The Assignment of an Exchangeable Low-Field NH Proton Resonance of Ribonuclease A to the Active-Site Histidine-119†

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ABSTRACT: High-resolution proton magnetic resonance spectra between 11 and 14 ppm of bovine ribonuclease A in H_2O show that a single resonance titrates with changes in pH. This resonance arises from a histidine ring nitrogen proton and exhibits an apparent pK_a of 5.8. This pK_a value can be correlated with that of only one of the four histidine ring C-2 protons, namely, that which has been assigned to the active site histidine-119. Additional evidence for the correlation is obtained from the selective shifts of the apparent pK_a values caused by temperature variation and by the binding of the inhibitors cytidine 2′-monophosphate, cytidine 3′-monophosphate, cytidine 5′-monophosphate, and inorganic phosphate. These results suggest that a specific histidine ring nitrogen proton of residue 119 is relatively inaccessible to solvent. Solvent inaccessibility of histidine residues as defined by proton magnetic resonance studies of proteins in H_2O is discussed.

High-resolution proton magnetic resonance (pmr) spectroscopy can provide information about the environment of individual atoms within protein molecules if the resonances can be resolved and assigned to specific nuclei. For pmr studies of proteins, D_2O is routinely used as the solvent in order to avoid a dominant H_2O proton resonance and to simplify the protein pmr spectrum by exchanging deuterons for protons bound to oxygen and nitrogen atoms. Nonetheless, recent reports show that individual resonances of exchangeable protons can be resolved at low field between 10 and 15 ppm in the pmr spectra of lysozyme (Glickson *et al.*, 1971), myoglobin (Patel *et al.*, 1970a; Sheard *et al.*, 1970), hemoglobin (Patel *et al.*, 1970b), and RNase A[1] (Patel *et al.*, 1972) in H_2O solutions. Several of these low-field resonances in lysozyme have been assigned to specific indole NH protons of tryptophan residues (Glickson *et al.*, 1971). Based on the proposed assignments of the four histidine ring C-2 proton resonances to specific histidine residues (Meadows *et al.*, 1968), we report here the assignment of an imidazole NH proton resonance of bovine pancreatic RNase A to the active-site histidine-119 on the basis of the determination of its apparent pK_a in the free enzyme at different temperatures and in the enzyme complexed with each of four different inhibitors.

Materials and Methods

Bovine pancreatic RNase A was obtained from Worthington Biochemical Corp. (Rutherford, N. J.) as a phosphate-free powder. Concentrated solutions of RNase A were dialyzed extensively against deionized water and then lyophilized. Solutions for pmr studies were then made by dissolving the RNase A to 100 mg/ml in H_2O containing 0.1 M NaCl. Concentrated HCl and NaOH were used for pH adjustments. 2′-CMP, 3′-CMP, and 5′-CMP were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Pmr spectra were recorded on a Varian HR 220 nmr spectrometer. A Varian C1024 time-averaging computer was used to accumulate spectra for 1–5 hr at each pH value. The probe temperature was 22° unless otherwise noted. The chemical shift values are given relative to external tetramethylsilane in carbon tetrachloride solution.

pH values were measured in the pmr sample tube using a long thin combination electrode and a Radiometer Model 26 pH meter. The pH was determined before and after each spectrum was recorded, with these values differing by less than 0.04 pH unit. The final pH value is quoted in this paper. The apparent pK_a values exhibited by the imidazole NH resonance were generally determined as the pH at half-maximal chemical shift change. However, the titration data obtained in the presence of 0.1 M P_i were computer curve fitted to yield apparent pK_a values of the several titrating groups (Shrager *et al.*, 1972).

Results and Discussion

Three pmr spectra of RNase A in H_2O at three different pH values are shown in Figure 1. Pmr spectra of RNase A in D_2O at pH 5 or 9 contain no resonances between 10 and 13 ppm, and, therefore, all resonances in Figure 1 belong to exchangeable protons. One resonance shifts from 12.9 ppm at pH 4.4 to 11.1 ppm at pH 8.9, and it follows a simple titration curve with an apparent pK_a of 5.8. From the observed pK_a, the chemical shift values and the total change in chemical shift due to pH changes in model compounds, this resonance has been assigned by Patel *et al.* (1972) to an imidazole NH proton which exchanges very slowly with solvent H_2O. We have found that the pH dependences (see below, Figure 3) of the other exchangeable resonances shown in Figure 1 are generally similar to the results of Patel *et al.* (1972).

To which one of the four histidine residues of RNase A does the slowly exchanging NH proton belong? The pmr titration curves of the four imidazole C-2 proton resonances have been described, and an assignment of each resonance to a corresponding histidine residue in the RNase structure has been proposed (Meadows *et al.*, 1968). Therefore, direct comparison of the pK_a value exhibited by the imidazole NH proton resonance to the pK_a values exhibited by the imidazole C-2 proton resonances in H_2O should allow assignment of the NH proton resonance to a specific histidine residue. In Figure 2, that direct comparison is made by normalizing the pH-

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[1] Abbreviation used is: RNase A, bovine pancreatic ribonuclease A.

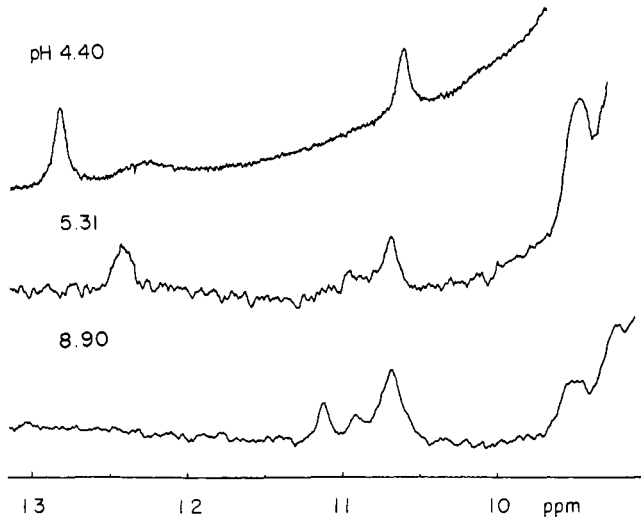


FIGURE 1: Pmr spectra of RNase A in H_2O,–0.1 M NaCl, 22°, at pH 4.4, 5.3, and 8.9. Different numbers of scans were averaged to obtain each spectrum using the Varian C1024 computer.

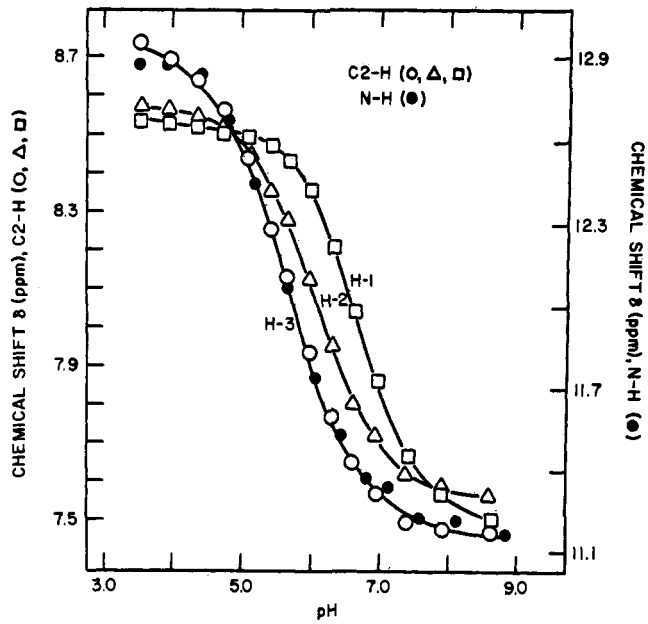


FIGURE 2: Pmr data for titration of histidine residues in RNase A in H_2O, 0.1 M NaCl, 22°. Chemical shift changes of the NH resonance are normalized with the chemical shift changes of the C-2–H resonances to facilitate comparison. The designations of the C-2 proton resonances as H-1, H-2, and H-3 follow the convention of Meadows *et al.* (1968).

dependent chemical shift values of the NH and the C-2 proton resonances. The titration of the NH proton resonance coincides exactly with the titration of the C-2 proton resonance, H-3, which has been assigned to histidine-119 in the active site of RNase (Meadows *et al.*, 1968).[2] Assuming the proposed assignment for the C-2 proton resonances are correct, the slowly exchanging imidazole NH proton is assigned to histidine-119.

A comparison of the pH dependence of the imidazole NH resonance with that of the C-2 proton resonances was conducted at 33° in order to compare the apparent pK_a values under a different set of solution conditions. At 33°, 0.1 M NaCl, the apparent pK_a value of both the imidazole NH resonance and the C-2 proton resonance, H-3, was 5.5 while the C-2 proton resonances H-1 and H-2 exhibited apparent pK_a values of 6.85 and 6.2, respectively. Thus, again the apparent pK_a value of the imidazole NH resonance correlates only with that of the C-2 proton resonance of histidine-119.

Additional evidence for assignment of the imidazole NH proton resonance to the histidine residue which exhibits the C-2 proton resonance, H-3, *i.e.*, to histidine-119, comes from studying the binding of the inhibitors 2′-CMP, 3′-CMP, 5′-CMP, and inorganic phosphate. Meadows *et al.* (1969) showed that the binding of these inhibitors causes selective shifts of the imidazole C-2 proton titration curves. As seen in Figure 3, the different cytidine mononucleotide inhibitors cause selective shifts of the imidazole NH resonance titration curve. At pH 2.5, where inhibitor binding is negligibly small, the imidazole NH proton resonates at 12.9 ppm. As the binding of inhibitor increases when the pH increases from 2.5 to 4.5, the chemical shift of the imidazole NH proton in the protonated histidine moves downfield by 0.4 ppm for 3′-CMP and 5′-CMP and by more than 0.8 ppm for 2′-CMP. The apparent pK_a values and the changes in chemical shift of the protonated histidine exhibited in Figure 3 are tabulated in Table I for comparison with the values of the imidazole C-2 proton resonances (Meadows *et al.*, 1969). The different apparent pK_a values of the imidazole NH resonance in the different RNase–inhibitor complexes correlate only with those of histidine-119. Inhibitor binding causes a downfield shift of the NH resonance. It should also be noted that inhibitor binding causes downfield shifts in the C-2 proton resonance of the protonated histidine-119, but upfield shifts in the C-2 proton resonance of the protonated histidine-12. Both the

TABLE I: Comparison of Apparent pK_a Values of RNase Imidazole NH and C-2 Proton Resonances in Complexes with CMP Inhibitors.[a]

	NH Resonance[b]		H-3 Resonance[c] His-119[d]		H-2 Resonance[c] His-12[d]	
Inhibitor	pK_a	$\Delta\delta$ (ppm)	pK_a	$\Delta\delta$ (ppm)	pK_a	$\Delta\delta$ (ppm)
3′-CMP	7.2	−0.4	7.4	−0.2	8.0	+0.1
2′-CMP	>8.0	−0.86	>8.0	−0.25	8.0	+0.08
5′-CMP	6.2	−0.4	<7.0	(0)[e]	8.0	+0.1

[a] All pH titrations were performed in the presence of 0.04 M inhibitor. $\Delta\delta$ is the maximum observed change due to inhibitor in the chemical shift of the protonated imidazole. [b] 22°, 0.1 M NaCl, H_2O this work. [c] 32°, 0.2 M NaCl, D_2O (Meadows *et al.*, 1969). [d] These assignments of resonances H-2 and H-3 are given by Meadows *et al.* (1968). [e] An accurate $\Delta\delta$ was not reported as no data were given below pH 5.5 by Meadows *et al.* (1969).

[2] The data between pH 3 and 7 for the NH proton resonance in H_2O can be adequately described by a simple proton association equilibrium with a pK_a of 5.8, a value which is similar to the overall pK_a of the C-2 proton resonance, H-3. In the acid region, however, the C-2 proton resonance shows deviations from the simple equilibrium indicating the interaction of two titrating groups with pK_a values in D_2O of 4.6 and 6.1 (Schechter *et al.*, 1972). The reason why the NH proton resonance is less sensitive than the C-2 proton to the acid perturbation (see also Figure 4) is not known.

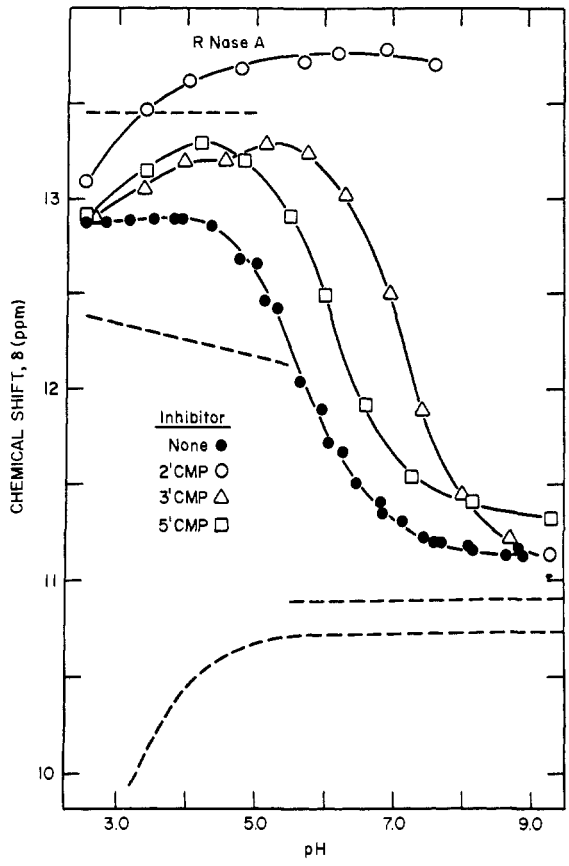


FIGURE 3: Effects of 2′-CMP, 3′-CMP, and 5′-CMP on the pH titration of the histidine NH resonance of RNase A. Nucleotides were 0.04 M. The dashed lines indicate the location of resonances which were unaffected by the cytidine mononucleotides. The resonances near 13.4 and 12.3 ppm broadened and finally disappeared as the pH increased above pH 4.5. The resonance at 10.9 ppm broadened and finally dsiappeared as the pH decreased below pH 7.

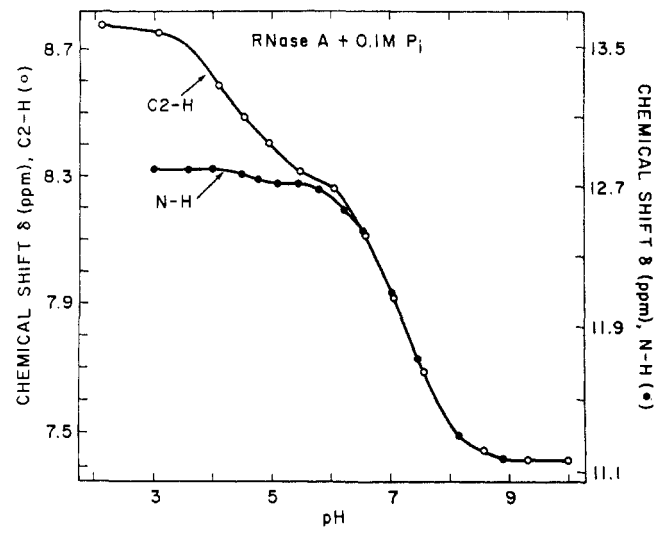


FIGURE 4: Effect of 0.1 M phosphate on the titration of the histidine NH resonance and the C-2 proton resonance, H-3, of RNase A. Chemical shift changes of the NH resonance are normalized with the chemical shift changes of the C-2 proton resonance, H-3. to facilitate comparison.

imidazole NH proton resonance and the histidine-119 C-2 proton resonance (Meadows *et al.*, 1969) are sharp in the presence of 3′-CMP, indicative of fast exchange, whereas each shows marked broadening with 2′-CMP above pH 7.0. For example, the imidazole NH resonance exhibits a half-width of 120 Hz at pH 7.6 in the presence of 2′-CMP compared to 20-Hz half-width at pH 2.5. Such exchange broadening implies that the lifetime of the protonated histidine–2′-CMP complex may be on the order of 0.5 msec (Meadows *et al.*, 1969).

A further comparison of the imidazole NH resonance with the C-2 ring proton resonance, H-3, is shown in Figure 4 which presents pH titration data obtained in the presence of 0.1 M phosphate which shifts the histidine titration curves (Cohen *et al.*, 1973) due to binding at the active site (Meadows *et al.*, 1969). Each curve in Figure 4 shows the identical histidine apparent pK_a of 7.2 as well as an additional inflection with an apparent pK_a of 4.4. The C-2 proton resonance, H-2, exhibits an apparent pK_a of 7.6 in the presence of 0.1 M phosphate (Cohen *et al.*, 1973). Thus, in the presence of 0.1 M phosphate, the imidazole NH proton resonance is identifiable only with the imidazole C-2 resonance, H-3, *i.e.*, with histidine-119.

The "static" accessibility of specific atoms in protein molecules to solvent molecules has been estimated by Lee and Richards (1971) who performed computer calculations of the exposed surface area of atoms in the X-ray crystallographic structures of RNase S, lysozyme, and myoglobin. Such calculated solvent accessibilities are termed "static" since they contain no information about local vibrations or flexibilities. In contrast, pmr spectroscopic studies in H_2O can be related to the "dynamics" of solvent accessibility of individual exchangeable protons.[3] Moreover, the effects of "motility" (Linderstrom-Lang and Schellman, 1959; Schechter *et al.*, 1969) on specific histidine residues in a protein can be studied using pmr spectroscopy, as will now be discussed.

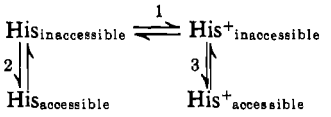
The exchange of NH protons of free imidazole or of free histidine with H_2O in aqueous solution is rapid (Eigen *et al.*, 1960) on the pmr time scale.[4] Thus, NH resonances of histidine residues in proteins are not usually observed since the NH protons exchange rapidly with H_2O. However, if the residence time of the NH proton on the imidazole group is much greater than 0.6 msec, the NH resonance may be observed (Sheard *et al.*, 1970). Thus, in those cases where the NH resonance is seen, the relatively long residence time would indicate that the particular NH proton whose resonance is observed is relatively inaccessible to water in the protein structure.

In a protein, a histidine residue with an imidazole NH proton judged "statically" inaccessible to solvent on the basis of its X-ray crystallographic structure could undergo small, rapid, reversible conformational changes whereby the NH

[3] The kinetics of proton exchange of individual tryptophan NH protons in lysozyme have been reported by Glickson *et al.* (1971).

[4] The pmr time scale is defined relative to the reciprocal of the frequency difference between two proton resonances. For example, for the exchange of the imidazole NH proton with water this frequency difference is approximately 1600 Hz. Thus, "rapid on the pmr time scale" implies an exchange is much more rapid than 0.6 msec whereas "slow on the pmr time scale" implies an exchange much slower than 0.6 msec. A detailed treatment of the behavior of proton resonances when a proton is undergoing exchange between two different chemical environments has been given by Becker (1969).

proton is briefly exposed to solvent. For such a situation, consider the simplified scheme



where 1 denotes the equilibrium of histidine in a conformation which is inaccessible to solvent between its neutral and protonated form, and where 2 and 3 denote, respectively, the equilibrium of neutral and of protonated histidine between the solvent inaccessible and solvent accessible conformations. The equilibration between the neutral and protonated forms of the histidine in its solvent accessible conformation, which is rapid (Eigen *et al.*, 1960), is not depicted. Pmr results can be used to set upper or lower limits on the lifetimes of each of the depicted histidine species. For example, since the NH resonance from both the neutral and the protonated forms of histidine-119 are seen, the half-time required for equilibration of 2 and of 3 must be very much more than 0.6 msec.[4] However, since a continuous pmr titration curve of the NH resonance of histidine-119 is observed, the half-time required for equilibration of 1 is much less than a millisecond. Thus, histidine-119 possesses one NH proton which exchanges slowly on the pmr time scale, for which the NH resonance is seen, and one NH proton which exchanges rapidly, for which no NH resonance can be seen. This implies that one ring NH proton of histidine-119 is relatively inaccessible to solvent molecules while the other ring NH proton is quite accessible to solvent molecules.

It is interesting to consider the possible case in which the half-times for equilibration of 1, 2, and 3 are all slow on the pmr time scale, *i.e.*, require more than 10 msec. In such a case a continuous pmr titration curve of the NH resonance would not be observed. Rather, one would see NH resonances of the protonated imidazole near 13 ppm at pH values below its pK_a while resonances near 11 ppm due to the neutral form of imidazole would be seen at pH values above its pK_a. In this regard, the dashed lines in Figure 3 indicate the presence of a resonance near 13.4 ppm at pH values below 5.5 and another resonance near 10.9 ppm at pH values above 7.0. These resonances might be due to one or more histidine residues whose imidazole NH protons would be judged by pmr as inaccessible to H_2O. The crystallographic structure of RNase S shows that histidine-48 is buried (Lee and Richards, 1971), and it shows very slow exchange between neutral and protonated species as judged from the pH dependence of its C-2 proton resonance (Meadows *et al.*, 1968). Thus, the resonances at 13.4 and 10.9 ppm might arise from histidine-48. Other pmr spectral patterns would result from alternative combinations of exchange rates.

In summary, the apparent pK_a value of the imidazole NH resonance coincides only with that of the C-2 proton resonance of histidine-119 under a variety of conditions. It is, therefore, concluded that the imidazole NH resonance belongs to histidine-119 in the active site of RNase A. This NH resonance of an active-site residue in RNase A provides another probe for pmr studies of structure–function relations in the catalytic site of this protein.

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