Correlation Proton Magnetic Resonance Studies at 250 MHz of Bovine Pancreatic Ribonuclease. I. Reinvestigation of the Histidine Peak Assignments†

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ABSTRACT: The deuterium exchange kinetics of the C(2) protons of the four histidine residues of native bovine pancreatic ribonuclease A have been followed at pH* 6.5 and 8.0 by proton magnetic resonance spectroscopy (^{1}H NMR). Comparison of the order of exchange of the histidine peaks with tritium exchange rates into individual histidine residues [Ohe, M., Matsuo, H., Sakiyama, F., and Narita, K. (1974), *J. Biochem. (Tokyo) 75,* 1197] supports the previous assignment of histidine NMR peaks H(1) and H(4) to histidine-105 and histidine-48 but requires reassignment of peaks H(2) and H(3) to histidine-119 and histidine-12, respectively. Ribonuclease A samples having differentially deuterated histidines have been used to verify the existence of crossover points in the histidine proton magnetic resonance titration curves and to observe the discontinuous titration curve of histidine-48. Proton magnetic resonance peaks have been assigned to the C(4) protons of the four histidine residues of ribonuclease A on the basis of their unit proton areas and by matching their titration shifts with the more readily visible C(2)-H peaks of the histidines. The pK' values derived from the C(4)-H data agree, within experimental limits, with those derived from C(2)-H data. The C(4)-H peaks were assigned to histidine-12, -48, -105, and -119 of ribonuclease A on the basis of their pH dependence, pK' values, shifts of their pK' values in the presence of inhibitor cytidine 3′-phosphate, and by comparison with the assignments of the histidine C(2)-H peaks above.

In 1962 Wishnia and Saunders demonstrated the possibility of using proton magnetic resonance (^{1}H NMR) spectroscopy to study deuterium exchange in proteins. They followed the intensity from overlapping resonances in the N-H region of spectra of bovine pancreatic ribonuclease A (RNase A)[1] as a function of time. Approximately 24 protons appeared to exchange more slowly than the others, and it was suggested that these correspond to slowly exchanging guanidino protons of the arginine residues. A more recent study at 60 MHz of the N-H protons of RNase A was carried out by Bradbury and King (1972).

With the introduction of high resolution NMR spectrometers using superconducting solenoids, it has become possible to resolve NMR peaks from single exchanging sites. Thus NMR spectroscopy provides an advantage over conventional hydrogen exchange techniques in that the exchange kinetics at single sites can be determined. Glickson and coworkers (1969, 1971) succeeded in resolving peaks corresponding to five of the six tryptophan N-H protons of lysozyme; and the deuterium exchange kinetics of these peaks were used as evidence for their assignment to specific tryptophan residues. Recently, Karplus et al. (1973) and Masson and Wüthrich (1973) have resolved up to 15 single-proton resonances in the spectral region δ −11 to −7.5 of bovine pancreatic trypsin inhibitor (Kunitz).

At pH values above 5, the carbon-bound C(2)-H proton of the histidine ring exchanges at a rate comparable to those of the slowly exchanging N-H protons (Meadows et al., 1968). Meadows and coworkers (1968) used this exchange reaction in their assignment of the NMR peak corresponding to the C(2)-H of His[12] of RNase A. The deuterium exchange kinetics of histidine C(2)-H protons of native proteins have been studied by Bradbury and Chapman (1972), and by Markley (1973a,b) and Markley and Cheung (1973) who showed that differential exchange of the histidine C(2)-H peaks of several native proteins can be achieved. Hydrogen exchange of the histidine C(2)-H protons of histidine in native proteins has also been followed by tritium exchange. Narita and coworkers have recently investigated the tritium exchange reaction in lysozyme (Matsuo et al., 1972), and ribonuclease A (Ohe et al., 1974) as an alternative method to ^{1}H NMR spectroscopy for determining the pK' values of histidine residues in proteins. We have suggested the combination of deuterium and tritium exchange studies as a technique for the unambiguous assignment of histidine ^{1}H NMR peaks in protein spectra (Markley and Cheung, 1973).

The present experiments were undertaken to test present techniques for the assignment of histidine C(2)-H and C(4)-H NMR peaks in proteins. The results reveal errors in the previous assignments of histidine peaks of ribonuclease A (Meadows et al., 1968; King and Bradbury, 1971; Bradbury and Chapman, 1972).

Experimental Section

Materials. Bovine pancreatic ribonuclease A (Worthington RAF Lot 53 H372, lyophilized, phosphate free) was used without further purification. 99.8% D_2O (Bio-Rad)

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[1] Abbreviations used are: RNase A, bovine pancreatic ribonuclease A; RNase S, ribonuclease S, the subtilisin cleavage product of RNase A; pH*, uncorrected pH meter reading of a D_2O solution made with a glass electrode standardized in H_2O buffers.

was used in all exchange experiments. KOD and DCl used for pH adjustment were from Merck of Canada. All other chemicals used were reagent grade.

Instrumentation. ^{1}H NMR spectra were obtained with a 250-MHz superconducting spectrometer system (Dadok et al., 1970) using the correlation technique (Dadok and Sprecher, 1974). The spectra are the result of 500, 1.5-sec scans in the low-field direction of a 1.5-kHz region downfield from the water peak. A time constant of 0.57 msec was used with the Princeton Applied Research phase detector amplifier, and the data were smoothed using a 1.5-Hz digital filtering factor. The resultant "instrumental" line broadening is estimated to be 2 Hz. The high frequency noise appearing at the low-field end of some spectra is an artifact resulting from unequal intensity at the extremes of the correlation spectrum. This noise can be filtered out at the expense, however, of the resolution. The sample temperature was 30°. An internal proton lock to the residual HDO peak was used to stabilize the field.

Procedures. The pH measurements and adjustments were carried out as previously described (Markley, 1973a). The notation pH* is used to indicate direct pH meter readings of D_2O solutions uncorrected for the deuterium isotope effect at the glass electrode using electrodes standardized in H_2O buffers.

The concentration of RNase A in all solutions used for deuterium exchange and for NMR spectroscopy was 40 mg/ml. Six deuterium exchange procedures with RNase A were studied. For clarity, these will be referred to as procedures A–G. (A) RNase A was dissolved in 0.3 *M* NaCl in D_2O; the pH* was adjusted to neutrality; and NMR spectra were taken immediately (within 10 min). (B) RNase A was dissolved in D_2O (resulting pH* 9.2), and the solution was lyophilized. This procedure was repeated twice. Then the lyophilized material was dissolved in 0.3 *M* NaCl in D_2O and the pH* adjusted. (C) RNase A was dissolved in D_2O containing 0.3 *M* NaCl, and the pH* was adjusted immediately to 6.5. The solution was incubated at 40° for a specified period of time. (D) RNase A was dissolved in D_2O, and the pH* was adjusted to 6.5. This solution was heated at 60° for the time specified, cooled, and lyophilized. The material was then dissolved in 0.3 *M* NaCl in D_2O. (E) RNase A was dissolved in D_2O, and the pH* was adjusted to 3.0. The solution was heated for 1 hr at 60°, cooled, and lyophilized. (F) The pH* of an RNase A solution prepared by procedure E was adjusted to 8.0. The solution was incubated at 40° for a specified time. (G) The pH* of an RNase solution prepared by procedure E was adjusted to 6.5. The solution was incubated at 40° for a specified time.

Acetate was added as a solution of 2.2 *M* potassium acetate in D_2O prepared by neutralizing acetic acid-*d* (Aldrich) with KOD (Merck of Canada). Cytidine-3′-phosphoric acid (3′-CMP, Sigma) was neutralized with KOH and lyophilized three times from D_2O before use. It was added as a 0.016 *M* solution in D_2O. Concentrations of 3′-CMP and RNase A were determined spectrophotometrically.

In the histidine C(2)–H deuterium exchange studies, aliquots of solutions F and G were withdrawn at various times (from zero time to 7.71 days), and the exchange reaction was stopped by lowering the pH* to 3.00 and then freezing the samples. The solutions were thawed and the pH* was checked before taking NMR spectra.

NMR spectra were taken with 300-μl protein solutions in 5-mm o.d. tubes (Wilmad 528 PP). A sealed coaxial capillary (Wilmad 520-3) containing 5% $(CH_3)_4Si$ in CCl_4 was used as an external reference. All chemicals shifts are reported from 5% $(CH_3)_4Si$ in CCl_4. These may be converted to chemical shifts from pure $(CH_3)_4Si$ by the equation

$$\delta_{(CH_3)_4Si} = \delta_{5\%\ (CH_3)_4Si\ in\ CCl_4} + 0.463 \quad (1)$$

Deuterium exchange of the histidine C(2)–H protons was followed by measuring the peak heights of the histidine peaks rather than their areas as was done previously (Markley and Cheung, 1973). Peak H(1′), the C(4)–H of His^{105} (Meadows et al., 1968), was used as an internal intensity standard. It was assumed that this proton does not exchange under the conditions used. The exchange data were fitted to a single exponential decay curve using a nonlinear least-squares program T1FIT written by Claudette Lederer of the University of California, Berkeley, and modified for use at Purdue by Dr. William R. Finkenstadt. NMR titration data were fitted to the Hill equation by a nonlinear least-squares program written by Dr. Finkenstadt (Markley, 1973a).

Results

Comparison of Various Exchange Procedures. RNase A in D_2O contains approximately 42 N–H protons in the spectral region δ −10.0 to −7.1 that exchange slowly with solvent when the protein is in its native configuration at 25° and neutral pH (exchange procedure A). After lyophilization three times (exchange procedure B) from D_2O at pH 9 the number of N–H protons remaining is approximately 37. These exchange with a half-time of several days or more between pH* 1 and 9. The N–H peaks exchange at very slow rates (comparable to the histidine C(2)–H peaks) if exchange is continued at 40° for prolonged periods of time (exchange procedure C).

Nearly all N–H peaks are removed by heating RNase A in D_2O to 60°. The histidine C(2)–H protons also exchange if the heating is carried out at pH* 6.5 (exchange procedure D); however, if the heating is carried out at pH* 3.0 the N–H peaks may be removed without appreciable exchange of the histidine C(2)–H peaks (exchange procedure E).

It was of interest to determine whether the procedure used to exchange N–H and histidine C(2)–H protons alters the structure of RNase A, and, in particular, the environment and pK' values of the histidines. In order to test this, two independent titration studies were carried out with a sample of RNase A lyophilized from D_2O (procedure B) and a sample exchanged for 12 days at pH* 6.5 and 40° (procedure C). The histidine NMR titration curves obtained from these two samples were indistinguishable within experimental limits.

The exchange procedures that involve heating RNase A to 60° (procedures D and E) also appear to have no effect on the histidine chemical shifts as judged by comparing spectra of exchanged samples with nonexchanged samples at several pH values. Benz and coworkers (1972) have exchanged RNase A in D_2O at 65° and pH 7 for 5–10 min to remove N–H peaks from the histidine region, and have found no significant effect of the exchange treatment on ^{1}H NMR spectra or enzymatic activity of RNase A.

Kinetics of Deuterium Exchange of the Histidine C(2)-H Protons of RNase A. Representative ^{1}H NMR spectra of samples used to investigate the kinetics of histidine C(2)–H exchange in native RNase A are shown in Figure 1. RNase A was first exchanged by procedure E to remove all N–H peaks (Figure 1a). Exchange of the histidine C(2)–H peaks

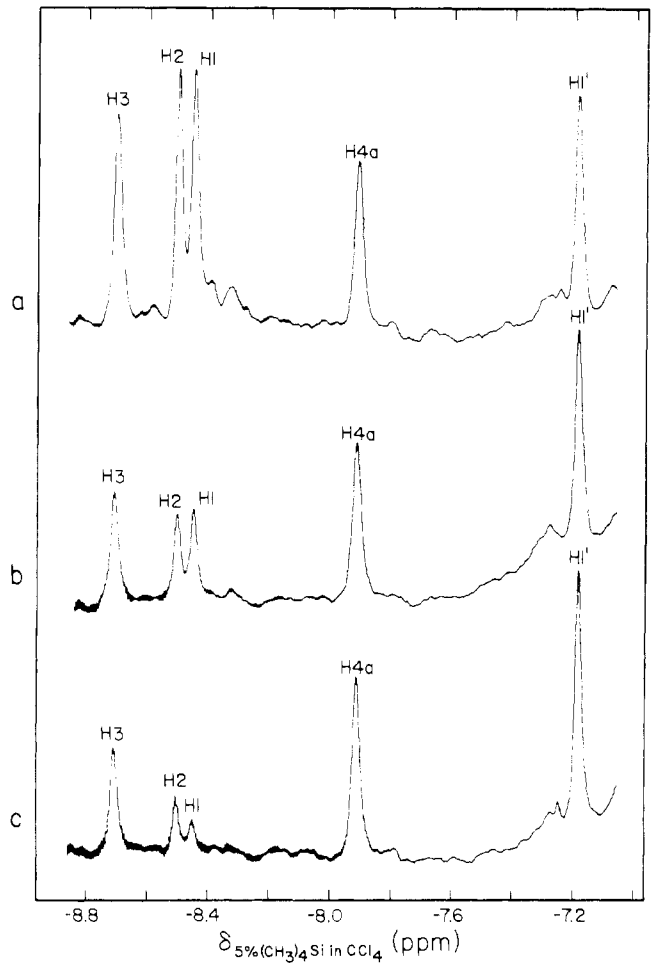


FIGURE 1: Effect of deuterium exchange on the histidine region of ^1H NMR spectra of RNase A. All spectra were taken with 40-mg/ml RNase A solutions in D_2O at pH* 3.0. (a) RNase A exchanged 2 hr at pH* 3.0 and 60° in D_2O to remove N-H peaks (procedure E). The intensities of the histidine peaks appear not to be affected by this exchange procedure. This spectrum represents zero time in the histidine exchange studies. (b) Effect of additional exchange in D_2O for 5 days at pH* 6.5 and 40° (procedure G). (c) Effect of exchange in D_2O for 5 days at pH* 8.0 and 40° (procedure F).

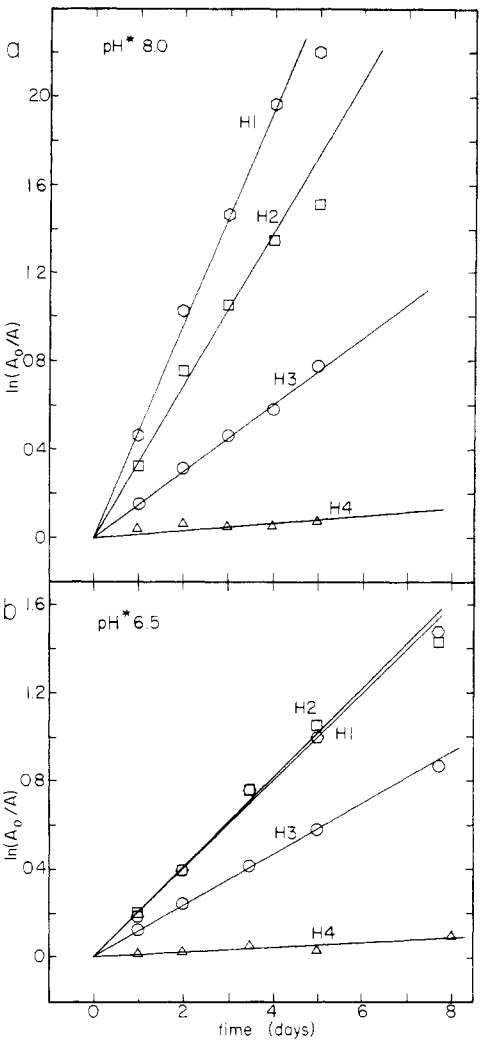


FIGURE 2: First-order plot of the ^1H NMR deuterium exchange data for the histidine C(2) protons of RNase A. A_0 is the intensity (peak height) at zero time, and A is the intensity at time t. (a) Exchange at pH* 8.0 and 40° (procedure F). (b) Exchange at pH* 6.5 and 40° (procedure G). The lines represent nonlinear least-squares fits of the data to single exponentials. Exchange rates are given in Table I.

at 40° was carried out at pH* 8.0 or 6.5. Then the pH* of all samples was adjusted to 3.0 to stop the exchange reaction. Spectra of RNase A exchanged for 5 days at these pH* values are shown in Figure 1b and c, respectively. By inspection of the spectra, the order of exchange of the histidine C(2)-H protons is H(2) ≈ H(1) > H(3) > H(4) at pH* 6.5 and H(1) > H(2) > H(3) > H(4) at pH* 8.0. First-order plots of the exchange data are shown in Figure 2. A good fit was obtained by least-squares analysis using a single exponential. The fitted rate constants and half-times for exchange are shown in Table I. The exchange rates are in essential agreement with those obtained in an independent experiment at 100 MHz (Markley and Cheung, 1973) using a slightly lower ionic strength (0.2 *M* NaCl instead of 0.3 *M* NaCl).

Reassignment of the Histidine C(2)-H Peaks of Ribonuclease A. Ohe et al. (1974) have very recently measured the tritium exchange kinetics of the histidine residues of RNase A. They studied the rate of tritium incorporation as a function of pH and derived p*K'* values for the histidine residues from these data that are in general agreement with those derived from NMR data. Their results at pH 6.5 and 8 (Table II) are very similar to our NMR exchange data (Markley and Cheung, 1973; and present work). Their exchange was carried out at 36.5°, and the exchange rates are

Table I: Deuterium Exchange Kinetics of the Histidine C (2) Ring Protons of Native Bovine Pancreatic Ribonuclease A.

NMR Peak (Residue)	Half-Time for Exchange (days)	Pseudo-First-Order Rate Constant ($sec^{-1} \times 10^6$)
pH* 6.5		
H(1) (105)	3.5 ± 0.1	2.32 ± 0.06
H(2) (119)[a]	3.4 ± 0.1	2.36 ± 0.07
H(3) (12)[a]	5.9 ± 0.1	1.35 ± 0.02
H(4) (48)	61 ± 12	0.13 ± 0.02
pH* 8.0		
H(1) (105)	1.43 ± 0.03	5.6 ± 0.1
H(2) (119)[a]	2.02 ± 0.07	4.0 ± 0.2
H(3) (12)[a]	4.55 ± 0.07	1.76 ± 0.03
H(4) (48)	43 ± 8	0.19 ± 0.03

[a] Assignments based on the present results in comparison with the tritium exchange kinetics of Ohe et al. (1974).

Table II: Selected Tritium Exchange Data for the Histidine Residues of Native Bovine Pancreatic Ribonuclease A at 36.5° Taken from Ohe et al. (1974).

	Histidine Residue	$t_{1/2}$ (days)
pH 6.5	12	11
	48	73
	105	5.3
	119	5.3
pH 8.0	12	9.3
	48	57.7
	105	3.1
	119	4.9

slightly lower than ours at 40°. Combination of the two experiments confirms the assignment of the slowest exchanging peak (H(4)) to His[48] and the fastest exchanging peak (H(1)) to His[105] in accordance with the previous assignment of Meadows et al. (1968). However, the combined data do not support the current assignment of peak H(2) to His[12] and H(3) to His[119], but suggest instead that these assignments should be reversed.

Titration Curves for the C(2)-H and C(4)-H Peaks of the Four Histidines of Ribonuclease A. Results of the 250-MHz NMR titration study of RNase A are shown in Figure 3 and Table III. The titration curves are least-squares computer fits to the data. The pK' values derived from curves H(1), H(2), and H(3) are similar to previous results (Bradbury and Scheraga, 1966; Meadows et al., 1968; Rüterjans and Witzel, 1969; King and Bradbury, 1971; Schechter et al., 1972; Westmoreland and Matthews, 1973). The magnitudes of the titration shifts and Hill coefficients for the transitions (Markley, 1973a) are reported here for the first time (Table III). The precision of the data did not warrant calculation of Hill coefficients for curves H(2′) or H(3′).

A sample of RNase A prepared by deuterium exchange by procedure F for 5 days and having differentially exchanged histidines was used to verify the crossover points in the histidine titration curves shown in Figure 3. Spectra of the differentially exchanged RNase A sample at three pH* values are presented in Figure 4. At pH* 8.20 the order of peaks from low to high field is H(2), H(1), and H(3) + H(4b) superimposed. At pH* 6.01, the order is H(1), H(2), H(3); peaks H(4a) and H(4b) disappear and could not be resolved at this pH* (Markley, 1975). Below the crossover at pH* 5, the order of peaks is H(3), H(2), H(1), H(4a). These results confirm the crossover points in the histidine titration curves arrived at by Schechter et al. (1972) on the basis of connecting NMR data points to give smooth titration curves.

Reassignment of the Histidine C(4)-H Peaks of RNase A. His[105]. Peak H(1′) is easily resolved and was assigned previously by Meadows et al. (1968) to the C(4)-H of His[105]. Curve H(1′) has a pK' of 6.66 which is in agreement with the pK' of curve H(1) (His[105], pK' = 6.72). None of the other histidines has such a high pK' value.

His[48]. The spectra reveal that the titration curve of the C(2)-H of His[48] is discontinuous (peaks H(4a) and H(4b) in Figure 3). The origin of this discontinuity is discussed in the following paper (Markley, 1975). The behavior of these peaks is shown in Figures 5 and 6. As the pH* is lowered from 8, peak H(4b) broadens and loses intensity (Figure 5), and a new peak H(4a) appears downfield as the pH* is lowered farther below 5 (Figure 6). There is no interference

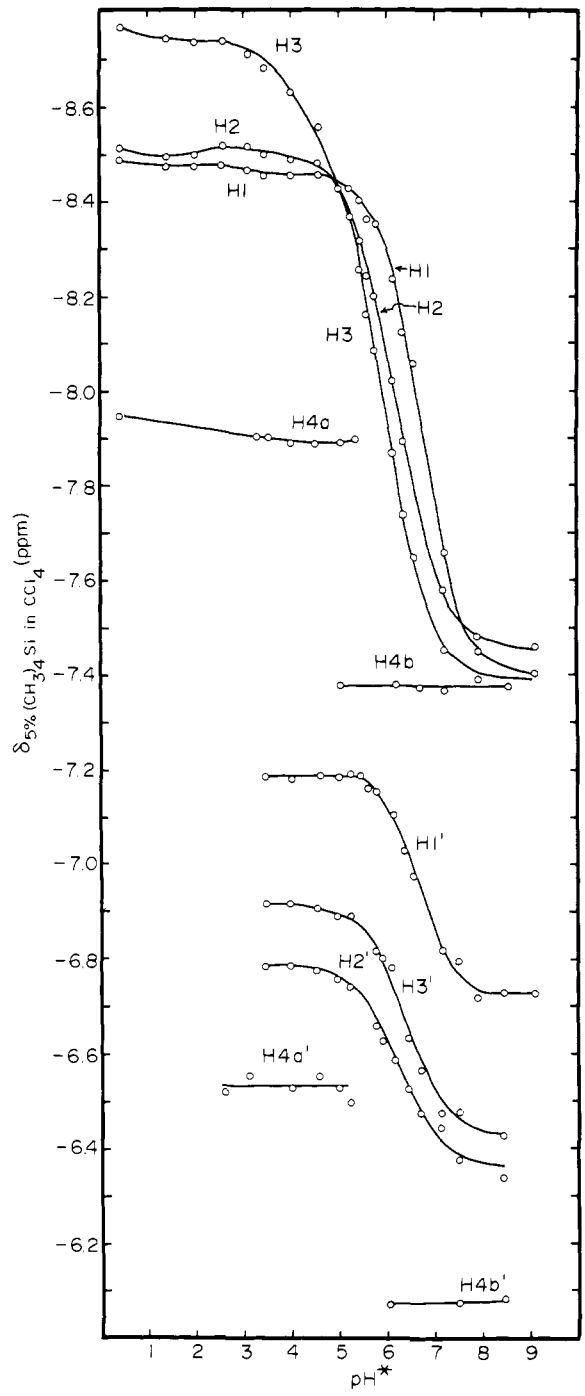


FIGURE 3: The 250-MHz proton NMR titration curves of the histidine residues of bovine pancreatic ribonuclease A; 40 mg/ml of enzyme in 0.3 *M* NaCl in D_2O, 30°. Assignments: H(1) and H(1′), C(2)-H and C(4)-H of His[105]; H(2) and H(2′), C(2)-H and C(4)-H of His[119]; H(3) and H(3′), C(2)-H and C(4)-H of His[12]; H(4a) and H(4b), C(2)-H and C(4)-H of His[48] in the acid-stable conformational form of the enzyme; H(4b) and H(4b′), C(2)-H and C(4)-H of His[48] in the base-stable conformational form.

from other peaks in these spectra since the other C(2)-H histidine peaks H(1), H(2), and H(3) and all N-H peaks have been removed by exchange according to procedure C. Peaks H(4b′) (Figure 5) and H4a′ (Figure 6) are assigned to the C(4)-H of His[48] because their intensities follow those of peaks H(4a) and H(4b) as a function of pH.

Confirmatory evidence for this assignment comes from the effect of added acetate on the spectra of RNase A. It is known that the C(2)-H of His[48] of RNase A in 0.2 *M* ace-

Table III: Least-Squares Analysis of ^{1}H NMR Titration Data for the Histidine Residues of Bovine Pancreatic Ribonuclease A.

	pK'			Chemical Shift ppm from 5% $(CH_3)_4Si$ in CCl_4			
	Hill Coefficient Fixed at 1	Variable Hill Coefficient	Fitted Value of Hill Coefficient	δ_{H^+}	δ_{H^0}	$\Delta\delta_{H^0,H^+}$	Variance $\times 10^4$
	a. Ribonuclease A in 0.3 *M* Sodium Chloride in D_2O, Histidine C(2)–H Peaks						
H(1) (His^{105})	6.72 ± 0.02	6.72 ± 0.02	0.94 ± 0.03	−8.46 ± 0.01	−7.39 ± 0.01	1.07 ± 0.02	1.6
H(2) (His^{119}, single pK')	6.19 ± 0.04	6.19 ± 0.02	0.79 ± 0.03	−8.52 ± 0.01	−7.45 ± 0.01	1.07 ± 0.02	1.3
H(3) (His^{12}, single pK')	5.79 ± 0.07	5.79 ± 0.02	0.67 ± 0.02	−8.73 ± 0.01	−7.35 ± 0.01	1.38 ± 0.02	1.8
	b. Ribonuclease A in 0.3 *M* Sodium Chloride in D_2O, Histidine C(4)–H Peaks						
H(1′) (His^{105})	6.67 ± 0.03	6.67 ± 0.03	0.95 ± 0.06	−7.20 ± 0.01	−6.72 ± 0.01	0.47 ± 0.02	1.0
H(2′) (His^{119})	6.21 ± 0.05			−6.78 ± 0.01	−6.36 ± 0.01	0.42 ± 0.02	2.8
H(3′) (His^{12})	6.38 ± 0.07			−6.91 ± 0.01	−6.42 ± 0.02	0.49 ± 0.03	7.2
	c. Ribonuclease A in 0.2 *M* Sodium Acetate in D_2O, Histidine C(4)–H (H(4)) and C(2)–H (H(4′)) Peaks						
H(4) (His^{48})	6.31 ± 0.08			−7.95 ± 0.02	−7.36 ± 0.02	0.59 ± 0.04	5.8
H(4′) (His^{48})	6.25 ± 0.09			−6.53 ± 0.02	−6.06 ± 0.02	0.46 ± 0.04	4.6

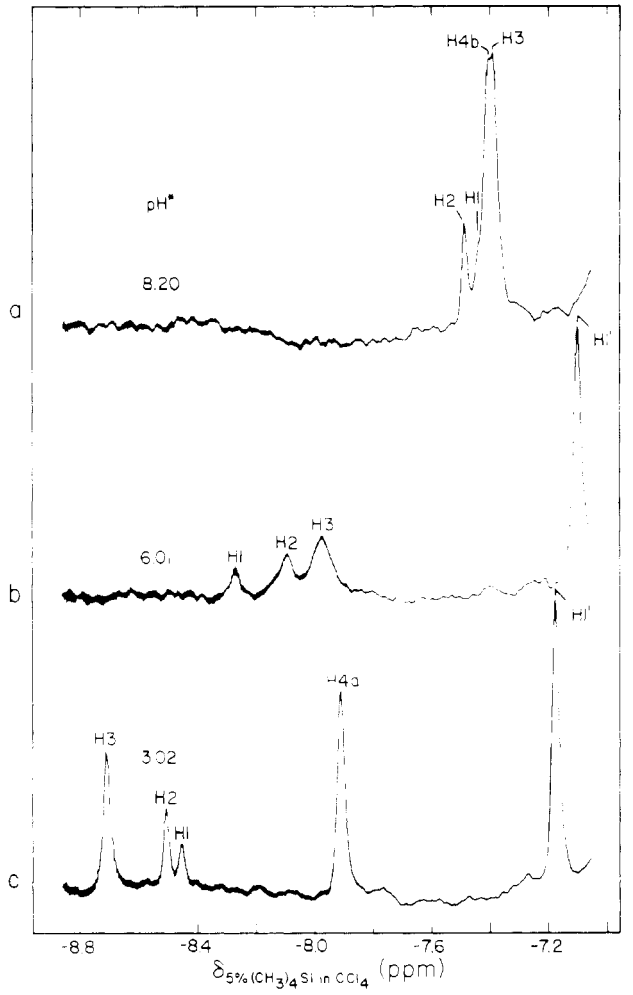


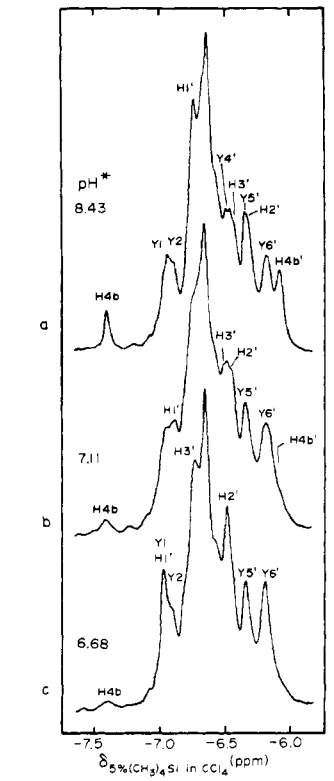
FIGURE 4: The use of differential exchange to determine crossover points in the histidine NMR titration curves of RNase A (see Figure 3). ^{1}H NMR spectra of RNase A prepared by exchange procedure F (exchange in D_2O for 5 days at pH* 8.0 and 40°) at three pH* values. (a) pH* 8.20; (b) pH* 6.01; (c) pH* 3.02. Peak H(4) from His^{48} is not visible in the spectrum at pH* 6.01. This peak broadens and disappears at intermediate pH values.

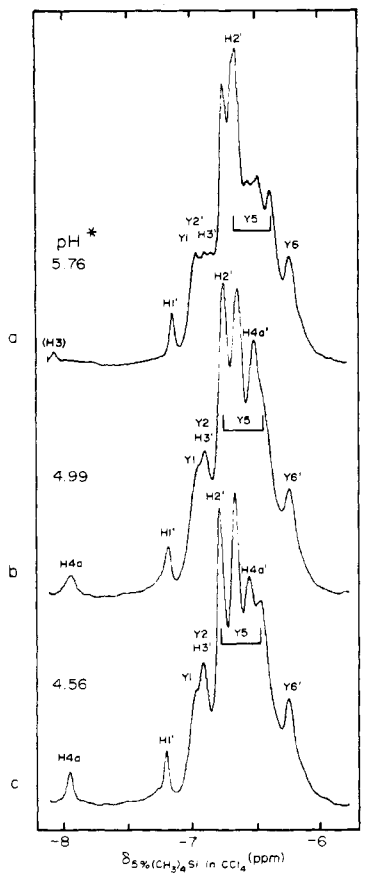
tate yields a continuous titration curve and is sharp throughout the titration region (Meadows et al., 1967; Roberts et al., 1969). The effect of added acetate on the peaks assigned to His^{48} is shown in Figure 7. Both peaks H(4a) and H(4a′) sharpen as acetate is added, and both peaks titrate with similar p(K′) values as is shown in Figure 8 and Table III.

His-12 and -109. Two other peaks in the aromatic region having single proton intensities, H(2′) and H(3′), are observed to shift with pH (Figures 5 and 6). Peaks H(2′) and H(3′) could not be correlated immediately with peaks H(2) and H(3) on the basis of their titration curves (Figure 3) because of the similarity of the pK' values of these residues (Table III). It is known that the pK' values of titration curves H(2) and H(3) diverge in the RNase A–3′-CMP complex: the pK' values of both residues are raised in the complex, but the pK' of H(2) is raised to a greater extent (Meadows and Jardetzky, 1968). The His C(4)–H titration curves of RNase A in the presence of a twofold excess of 3′-CMP are shown in Figure 9 (solid lines). The titration curves in the absence of 3′-CMP (dashed lines) are given for reference. As expected (Meadows and Jardetzky, 1968) the titration curves for His^{105} (H(1′)) and His^{48} (H(4a′) and H(4b′)) are not perturbed in the complex, whereas those of the two active site histidines are. The curve labeled H(2′) shows the largest pK' displacement and is thus correlated with residue H(2) (His^{119}); curve H(3′), therefore, corresponds to H(3) (His^{12}).

Discussion

Exchange of N–H Peaks. The number of slowly exchanging protons of RNase A resolved in this study (~42) is larger than the number (14) reported in a previous NMR study (Wishnia and Saunders, 1962). A possible explanation for the difference may be that much of the intensity of the N–H peaks was lost in the base line in the previous study at 60 MHz. Numerous N–H peaks of RNase A and other proteins are sharper and much more visible in spectra taken at 250 MHz than at 100 MHz. This suggests that some N–H peaks in proteins are exchange broadened, presumably as the result of exchange among multiple solvent-inaccessible environmental states. One striking feature of the spectra is the lack of slowly exchanging protons with chemical shifts within the aromatic envelope (δ −7 to −6). This contrasts sharply with spectra of the proteinases, porcine trypsin (Porubcan and Markley, unpublished) and staphylococcal protease (Markley et al., 1975), which contain a large number of slowly exchanging protons with





FIGURES 5 and 6: The 250-MHz ^1H NMR spectra of RNase A [exchanged in D_2O by procedure D to remove N-H and histidine peaks H(1), H(2), and H(3)] (Figure 5, top; Figure 6, bottom). Assignments: H(3), residual intensity from the C(2)-H peak of His[12]; H(4a) and H(4a'), C(2)-H and C(4)-H peaks of His[48] in its acid-stable state; H(4b) and H(4b'), C(2)-H and C(4)-H peaks of His[48] in its base-stable state; H(1'), H(2'), and H(3'), C(4)-H peaks of His[105], His[119], and His[12]; Y(1), Y(2), Y(5), and Y(6), tyrosine resonances. pH* values: (5a) 8.43; (5b) 7.11; (5c) 6.68; (6a) 5.76; (6b) 4.99; (6c) 4.56.

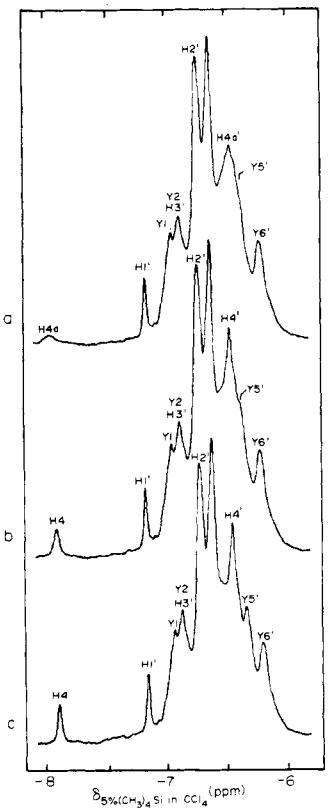


FIGURE 7: Effect of the addition of acetate on the low-field ^1H NMR region of 40 mg/ml of RNase A in D_2O at pH 5.4, 30°. Acetate concentration: (a) 0; (b) 0.025 *M*; (c) 0.14 *M*.

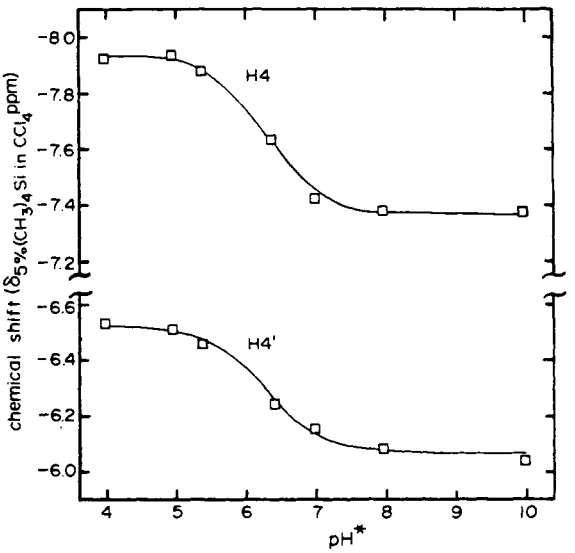


FIGURE 8: ^1H NMR titration curves of the C(2)-H (H(4)) and the C(4)-H (H(4')) of His[48] of RNase A in 0.2 *M* sodium acetate. The titration parameters are given in Table III.

chemical shifts in this region. In RNase A only one proton in this region at δ −6.9 appears to exchange under the conditions studied.

The number of slowly exchanging protons found in the present study corresponds closely to the number of class IV exchangeable protons (half-times greater than 6–22 hr at 25°) previously determined by classical hydrogen exchange techniques (Hvit, 1955; Haggis, 1957; Schildkraut and Scheraga, 1960). However, several studies indicate the existence of 25–50 class III exchangeable N-H protons with

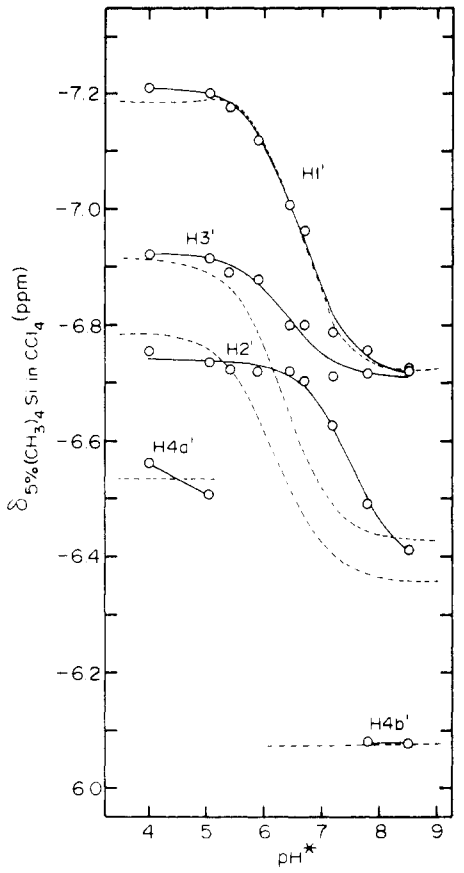


FIGURE 9: ^{1}H NMR titration curves of the histidine C(4)-H peaks of RNase A in the presence of a twofold molar excess of the inhibitor 3′-cytidine monophosphate (—). Titration curves in the absence of inhibitor (- - -) are shown for reference. Assignments are as in Figure 3.

half-times of 2–22 hr (summarized in Richards and Wyckoff, 1971) that should have appeared in spectra of samples prepared by procedure A. The ^{1}H NMR spectra indicate an upper limit of three or four protons in the spectral region δ −10 to −5 that exchange at this rate. The remaining class III protons in this sample may have been lost rapidly at pH* 9.2, the pH* at which the RNase A was dissolved in D_2O.

Exchange of Histidine C(2)-H Peaks. The C(2) hydrogen of the imidazole ring of histidine is much more labile than any other carbon-bound hydrogens present in amino acids. The exchange rate of this hydrogen is intermediate between those of the rapidly exchanging N-H, O-H, and S-H hydrogens and other C-H hydrogens that exchange under more vigorous conditions [e.g., α- and β-C-H and aromatic C-H (see, e.g., Markley, 1972)]. Imidazole (Harris and Randall, 1965), histidine, and histidine derivatives (Matsuo et al., 1972) exhibit pseudo-first-order exchange kinetics having a sigmoidal pH dependence. It has been proposed (Harris and Randall, 1965; Olofson et al., 1964; Vaughan et al., 1970) that the exchange is base catalyzed (by OD^- or D_2O) but that only the acidic (cationic) form of imidazole reacts at an appreciable rate to form the postulated ylide intermediate. Model imidazole compounds (Im) follow the following equation for pseudo-first-order exchange (Vaughan et al., 1970; Markley and Cheung, 1973):

$$k_{obsd} = \frac{k_a[D_2O][D^+] + k_bK_w}{K_a + [D^+]} \quad (2)$$

where $K_a = [Im][D^+]/[Im^+]$, $K_w = [D^+][OD^-]$, and k_a and k_b are respectively the rate constants for the reactions catalyzed by D_2O and OD^-. The constants k_a and k_b found for *N*-acetylhistidine are shown in Table IV. An attempt was made to obtain values for these constants for the individual histidine peaks H(1), H(2), and H(3) of RNase A by solving eq 2 using the observed exchange rates at pH* 6.5 and 8.0 and the macroscopic dissociation constants for the histidines derived from NMR data (Table III this paper; and Table Id, Markley and Finkenstadt, 1975). The small increase in exchange rate of peak H(4) with increasing pH is not significant and precludes such an analysis for His^{48}. The results are given in Table IV. As is generally true in other proteins (Markley and Cheung, 1973) the exchange rate k_b is higher for histidine C(2)-H protons in proteins than in model compounds. This suggests that other groups on the protein surface assist in catalysis.

Table IV: Analysis of Histidine C(2)-H Deuterium Exchange Data by the Equation of Vaughan et al. (1971) [Eq 2 in Text].

Exchanging Species	k_a (sec^{-1})	k_b (sec^{-1})
N-Acetylhistidine[a]	4×10^{-9}	38
RNase A		
H(1) (His^{105})	3.4×10^{-9}	110
H(2) (His^{119})[b]	0	260
H(3) (His^{12})[b]	0	200

[a] Data from Markley and Cheung (1973). [b] Assignments according to present work.

The exchange rate of peak H(4) (His^{48}) is much slower than those of the other C(2)-H histidine peaks of RNase A; it is in fact the slowest histidine exchange rate measured in all proteins that have been studied (Markley and Cheung, 1973). This is in agreement with chemical evidence (see Richards and Wyckoff, 1971) and X-ray data (Kartha et al., 1967) which indicate that His^{48} of RNase A has a limited accessibility to the solvent. The discontinuous titration curves for His^{48} (Figure 3) also indicate that the imidazole N-H groups are not freely accessible to solvent (Markley, 1975).

Assignment of Histidine C(2)-H Peaks. Comparison of the order of deuterium exchange of the histidine C(2)-H peaks of RNase A determined by NMR spectroscopy (Figure 2) with the kinetics of tritium incorporation into the individual histidine residues of RNase A (Ohe et al., 1974; data reproduced in Table II) yields the following assignments: H(1) = His^{105}; H(2) = His^{119}; H(3) = His^{12}; H(4) = His^{48}. The assignments of peaks H(1) and H(4) to histidine-105 and -48, respectively, are in agreement with previous results (Meadows et al., 1968). However, the present assignments of peaks H(2) and H(3) to histidine-119 and -12 are the reverse of previous assignments (Meadows et al., 1968; Bradbury and Chapman, 1972).

There are possible ambiguities in the previous assignments of peaks H(2) and H(3). Meadows et al. (1968) assigned peak H(2) by comparing spectra of RNase S with spectra of RNase S′ reconstituted from S protein plus S peptide in which His^{12} had been deuterated by exchange in D_2O. The missing peak in spectra of the RNase S′ (reconstituted) corresponded to peak H(2) of their RNase S control. A subsequent investigation of the chemical shifts of the histidine peaks of RNase S demonstrated, however, that the control sample used by Meadows et al. (1968) must have contained phosphate which shifted peaks H(2) and H(3) downfield. If the reconstituted RNase S′ contained less

phosphate than the control, the original assignment would be in error. The later assignment of Bradbury and Chapman (1972) involved differential deuterium exchange of the histidines of RNase A followed by conversion to RNase S and separation of S protein and S peptide. Their assignment of peak H(2), which exchanges more rapidly than peak H(3), to His^{12} was based on their inability to resolve the histidine peak in the spectrum of S peptide rather than on actual peak area measurements.

The present assignments of peaks H(2) and H(3) are based on exchange rates obtained from a series of consistent measurements. Thus they are probably more reliable than the earlier results. The present assignments would be in error only if there exist large selective isotope effects on the exchange rates of histidine-12 and -119 of RNase A in D_2O as compared to H_2O. The virtual identity of X-ray diffraction patterns of RNase A crystallized from H_2O and D_2O solutions (Bello and Harker, 1961) and the similarity of NMR titration curves of histidine-12 and -119 obtained in H_2O and D_2O (Roberts et al., 1969) suggest that such an isotope effect is unlikely.[2]

Assignment of Histidine C(4)-H Peaks. There exist several points of disagreement between the results of these experiments and those of a previous assignment of the histidine C(4)-H peaks of RNase A based on difference spectra obtained at 100 MHz (King and Bradbury, 1971). In that study, the resonance assigned to His^{48} exhibited a titration shift between pH* 5.5 and 6.5 and a discontinuous *downfield* shift between pH* 6.5 and 7.5; and the peaks previously assigned to His^{12} and His^{119} showed a pronounced inflection at pH* 5. As is discussed in the following paper (Markley, 1975), the largest peaks in pH difference spectra of RNase A in the pH region around pH* 5.5 correspond to a tyrosine residue (Y(5) in Figure 6). The peaks previously assigned to the C(4) protons of His^{12} and His^{119} at low pH (King and Bradbury, 1971) match these tyrosine peaks.

The chemical shifts of the His C(4)-H resonances of RNase A appear to be more responsive to the local environment than the chemical shifts of the C(2)-H resonances. There is a much greater disparity among the δ_{H^+} and δ_{H^0} values for the His C(4) protons (Table III) than among these values for the His C(2) protons (see Table I in Markley, 1973a). The C(4) protons are closer to the protein backbone and should be influenced more strongly than the C(2) protons by local conformational differences (King and Bradbury, 1971). On the other hand, the titration shifts ($\Delta\delta_{H^+,H^0}$) of the C(4)-H resonances (with the exception of His^{48}) are approximately half those of the C(2)-H resonances. Thus more precise titration data may be obtained from C(2)-H chemical shifts than from C(4)-H chemical shifts.

Comparison of C(2)-H and C(4)-H data can yield additional information about the environment of a given histidine residue than either set of data alone. For example, the abnormal chemical shift of His^{48} in its acid-stable form may be explained either by an effect such as hydrogen bonding, which should influence the titration shift ($\Delta\delta_{H^+,H^0}$) of both the C(2)-H and C(4)-H, or by a local shielding of the C(2) proton alone. The normal titration shift observed for the C(4)-H of His^{48} (Table III) suggests that the latter explanation is correct.

[2] After this manuscript was submitted, the author learned that D. J. Patel, L. L. Canuel, C. Woodward, and F. A. Bovey (personal communication) and C. R. Matthews and J. S. Cohen (personal communication) have independent data which support the revised assignment of peak H(3) to His^{12} of RNase A.

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Correlation Proton Magnetic Resonance Studies at 250 MHz of Bovine Pancreatic Ribonuclease. II. pH and Inhibitor-Induced Conformational Transitions Affecting Histidine-48 and One Tyrosine Residue of Ribonuclease A†

John L. Markley

ABSTRACT: The microenvironment of histidine-48 of bovine pancreatic ribonuclease A was investigated by proton magnetic resonance spectroscopy (^{1}H NMR) using partially deuterated enzyme in which resolution of the C(2)-H resonance of histidine-48 was simplified. The NMR titration curves at 100 and 250 MHz of histidine-48 of ribonuclease A are discontinuous both for the enzyme alone in 0.3 *M* chloride and for its complex with cytidine 3′-phosphate. This suggests that titration of histidine-48 occurs only as the result of a slow conformational transition. The sum of the peaks corresponding to histidine-48 in the acid-stable and base-stable forms of the enzyme is less than one proton in the transition region, which indicates that there exists at least one intermediate conformational form of the enzyme. The transition from the acid-stable form to an intermediate form has a pH_{mid} of 5.6, and the transition from an intermediate form to the base-stable form has a pH_{mid} of 6.9. In ribonuclease S and in ribonuclease A in the presence of 0.3 *M* acetate, the titration curve of histidine-48 is continuous, and the area of the peak is uniform throughout the titration. Proton NMR difference spectra at 100 and 250 MHz reveal a pH-induced conformational change with a pH_{mid} of 5.7 that affects the chemical shift of a single tyrosine residue. This conformational transition is absent in ribonuclease S and is altered in ribonuclease A by the presence of either acetate or cytidine 3′-monophosphate. It is postulated that the same conformational transition is responsible for both the tyrosine perturbation and the disappearance of the histidine-48 peak observed in the acid-stable form of the enzyme. It is proposed that the perturbed tyrosine is tyrosine-25. The transition with pH_{mid} 5.6 is attributed to dissociation of aspartic acid-14, and the transition with pH_{mid} 6.9 is assigned to dissociation of histidine-48. A peak in the aromatic region that moves upfield on addition of the competitive inhibitor cytidine 3′-monophosphate is assigned to a tyrosine, and evidence is presented that this tyrosine is tyrosine-25. Inhibitor binding appears to induce a conformational change in the histidine-48/tyrosine-25 region which is remote from the active site.

One of the curiosities of previous proton magnetic resonance (^{1}H NMR) studies of ribonuclease is the behavior of the resonance assigned (Meadows et al., 1967, 1968; Markley, 1975) to His[48]. In solutions of bovine pancreatic ribonuclease A (RNase A)[1] in NaCl (0–0.3 *M*), the C(2)-H peak of His[48] broadens and disappears as the pH is raised above 5 (Meadows and Jardetzky, 1968; Roberts et al., 1969). This result has been confirmed in a number of laboratories (Rüterjans and Witzel, 1969; King and Bradbury, 1971; Schechter et al., 1972; Westmoreland and Matthews, 1973; Sacharovsky et al., 1973; Migchelsen and Beintema, 1973) although there is widespread disagreement whether or not the peak exhibits a titration shift before its disappearance.

In contrast, the C(2)-H peak of His[48] is sharp and visible and yields a normal NMR titration curve in RNase A in the presence of 0.2 *M* acetate (Meadows et al., 1967), in RNase S (Meadows et al., 1968; Cohen et al., 1973), and rat ribonuclease A (Migchelsen and Beintema, 1973). Meadows and Jardetzky (1968) and Roberts et al. (1969) attributed the disappearance of the C(2)-H peak of RNase A to exchange broadening resulting from the decreased rate of exchange of this group between two different environments above pH 5. King and Bradbury (1971) proposed, on the other hand, that the disappearance results from dipolar broadening caused by the immobilization of the C(2)-H (but not the C(4)-H) of His[48] near another proton. More recent evidence (Markley, 1975) indicates that both the

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[1] Abbreviations used are: RNase, bovine pancreatic ribonuclease; pH*, uncorrected pH meter reading of a D_2O solution made with a glass electrode standardized in H_2O buffers; 3′-CMP, cytidine 3′-monophosphate.