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Nuclear Magnetic Resonance Studies of the Structure and Binding Sites of Enzymes. VII. Solvent and Temperature Effects on the Ionization of Histidine Residues of Ribonuclease*

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ABSTRACT: The chemical shift of the nuclear magnetic resonance absorption of the C_2 hydrogen of histidine is a measure of the protonation of the imidazole ring. By this means the titration curves of the four histidine residues of ribonuclease have been obtained under a variety of conditions. Comparisons are made between the titration curves obtained in H_2O or D_2O and 0.2 M sodium acetate or 0.2 M sodium chloride, and thermodynamic data are reported for the ionization of histidine residues 12, 119, and 105. Evidence for an isomerization of the enzyme involving histidine residue 48 is discussed.

The resolution of the nuclear magnetic resonance absorptions of the imidazole C_2 hydrogens of the four histidine residues in RNase[1] (Meadows *et al.*, 1967) and their assignment to specific residues in the amino acid sequence of the enzyme (Meadows *et al.*, 1968) have been reported. These histidine C_2–H peaks shift position as a function of pH, reflecting the protonation state of each imidazole ring (Meadows *et al.*, 1967, 1968). Thus, it is possible for the first time to determine the microscopic hydrogen ion dissociation constants of individual amino acids in a protein and to study the effects of solvent and temperature upon them.

Materials and Methods

Lyophilized, phosphate-free RNase (Worthington) was used without further treatment. Solutions of 0.2 M sodium chloride or sodium acetate were made up in 99.85% D_2O (Bio-Rad) unless otherwise stated, using NaCl (Merck reagent grade) or CD_3COOD and NaOD (Merck of Canada). H_2O solutions were made up in distilled, deionized water. RNase was dissolved in these solutions to give a final concentration of 0.0065 M

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[1] Abbreviation used: RNase, bovine pancreatic ribonuclease A (EC 2.7.7.16).

TABLE I: The pK Values of the Histidine Residues of RNase.[a]

Histidine Residue	0.2 M Sodium Acetate[b] 32°	0.2 M Sodium Chloride 10°	20°	32°	41°
12	6.2	6.46	6.35	6.23	5.77
48	6.4	*c*			
105	6.7	7.10	6.85	6.65	6.45
119	5.8	6.06	5.90	5.70	5.45

[a] Estimated accuracy ±0.05. [b] Meadows *et al.* (1968). [c] Not observable; see text.

(based on a molar extinction of 11,900 at 278 mμ; D. F. Veber and S. L. Varga, personal communication). The pH of the solutions was adjusted with 1.0 or 0.1 M NaOD or DCl and measured with a Radiometer Model 26 pH meter equipped with a combination microelectrode and a water-jacketed measuring vessel. The pH was measured both before and after the spectra were run, and the results were accepted only if the two measurements agreed to within 0.04 pH unit.

Nmr spectra (100 Mc) were obtained with a Varian HA-100 spectrometer with a V4343 variable-temperature controller. Probe temperature was 32° unless otherwise stated; the variable-temperature controller was calibrated with dry methanol. Sweep rate was 1 cps/sec, and spectra were averaged over 20–100 sweeps with a C1024 computer of average transients. Chemical shifts are given as cycles per second downfield from the external standard hexamethyldisiloxane.

Results and Discussion

Here, as in previous reports from this laboratory (Meadows *et al.*, 1967, 1968), pD values in D_2O are given as uncorrected glass-electrode meter readings, and the pK values are calculated from these figures. RNase has now been titrated in 0.2 M acetate in H_2O, and it has been found that the titration curves of all four histidine residues in H_2O and in D_2O are superimposable over the whole pH range 4.5–8.5. Since the isotope effect on the glass electrode of about 0.4 pH unit (Glasoe and Long, 1960) would produce an apparent shift in the titration curves toward low pH, the deuterium isotope effect on the ionization equilibrium of the histidines, which produces a shift in the opposite direction, must also be approximately 0.4 pH unit. This is similar to the values of 0.54 found for histidine by Li *et al.* (1961) and 0.4–0.5 for a number of histidine derivatives (D. Meadows, 1967, unpublished data). It is clear, therefore, that the pK values obtained in D_2O are coincidentally identical with those in H_2O. Furthermore, since the apparent isotope effect on the pK's of all the histidines is identical, and since there are no chemical shift differences between H_2O and D_2O, it is most unlikely that there is any conformational difference in the enzyme between H_2O and D_2O which affects the environment of any of the histidine residues.

A comparison of the pK values of the histidine residues in 0.2 M sodium acetate and 0.2 M sodium chloride (both in D_2O) is given in Table I. The pK values of histidines-105 and -12 are essentially identical in both solvents, while that of histidine-119 is 0.1 pH unit lower in NaCl. This small difference is outside the experimental error, and may reflect a greater binding of acetate than of chloride in the vicinity of histidine-119 at about pH 6. This is in agreement with the relative effectiveness of acetate and chloride as inhibitors of the urea denaturation of RNase at pH 7 (Nelson *et al.*, 1962). Figure 1 shows the titration curves of histidine-119 in 0.2 M sodium chloride and 0.2 M sodium acetate, together with a theoretical curve calculated from the Linderström-Lang (1924) equation as modified by Cannan *et al.* (1942). The slopes of both experimental curves are close to that of the theoretical curve down to pH 5.0; the deviation of the curve obtained in NaCl below this pH may reflect the increase in binding of chloride ion which is known to occur below pH 5.0 (Loeb and Saroff, 1964) and which would be expected to produce an upfield shift of the histidine nmr absorption. The titration curves of the other three histidine residues have slopes essentially identical with those of

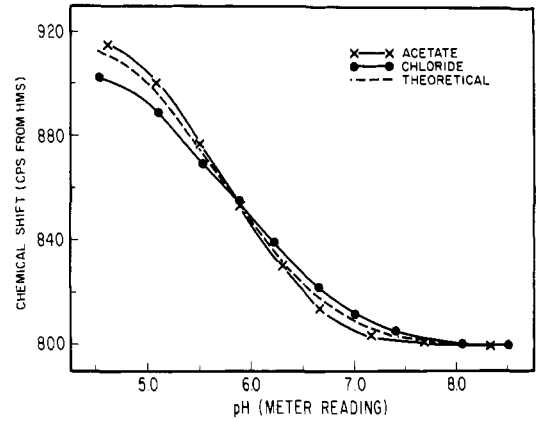

FIGURE 1: Chemical shift of the imidazole C_2 proton of histidine-119 of RNase in 0.2 M sodium chloride (●—●) and 0.2 M sodium acetate (X—X) as a function of pH. The middle curve (– – – –) is a theoretical one, calculated from Linderström-Lang's (1924) equation, using $\omega = 0.08$ and values of Z from Tanford and Hauenstein (1956). The theoretical curve has been adjusted to have a pK of 5.75; the pK values of the experimental curves are 5.8 (acetate) and 5.7 (chloride).

TABLE II: Thermodynamic Parameters for the Ionization of the Histidine Residues of RNase.[a]

Histidine Residue[a]	ΔH (kcal/mole)[b]		ΔF (kcal/mole)		ΔS (eu)	
	10–32°	32–41°	20°	41°	20°	41°
12	4.0	21.1	8.5	8.3	−15.3	+41.0
105	8.2	8.2	9.2	9.3	−3.5	−3.5
119	6.3	11.4	7.9	7.8	−5.5	+11.5

[a] In 0.2 M NaCl. In this solvent, the C_2–H peak of histidine-48 is too broad to be observable; see text. [b]Calculated from the slopes of the lines in Figure 3.

the corresponding theoretical curves over the whole pH range. Thus, if there is any specific interaction between these histidine residues and other groups titrating in this pH range, its effect is too small to be detectable from our data. In particular, a hydrogen bond between histidine-12 and histidine-119 is most unlikely. It is therefore justifiable to assume that the ionizations of the two histidine residues at the active site (12 and 119) are independent, and further that the change in chemical shift of the C_2–H in this pH range is due solely to the change in the protonation state of the imidazole ring.

The most striking difference between nmr spectra of RNase obtained in chloride and in acetate is in the behavior of the C_2–H absorption of histidine-48. In acetate this peak, though broad, can be followed throughout its titration range, while in chloride it is too broad to be observable above pH 5.5 (see Figure 2). There are two possible causes of this broadening: a decreased motional freedom of the imidazole of histidine-48 in chloride, or a decreased rate of exchange of this group between two different environments in chloride compared with acetate. In view of the effects of inhibitors on the line width of this peak (Meadows and Jardetzky, 1968; D. H. Meadows, G. C. K. Roberts, and O. Jardetzky, in preparation), the latter explanation is the more likely. We have suggested earlier (Meadows and Jardetzky, 1968) that these changes in the C_2–H nmr signal of histidine-48 reflect a conformational equilibrium involving that part of the peptide chain containing lysine-41 and histidine-48. The effect of acetate in increasing the rate of this exchange is most readily explained if the isomerization involves a change in the state of protonation of histidine-48, since acetate might be expected to accelerate the proton exchange rate by nucleophilic catalysis. In this connection it is interesting that the broad, pH-independent peaks in the region of the nmr spectrum of RNase between 775 and 950 cps (see Figure 2), which are probably non-exchanged peptide NH peaks, are more marked in chloride than in acetate. The idea of an isomerization of RNase involving the uptake and release of protons is in agreement with the temperature-jump experiments of French and Hammes (1965), who also suggested the involvement of a histidine residue.

The titration curves of the histidine residues of RNase have also been determined at 10, 20, and 41° in 0.2 M NaCl, and the pK values obtained are shown in Table I. The C_2–H resonances of histidines-119 and -48 are

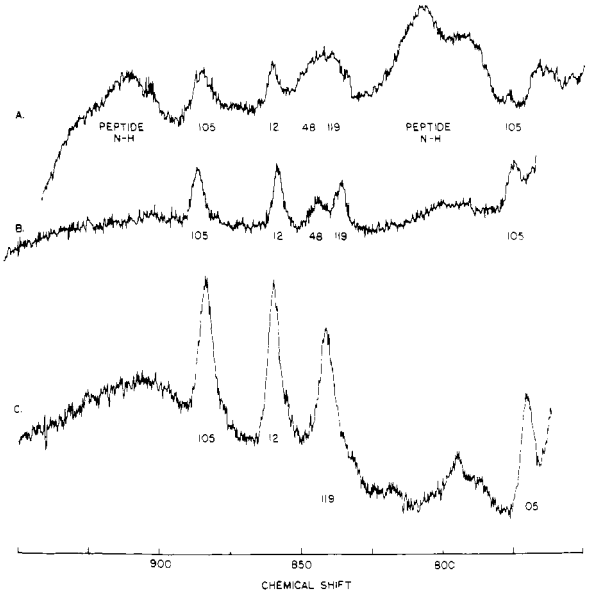

FIGURE 2: Histidine region of the nuclear magnetic resonance spectrum of ribonuclease. The C_2–H peaks of histidines-12, -48, -105, and -119 and the C_4–H peak of histidine-105 are indicated. (A) 0.2 M sodium acetate, H_2O, pH 6.10; (B) 0.2 M sodium acetate, D_2O, pH 6.10; and (C) 0.2 M sodium chloride, D_2O, pH 6.10.

broadened much more on reducing the temperature to 10° than are those of histidines-105 and -12. This observation is compatible with the idea that histidine-48 is exchanging between two different environments in the enzyme, and suggests that this may also be true of histidine-119.

The temperature dependence of the dissociation constants of histidine-12, -105, and -119 is shown in Figure 3, and the values of free energy, enthalpy, and entropy of ionization of these residues are given in Table II. Histidine-105 has values for pK, ΔH, and ΔS close to those found for imidazole and histidine (Kirby and Neuberger, 1938; Cohn and Edsall, 1943; Nozaki *et al.*, 1957), and the value of ΔH is constant over the temperature range 10–41°. These findings are consistent with the position of histidine-105 on the surface of the molecule, freely accessible to solvent (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967). In the temperature range 10–32°, both histidines-12 and -119 have lower values of pK and ΔH than the normal values found for histidine-105. The

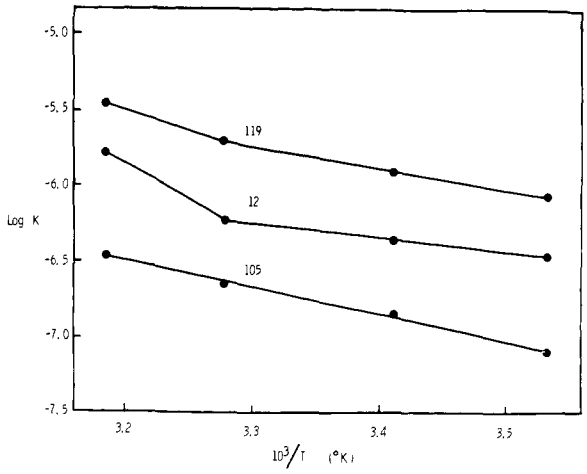

FIGURE 3: Temperature dependence of the dissociation constants of histidines-12, -105, and -119 of RNase. The estimated error in the measurements (±1° in T, ±0.05 in log K) is indicated approximately by the size of the symbols.

enthalpy of ionization of histidine-12 is in fact only half that of histidine-105, and this difference can be accounted for by the much larger negative value for ΔS shown by histidine-12. The interpretation of these differences is not straightforward, since the magnitudes of the various contributions to the entropy change are unknown. It seems likely, however, that the major contribution is the greater ordering of water molecules around the released proton than around the imidazolium ion, which has a much lower charge density and lower symmetry. If this is so, then it appears that histidine-12 is much less solvated than residues 119 or 105; this is confirmed by the crystal structure of the enzyme (H. Wyckoff, personal communication) in which histidine-12 is partially buried while histidines-105 and -119 appear to be freely accessible to water.

Between 32 and 41° the enthalpy of ionization of histidines-12 and -119 is much greater than that of histidine-105 over the same temperature range, and much greater than that of the same two histidines (12 and 119) at lower temperatures. The entropy of ionization of those residues calculated at 41° is quite large and *positive*, in contrast to the negative values of ΔS seen at lower temperatures. It seems likely that this marked change with increasing temperature represents a slight unfolding of the protein. Although the reversible thermal denaturation of RNase, as determined by changes in ultraviolet absorption and optical rotation, has a midpoint of about 60° in the pH range 5–6 (Hermans and Scheraga, 1961), there is some evidence for a conformational transition at lower temperatures. Hirs (1962) found that the chromatographic behavior of RNase on an ion-exchange column, using phosphate buffer (pH 6.0) as eluent, was markedly temperature dependent in the range 35–40°. The midpoint of the transition was approximately 38°. Hirs (1962) suggested that the transition involved a localized unfolding of the region of the molecule containing the phosphate binding site. Since phosphate appears to bind very close to histidines-12 and -119 (Kartha *et al.*, 1967; D. H. Meadows, G. C. K. Roberts, and O. Jardetzky, in preparation), it is likely that the marked change in the ΔH of ionization of these two residues reflects the same conformational change as that observed by Hirs (1962). Histidine-12 appears to be partially buried, while histidine-119 is not, and a greater effect of a slight unfolding in this region on histidine-12 would therefore be expected; this is in fact observed (Figure 3). The lack of any effect on histidine-105 of this presumed conformational transition supports the idea that the change is a localized one, though it is possible that histidine-105 is already so exposed to the solvent and removed from the influence of neighboring groups that unfolding of the peptide backbone has no effect. Further studies of the thermal unfolding of RNase are in progress to clarify the nature of these effects on the histidine residues.

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