporated or free.