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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†

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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.

C(2)-H and C(4)-H of His[48] of RNase A show the same discontinuous titration behavior.

The abnormally slow deuterium exchange kinetics of the C(2)-H of His[48] (Markley and Cheung, 1973) suggested the possibility of preparing a deuterium exchanged sample of RNase A that would have a simplified NMR spectrum in the His[48] C(2)-H region. A sample was prepared in which the only major peak in the histidine C(2)-H region was from His[48]. This partially exchanged sample permitted a detailed study of the His[48] resonances without interference from other peaks.

The present results at 100 and 250 MHz are not compatible with either a simple exchange broadening or dipolar broadening mechanism for the His[48] peaks. The data reveal the existence of two slow conformational transitions in RNase A having pH_{mid} values of 5.6 and 6.9 that affect the environment of His[48]. NMR difference spectroscopy (King and Bradbury, 1971; Markley, 1973) of the aromatic envelope shows that a tyrosine residue is perturbed by the transition occurring at lower pH. These conformational transitions are absent or modified in RNase S and in RNase A in the presence of acetate. The results are discussed in relation to previous studies of conformational equilibria in RNase A and S.

Experimental Section

Materials. Deuterium exchanged RNase A was prepared by procedure C as described in the preceding paper (Markley, 1975); the exchange time was 26 days. Other materials were the same as used previously (Markley, 1975).

Instrumentation. The 100-MHz ^1H NMR spectra were obtained with a Varian XL-100-15 spectrometer operating in the continuous-wave mode. The sample temperature was 32°. The spectra were the result of averaging 128 100-sec scans of a 250-MHz region using a Nicolet 1080 computer. The 250-MHz correlation ^1H NMR spectra were obtained as before (Markley, 1975).

Procedures. A description of the procedure used to obtain the 100-MHz difference spectrum of RNase A has been published (Markley, 1973). The 250-MHz spectra were stored on magnetic tape. Difference spectra were then formed using a computer program written for the Nicolet 1080 (D. M. LeMaster and J. L. Markley, unpublished) that displays equivalent regions of two spectra, corrects the base line, normalizes the areas of the two spectra, and subtracts one spectrum from the other. Chemical shifts are reported in ppm from external 5% $(CH_3)_4SI$ in CCl_4. These chemical shifts may be converted to ppm from external $(CH_3)_4Si$ by the equation

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

Results

Conformational Transitions Affecting Histidine-48. The histidine C(2)-H region of the 250-MHz ^1H NMR spectrum of extensively exchanged RNase A (26-day exchange by Procedure C, Markley, 1975) is shown in Figure 1. The exchange treatment has removed nearly all intensity from N-H peaks (with the exception of the extremely slowly exchanging peaks a and b) and from the C(2)-H peaks of His[105] (H(1), intensity 0.09 proton), His[12] (H(2), 0.08 proton) and His[119] (H(3), 0.11 proton). Thus the largest peaks in this region come from the C(4)-H peak of His[105] (H(1')), and from the C(2)-H peaks of the acid-stable (H(4a)) and base-stable (H(4b)) forms (maximum intensi-

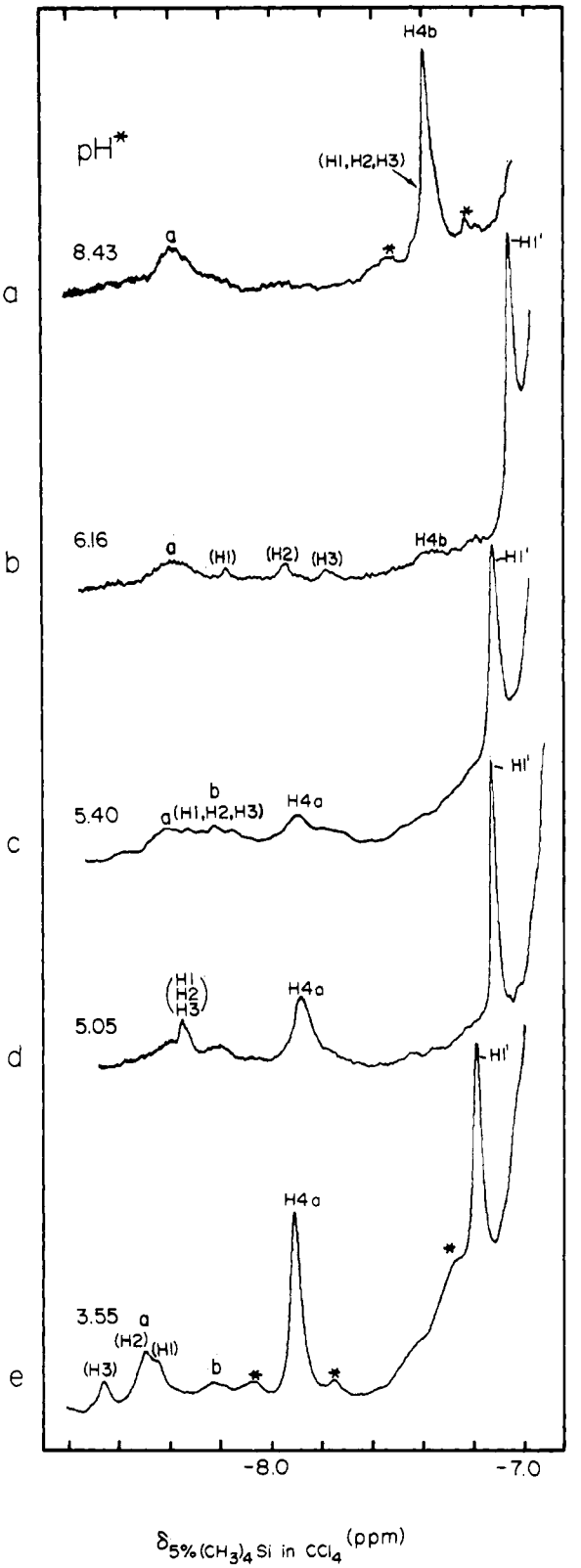

FIGURE 1: The 250-MHz correlation ^1H NMR spectra of the histidine C(2)-H region of bovine pancreatic ribonuclease A (40 mg/ml in 0.3 *M* NaCl in D_2O, 30°) showing the transition from the base-stable conformational form of His[48] (peak H(4b)) to the acid-stable form (peak H(4a)). The ribonuclease sample has been exchanged in D_2O to remove most of the intensity from the other histidine C(2)-H peaks: His[105] (H(1)), His[12] (H(2)), His[119] (H(3)). Peaks a and b are resonances from extremely slowly exchanging N-H protons. Peak H(1') is the C(4)-H of His[105] which does not exchange with D_2O under the conditions used. pH* values: (a) 8.53, (b) 6.16, (c) 5.40, (d) 5.05, and (e) 3.55. The asterisks (*) indicate spinning side bands.

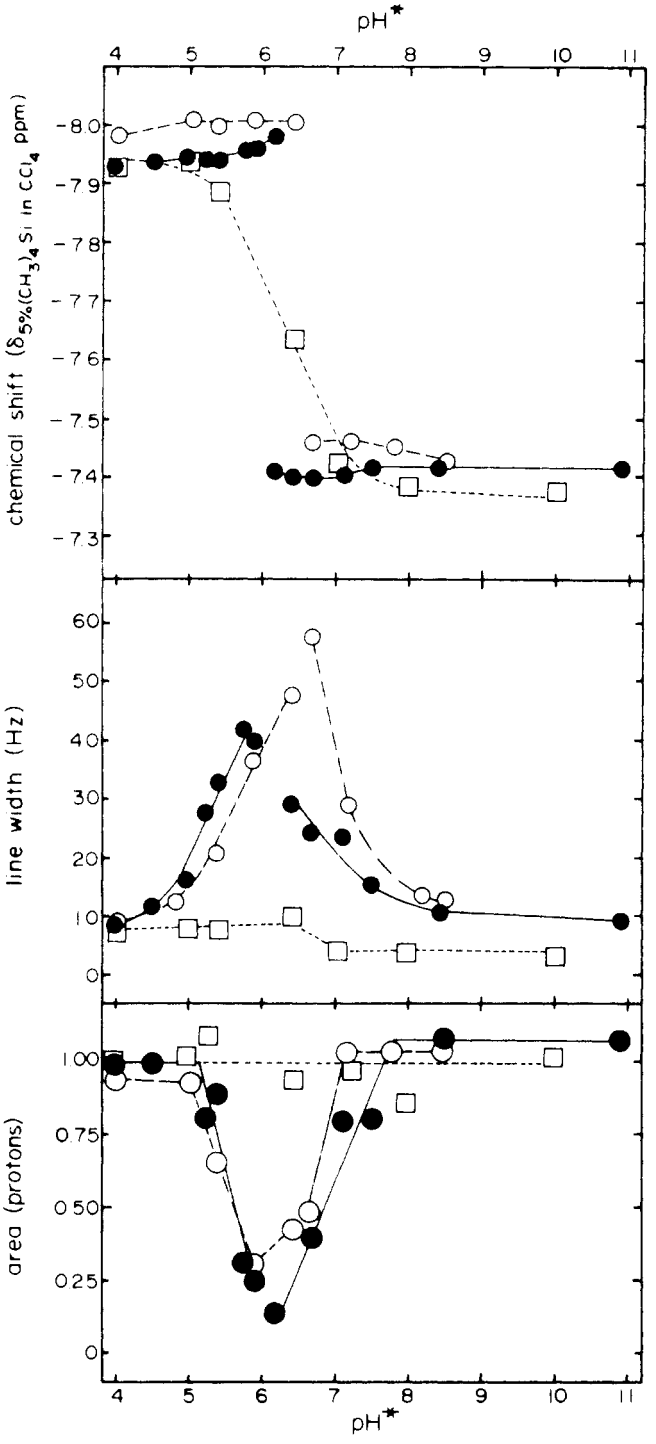

FIGURE 2: The 250-MHz NMR data for the transition from the acid-stable conformational form to the base-stable form of His[48] in ribonuclease A. (●) Ribonuclease A in 0.3 *M* NaCl; (○) ribonuclease A saturated with the inhibitor cytidine 3′-monophosphate; (squares) ribonuclease A in 0.2 *M* sodium acetate. The data were obtained using the exchanged sample of ribonuclease shown in Figure 1. The area measurements have been corrected for the 10% deuterium exchange of the C(2)-H peak of histidine-48.

ty 0.9 proton each) of His[48]. As the pH* is raised above 4 the C(2)-H peak of His[48] (H(4a)) broadens and loses intensity and finally disappears at pH* 6.1. Its chemical shift remains nearly constant throughout the transition. As peak H(4a) broadens and disappears, peak a sharpens and becomes more visible. This peak was mistakenly assigned to an intermediate form of His[48] in an earlier analysis of spec-

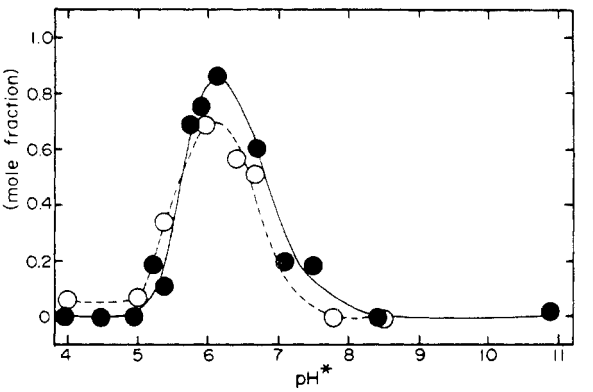

FIGURE 3: The total concentration of postulated intermediate conformational species of ribonuclease A lying between the acid- and base-stable forms of the enzyme as a function of pH*. (●) Ribonuclease A in 0.3 *M* NaCl in D_2O; (○) saturated ribonuclease A-cytidine 3′-monophosphate complex in 0.3 *M* NaCl in D_2O.

tra of less extensively exchanged RNase A (Markley, 1974). Both peaks a and b are now firmly assigned as N-H peaks because they disappear when RNase A is exchanged in D_2O under more vigorous conditions (Markley, 1975). Peak H(4b) of His[48] is readily resolved after exchange of the underlying N-H peaks; and it can be visualized best if the C(2)-H peaks of the other histidines are also removed by deuterium exchange. Peak H(4b) is visible in spectra above pH* 6.1. It grows and sharpens as the pH* is raised, but its chemical shift remains constant.

In order to investigate the mechanism for broadening of peaks H(4a) and H(4b), spectra were taken at 100 MHz of the same exchanged sample. If the broadening results from an intermediate rate of exchange between two or more states as postulated by Roberts et al. (1969), the line widths should be frequency dependent. The spectra (not shown) are very similar to those obtained at 250 MHz in that peak H(4a) broadens and disappears and peak H(4b) appears and sharpens as the pH* is raised. Peak H(4a) shifts upfield slightly before disappearing in the 100-MHz spectra. In this respect, the data at 100 MHz appear to represent intermediate exchange on the NMR time scale, whereas the results at 250 MHz appear to be more in the slow exchange limit.

The experiments at 250 MHz were repeated with a saturated complex of RNase A with 3′-CMP and with RNase A in 0.2 *M* acetate. The chemical shift, line width, and peak area data are compared in Figure 2. The NMR titration curve of His[48] is discontinuous for RNase in 0.3 *M* NaCl and for the RNase-3′-CMP complex of 0.3 *M* NaCl. Peak H(4a) is shifted downfield slightly in the complex as was previously noted by Meadows and Jardetzky (1968). Peak H(4b) is also shifted downfield in the complex. The line width of peak H(4a) is similar in RNase A and in the RNase A-3′-CMP complex, whereas peak H(4b) of the complex exhibits a much larger broadening at transition pH* values. In the presence of 0.2 *M* acetate, the NMR titration curve of His[48] is continuous; the line width is nearly constant (except for a slight decrease between pH* 6.5 and 7.0); and the area of the single peak is roughly constant at 1 proton.

At 250 MHz, the areas of the His[48] C(2)-H peaks H(4a) and H(4b) of RNase A could be determined in the transition regions with a fair degree of accuracy. Thus, if H(4a) and H(4b) represent the only two His[48] conformational forms of RNase A, the two areas should equal one proton (corrected for the 10% exchange of the His[48] C(2)-H). The

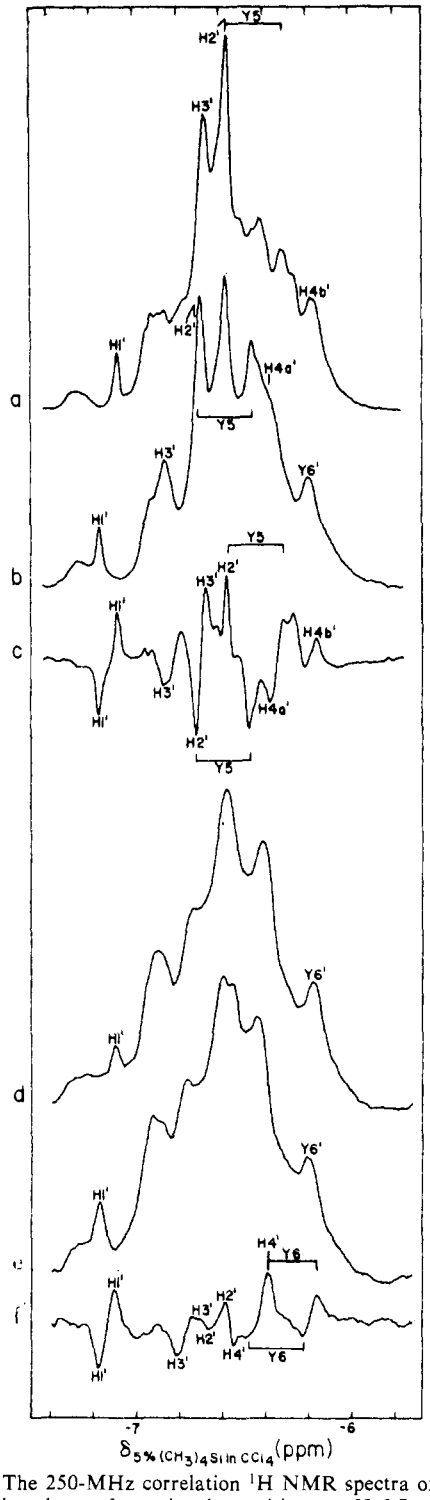

FIGURE 4: The 250-MHz correlation ^{1}H NMR spectra of ribonuclease illustrating the conformational transition at pH 5.7 affecting the chemical shift of a tyrosine residue (assigned to tyrosine-25) which is present in ribonuclease A but not in ribonuclease S. Ribonuclease A: (a) ribonuclease A, pH* 6.02; (b) ribonuclease A, pH* 5.02; (c) difference spectrum formed by subtracting spectrum b from a. Assignments: H(1′), H(2′), and H(3′) are the C(4)-H of histidines-105, 119, and 12; H(4a′) and H(4b′) are the C(4)-H of the acid-stable and base-stable forms of histidine-48; Y(5) is tyrosine-25; and Y(6′) is the meta proton resonance of a buried tyrosine. The largest contribution to the difference spectrum comes from the perturbation of the tyrosine doublet Y(5) upfield with increasing pH. Ribonuclease S: (d) ribonuclease S, 40 mg/ml, pH* 5.99; (e) ribonuclease S, pH* 4.99; (f) difference spectrum formed by substracting spectrum e and f. Assignments: the same as above except that there is only a single histidine-48 C(4)-H peak (H(4′)). The difference spectrum of ribonuclease S (f) may be accounted for mainly by titration shifts of the four histidine peaks. There appears to be a slight upfield shift in one tyrosine doublet (Y(6)) with increasing pH*.

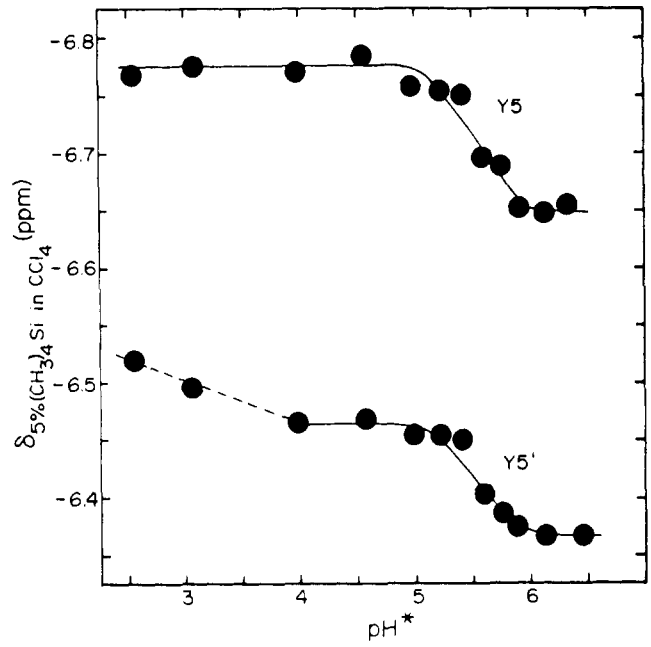

FIGURE 5: Plot of the chemical shifts of peaks assigned to tyrosine-25 (ortho protons Y(5), meta protons Y(5′)) as a function of pH*. The curves represent least-squares analysis of the data. The pK' value for the transition is 5.7. The fitted parameters are given in Table I.

sum of the two areas is clearly less than one proton in the transition region (Figure 2). Thus there must exist one or more intermediate conformational states of His^{48} in RNase A. The pH* dependence of the missing intensity, or concentration of postulated intermediate(s), is shown in Figure 3 for RNase A alone and for the RNase A-3′-CMP complex. The maximum concentration of intermediate(s) is at pH* 6.1. The intermediate is formed by a proton dissociation at pH* 5.6 and disappears as a result of a second proton dissociation at pH* 6.9. These two transitions appear to be very similar in RNase A alone and in the RNase A-3′-CMP complex.

Conformational Transitions Affecting a Tyrosine Residue. In order to determine if any aromatic residues are perturbed by the histidine transitions, ^{1}H NMR difference spectra were taken of the aromatic region. The 250-MHz spectra of RNase A at pH* 6.0 and 5.0 and their differences are shown in Figure 4a-c. The intensity of the difference spectrum is too great to be accounted for solely by shifts of the four histidine C(4)-H resonances. An estimate of the intensity of a single proton can be obtained from the C(4)-H peaks of His^{105} labeled H(1′). A good fit to the bulk of the difference spectrum may be obtained by assuming that a tyrosine doublet (Y(5)) shifts upfield as the pH* is raised. At 100 MHz the tyrosine spectrum is expected to be a quartet. Similar NMR difference spectra of RNase A taken at 100 MHz can be fitted by assuming that a tyrosine quartet shifts in an analogous manner.

Since it is known that the titration curve of His^{48} in RNase S is continuous (Meadows et al., 1968), it was of interest to investigate the tyrosine transition in RNase S. As is shown in Figure 4d—f, the difference spectrum is much smaller for RNase S. The RNase S difference spectrum can be explained almost entirely by titration shifts of histidine C(4)-H peaks. The only additional change appears to be a small upfield shift in the chemical shift of a tyrosine-labeled Y(6).

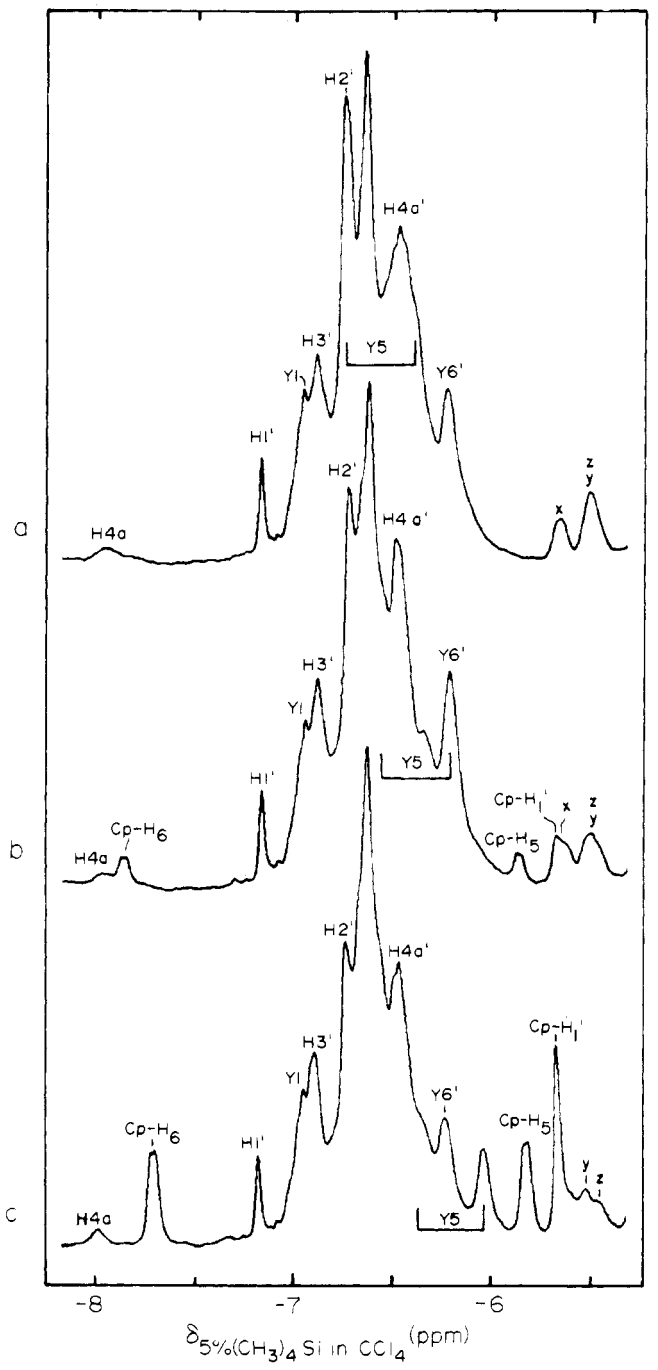

FIGURE 6: The 250-MHz correlation ^1H NMR spectra of ribonuclease A (40 mg/ml) which show the effect of adding the competitive inhibitor cytidine 3′-monophosphate at pH* 5.4. (The ribonuclease sample has been exchanged in D_2O as in Figure 1.) As the inhibitor is added, two peaks (Y(5)) assigned to tyrosine-25 move upfield. The peak (H(4a)) corresponding to the acid-stable form of histidine-48 moves slightly downfield. Notice also the divergence of peaks y and z tentatively assigned to backbone α-CH (Markley, 1975) as the inhibitor is added. Other assignments: H(1′), H(2′), H(3′), C(4)-H peaks of His^{105}, His^{119}, and His^{12}, respectively; H(4a′), the C(4)-H′ of the acid-stable form of His^{48}; Y(1) and Y(6), tyrosine peaks; Cp-H(6), Cp-4(5), and Cp-H(1′) are resonances from the inhibitor cytidine 3′-monophosphate (3′-CMP*). (a) 0 3′-CMP; (b) [3′-CMP]/[RNase A] = 0.5; (c) [3′-CMP]/[RNase A] = 2.3.

The chemical shifts of peaks assigned to the tyrosine doublet [Y(5) (ortho protons) and Y(5′) (meta protons)] of RNase A are plotted as a function of pH* in Figure 5. Both curves were fitted by nonlinear least-squares analysis to theoretical titration curves with a Hill coefficient of 1. The

Table I: Least-Squares Analysis of Data for the Spectroscopic Shift Affecting One Tyrosine Doublet (Peaks Y(5) and Y(5′)) Assigned to Tyrosine-25 of Ribonuclease A.

Peak	pK'	δ_{H^+}	δ_{H^c}	$\Delta\delta$	Variance × 10^4
Y(5)	5.73 ± 0.24	6.48 ± 0.01	6.326 ± 0.03	0.15 ± 0.04	2.2
Y(5′)	5.74 ± 0.22	6.78 ± 0.01	6.600 ± 0.03	0.18 ± 0.04	3.5

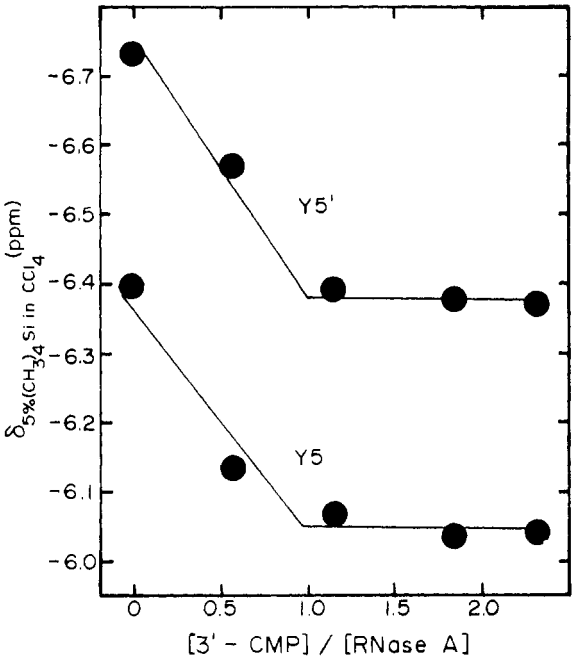

FIGURE 7: Chemical shifts of the peaks assigned to tyrosine-25 of RNase A as a function of added cytidine 3′-monophosphate (3′-CMP); 40 mg/ml of RNase A in 0.3 M NaCl in D_2O, pH* 5.4, 30°.

data were not good enough to warrant fitting the Hill coefficient to determine the cooperativity of the transition. The results are given in Table I. The pK' values based on Y(5) and Y(5′) both equal 5.7 ± 0.2.

Previous studies at 100 MHz (Meadows and Jardetzky, 1968; Meadows et al., 1969) have noted the upfield shift of a peak having an approximate intensity of five protons on the addition of cytidine containing inhibitors (cytidine, 2′-CMP, 3′-CMP, or 5′-CMP) at pH* 5.5. It has been suggested (Meadows, 1969; Meadows et al., 1969) that this peak corresponds to Phe^{120} and that the upfield shift results from the anisotropy of the cytidine ring. This effect was reinvestigated at 250 MHz (Figure 6). As 3′-CMP is added, a peak of two-proton intensity moves upfield starting from the position of the meta protons of tyrosine Y(5) (Figure 6a), up on top of peak Y(6′) (Figure 6b), and finally upfield from peak Y(6′) (Figure 6c). The two-proton intensity of the peak is readily apparent in Figure 6c by comparison with peak H(1′) (one proton). Other changes within the aromatic envelope suggest that another peak starting from the position of the ortho protons of tyrosine Y(5) also moves upfield roughly in parallel to the more visible peaks. The reasons for assigning these tyrosine peaks to the same tyrosine affected by the pH transition with pH_{mid} 5.7 are discussed below. The chemical shifts of the peaks assigned to tyrosine Y(5) are shown as a function of added 3′-CMP in

Figure 7. The chemical shifts are consistent with a 1:1 3′-CMP-enzyme complex.

The chemical shifts of the peaks assigned to tyrosine Y(5) are plotted as a function of pH* in Figure 8. The chemical shifts of these peaks in uninhibited RNase A are shown as dashed lines for reference. The tyrosine peaks (Y(5)) are shifted upfield in the complex in the entire pH* range studied (4–8). The upfield shift that occurs at pH* 5.7 in uninhibited RNase A appears to take place at about pH* 4.5 in the 3′-CMP inhibited enzyme. The peaks shift back downfield in 3′-CMP inhibited RNase A as the pH* is raised above 6.75.

Discussion

Proposed Mechanism for the Conformation Equilibria. The minimal mechanism required to explain the ^{1}H NMR data must include two slow protein conformational equilibrium steps and two proton dissociations. Since it is generally assumed that proton dissociation of the imidazole N–H is rapid (Eigen et al., 1960), a total of four species is required:

$$\underset{\text{H(4a)}}{\text{HEH}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{HE'H} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E'H} + \text{H}^+ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{E'} + \text{H}^+ \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underset{\text{H(4b)}}{\text{E''}} \qquad (2)$$

$$pK_2' = 5.6 \qquad pK_3' = 6.9$$

Only two of these species are observed in the ^{1}H NMR spectra of RNase A: the acid-stable form (peak H(4a)) corresponding to HEH, and the base-stable form (peak H(4b)) corresponding to E″. No peaks have been found that would correspond to one of the intermediates HE′H, E′H, or E′. Since the proton transfer steps (k_2, k_{-2}, k_3, k_{-3}) should be rapid, a single NMR peak is expected for these postulated species. At pH 6 this peak should have an intensity of nearly 1 proton (Figure 3), and it should lie in the normal histidine region of the spectrum. The failure to resolve such a peak may indicate that the mechanism proposed in eq 2 is inadequate. The resonance from the intermediate may be broadened beyond detection ($\Delta V_{1/2} > 100$ Hz) by additional chemical exchange processes, or there may be a number of intermediate species in slow exchange none of which predominates at a given pH. Either explanation requires a more complicated mechanism than that proposed in eq 2. The broadening of peaks H(4a) and H(4b) in the transition regions (Figure 2) presumably results from intermediate exchange on the NMR time scale of steps k_1, k_{-1}, k_4, and k_{-4}.

Proposed Assignments. Since the two slow equilibria K_1 and K_4 are perturbed by the proton dissociation steps, the pK' values of the latter steps may be estimated from the concentrations of species HEH (peak H(4a)) and E″ (peak H(4b)). The two pK' values are 5.6 and 6.9. One of these probably corresponds to the dissociation of His^{48}, and the other represents a group either in proximity to His^{48} or coupled to it by an allosteric linkage. A tyrosine residue Y(5) appears to be perturbed in free RNase A by the same transition at pH* 5.6 but not by the transition at pH* 6.9. Thus the tyrosine must be in the vicinity of His^{48} (or otherwise coupled to His^{48}) in the acid-stable conformation but not in the base-stable conformation.

Probable assignments of the critical tyrosine residue and the second dissociating group may be made on the basis of X-ray diffraction data for RNase A crystals (Kartha et al., 1967; Carlisle et al., 1974). His^{48} lies in a hydrophobic region bounded on one side by Tyr^{25} whose side chain hydroxyl is hydrogen bonded to the side chain carboxyl of Asp^{14}. Although more complicated mechanisms may be envisioned such as a cooperative conformational change involving simultaneous dissociation of His^{48} and Asp^{14}, the present data may be explained most simply by assigning the transition with a pH$_{mid}$ of 5.6 to dissociation of Asp^{14} and the transition with a pH$_{mid}$ of 6.9 to dissociation of His^{48}. This assignment is in better agreement with pK' values normally attributed to these groups in proteins than the reverse assignment, and this order of pK' values agrees with the assigned pK' values for Asp^{14} and His^{48} in RNase S (Cohen et al., 1973) and RNase A in the presence of acetate (see below). The tyrosine peak Y(5) affected by the transition with pH$_{mid}$ of 5.7 is assigned to Tyr^{25}. The ^{1}H NMR results confirm the observation of Donovan (1965) by ultraviolet difference spectroscopy of a tyrosine perturbation around pH 6 in RNase A. This difference spectrum now may be assigned to Tyr^{25}.

The aromatic peak of RNase A that shifts upfield in the presence of 3′-CMP and other cytidine containing inhibitors (Meadows and Jardetsky, 1968; Roberts et al., 1969) is assigned here to a single tyrosine. The peak has an area of

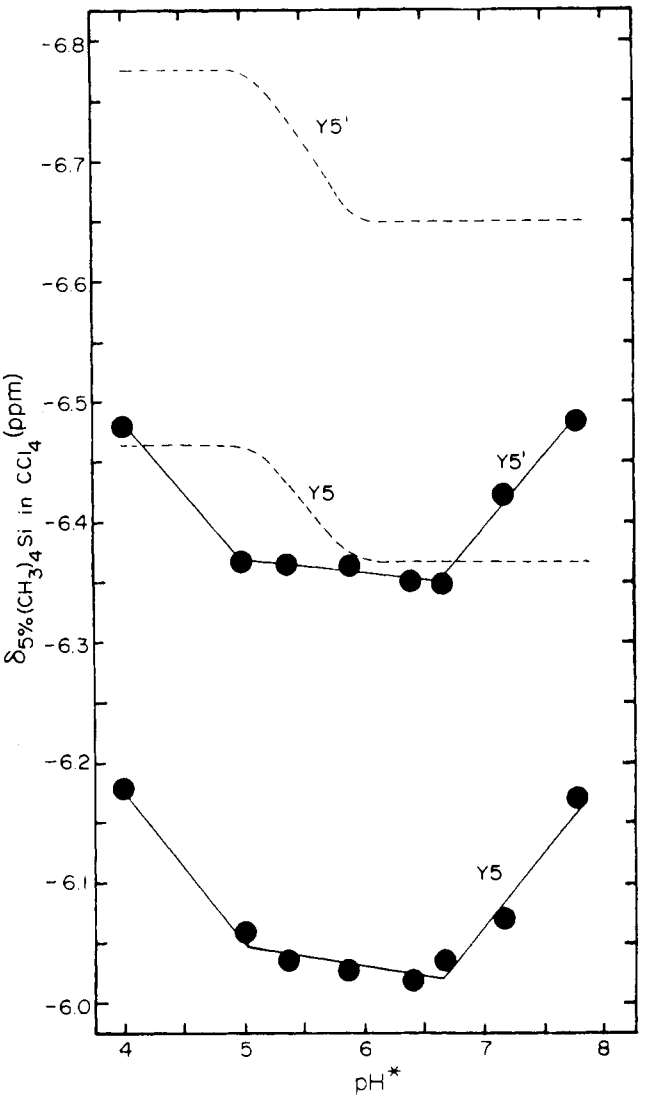

FIGURE 8: Chemical shifts as a function of pH* of the peaks assigned to tyrosine-25 in the 2:1 cytidine 3′-monophosphate–ribonuclease A complex. The dashed lines show the chemical shifts of the peaks in uninhibited ribonuclease A from Figure 5.

Table II: Proton Dissociation Constants of Aspartic Acid-14 and Histidine-48 Based on Proton NMR Data.

Enzyme Species	pK' Values	
	Asp^{14}	His^{48}
Bovine RNase A	5.7	6.9
Bovine RNase S[a]	4.2	6.1
Bovine RNase A–3′-CMP	5.7	6.9
Bovine RNase A + acetate	4–5	6.3
Rat RNase A[b]	?	7.6

[a] Based on data of Cohen et al. (1973). [b] Based on data of Migchelsen and Beintema (1973).

two protons, and another peak with two proton intensity shifts in parallel with it (Figures 7 and 8) as expected for a tyrosine ring that is free to rotate around its 1-3 carbon axis. These tyrosine peaks are equated with those tyrosine peaks assigned to Tyr^{25} because the peaks have identical chemical shifts in the absence of 3′-CMP (Figure 7) and because the pH 5.7 perturbation of the tyrosine peaks assigned to Tyr^{25} is altered in the presence of 3′-CMP (Figure 8).

Effect of Conversion of RNase A to RNase S. The ^1H NMR data indicate substantial structural differences between RNase A and RNase S in the region of His^{48} and Tyr^{25}. The tyrosine perturbation is absent in RNase S, and the His^{48} titration curve is continuous in RNase S. According to the recent X-ray results of Carlisle and coworkers (1974) the largest difference in the backbone hydrogen bonding pattern of RNase A and RNase S is centered near His^{48}. RNase A contains five backbone hydrogen bonds in this region that are not present in RNase S: His^{12}–O···N–Val^{47}; Asp^{14}–N···O–Val^{47}; Ser^{16}–N···O–His^{48}; Ser^{15}–O···N–Ala^{20}; Ser^{50}–O···N–Val^{54}. In addition, the side chain of His^{48} is more exposed and lies farther from the ring of Tyr^{25} in RNase S than in RNase A (Wyckoff et al., 1970). The additional hydrogen bonds present in RNase A probably are responsible for the existence of the slow conformational equilibria not present in RNase S. The altered position of the His^{48} side chain is consistent with the observation (Meadows et al., 1968; Cohen et al., 1973) that the chemical shift δ_{H^+} of His^{48} is more normal in RNase S than in RNase A. His^{48} in RNase S is affected by a spectroscopic perturbation with a pK' of 4.2 that has been assigned (Cohen et al., 1973) to the dissociation of Asp^{14}. Thus the data reveal that the pK' of Asp^{14} is lower and more normal in RNase S than in RNase A which is consistent with its being more exposed in RNase S (Table II).

Effect of Acetate on RNase A. The effect of acetate on the ^1H NMR spectra of RNase A is similar, in some respects, to the effect of conversion of RNase A to RNase S. The ^1H NMR titration curve of His^{48} of RNase A becomes continuous in the presence of acetate, and an inflection is observed with a pK' of 6.3 (Markley, 1975). It is known that high concentrations of acetate loosen the amino terminal peptide region of RNase A which includes Asp^{14} and Tyr^{25} (Fruchter and Crestfield, 1965), and a tyrosine perturbation by acetate has been detected previously by optical spectroscopy (Cann, 1971). It may be reasonable to expect, therefore, that at a lower concentration acetate destabilizes the structure of the His^{48} domain sufficiently to convert the slow equilibria to rapid equilibria on the NMR time scale. An increase in the rates of k_1, k_{-1}, k_4, and k_{-4} by a factor of 3–5, for example (plus an increase in the rates of any intervening slow steps), would be sufficient to explain the difference observed. Acetate apparently does not expose His^{48} fully since deuterium exchange experiments (Markley and Cheung, 1973) indicate that the C(2)–H of His^{48} is still inaccessible to the solvent in the presence of 0.2 *M* acetate.

The next question to consider is whether the pK' values of Asp^{14} and His^{48} are perturbed by 0.2 *M* acetate. Preliminary NMR data (J. L. Markley, unpublished) show that the upfield shift of Tyr Y(5) occurs between pH* 4 and 5 in the presence of acetate. Thus the pK' of Asp^{14} appears to be lowered by at least 0.5 pH* unit. The pK' of His^{48} is 6.3 in acetate which is closer to its value in RNase S (Cohen et al., 1973) than its value in RNase A alone (Table II).

Comparison with Other Studies. The above results are compatible in many respects with the conclusions of French and Hammes (1965) based on temperature-jump experiments. They found evidence for a slow isomerization of RNase A at neutral pH that is absent in RNase S. In D_2O, the isomerization appeared to be controlled by a single group with a pK of 6.5 which was assigned to His^{48}. The rates of isomerization in D_2O were found to be 183 sec^{-1} in one direction and 387 sec^{-1} in the reverse direction. It appears very likely that this conformational equilibrium is the same as one or more of the steps implicated by the NMR data. Using French and Hammes' (1965) rates the chemical shift differences between the exchange broadened species postulated in eq 2 (HEH and HE′H, or E′ and E″) may be calculated. They lie in the range 46–97 Hz, or 0.18–0.39 ppm at 250 MHz, which are reasonable chemical shifts for conformational perturbations of a histidine residue. The mechanism proposed by French and Hammes (1965) involves the isomerization of RNase A to a configuration in which the group with a pK of 6.5 (6.1 in H_2O) is exposed:

$$E_1H \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} E_2H \overset{k_{a1}}{\rightleftarrows} E_2 + H^+ \qquad (3)$$

This mechanism resembles the left-hand side of eq 2. The second slow equilibrium step (E′ ⇄ E″) may not have been observable in the temperature-jump studies. The pK' of 6.5 for the process in D_2O corresponds to an average of the two pK' values found by ^1H NMR spectroscopy.

French and Hammes (1965) concluded that the conformational change involves the breaking of a hydrogen bond because of the large deuterium isotope effect. Evidence for a change in the hydrogen-bonded structure of RNase A comes from the ^1H NMR experiments of Griffin et al. (1973) who found a discontinuity in the chemical shifts of three N–H peaks of RNase A in H_2O in pH 5.5. Two of these peaks were assigned to the titrating ring N–H of His^{48}. According to the present assignment of pK' values, the peaks should instead be assigned to hydrogen-bonded groups perturbed by the dissociation of Asp^{14}. For example, the peak that shifts upfield between pH* 3.0 and 5.5 may represent the titrating proton of Asp^{14}, and the other peaks at δ −13.4 and −10.9 may represent the hydroxyl proton of Tyr^{25} in the low pH and high pH conformational forms, respectively (Griffin et al., 1973, Figure 3). In any event, the results of Griffin et al. (1973) provide solid evidence for changes in the hydrogen-bonded sturcture of RNase A at the pH_{mid} of the first conformational transition. The N–H NMR region of RNase S in H_2O should show a very different pattern in this pH region.

Other lines of evidence have pointed to inhibitor-induced conformational changes in RNase A. These include optical

rotation studies (Deavin et al., 1966), comparison of deuterium exchange in the absence and presence of inhibitors (Nonnenmacher et al., 1971), and the observation that 2′-CMP significantly reduces the rate of conversion of RNase A to RNase S by subtilisin (Marcus et al., 1968). The present results are in agreement with those of del Rosario and Hammes (1970) on the binding of uridine (or cytidine) 2′,3′-cyclic phosphate, namely, that "formation of an initial enzyme–substrate complex [is] followed by a conformational change, with the enzyme–substrate complex able to undergo a conformational change similar to that which the free enzyme undergoes." In the previous studies of 3′-CMP binding to RNase A, however, it was concluded that the base-stable form binds the inhibitor more strongly (Hammes and Walz, 1969; Ernan and Hammes, 1966). This result is not compatible with the present finding that 3′-CMP does not affect pK_2' and pK_3' to a significant extent (Figure 2). On the other hand, k_4 and k_{-4} (eq 2) appear to be larger in the presence of 3′-CMP since peak H(4b) is broader (Figure 2).

In rat ribonuclease A three uncharged residues that lie adjacent to His^{48} in the bovine enzyme are replaced by charged residues (Beintema and Gruber, 1967, 1973):

cow	rat
Ser^{16}	Glu^{16}
Ser^{80}	Arg^{80}
Asn^{103}	Glu^{103}

The charged groups should greatly alter the environment of His^{48} and probably make it more exposed to the solvent. Migchelsen and Beintema (1973) have obtained NMR titration curves for the histidines of rat RNase A. The peak assigned to His^{48} has a continuous titration curve with a pK' of 7.6. The δ_{H^+} of His^{48} is abnormally shielded as in bovine RNase probably indicating that His^{48} is adjacent to Tyr^{25}. However, the His^{48} curve of rat RNase does not show a low pH* inflection that could be assigned to Asp^{14} (the curve was followed only down to pH* 4). It may be that Asp^{14} is exposed and has a more normal pK' of ≤ 4. The abnormally high pK' of His^{48} in rat RNase A may result from its interaction with the two additional negatively charged side chains of Glu^{16} and Glu^{103} present in the rat enzyme.

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