Nuclear Magnetic Resonance Titration Curves of Histidine Ring Protons

CONFORMATIONAL TRANSITION AFFECTING THREE OF THE HISTIDINE RESIDUES OF RIBONUCLEASE*

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NMR titration curves are reported for the 4 histidine residues of ribonuclease A in sodium acetate and for ribonuclease S in sodium acetate, phosphate, and sulfate solutions. Evidence is presented that the imidazole side chain of histidine residue 48 undergoes a conformational change, probably also involving the carboxyl side chain of aspartic acid residue 14. This group is considered to be responsible for the low pH inflection with pK, 4.2 present in the NMR titration curve of the C-2 proton resonance of histidine 48. The NMR titration curves of the active site histidine residues 12 and 119 also exhibit inflections at low pH values, although there is no carboxyl group within 9 Å of the imidazole side chain of histidine residue 12 in the structure of ribonuclease S determined by x-ray crystallography (Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970) J. Biol. Chem. 245, 305-328). Curve fitting was carried out on 11 sets of NMR titration data using a model in which the 3 histidine residues 12, 119, and 48 are assumed to be affected by a common carboxyl group. The results obtained indicate that such a model with fewer parameters gives as good a representation of the data as the model in which each histidine residue is assumed to interact separately with a different carboxyl group. Therefore, it is concluded that the ionization of aspartic acid residue 14 is indirectly experienced by the active site histidine residues through the conformational change at histidine 48. A model assuming mutual interaction of the active site histidine residues does not account for the low pH inflections in these curves.

Several NMR studies of the titration properties of the histidine ring C-2 proton resonances of ribonuclease have been reported (1-9). These have no doubt contributed to the general interest in the application of NMR spectroscopy to proteins. However, there has been a certain amount of confusion in the results, which may partly have arisen from the sensitivity of NMR titration curves as monitors of protein conformation to such variables as temperature and solution conditions (10-14). In order to clarify the nature of the four NMR titration curves of ribonuclease A, and its derivative ribonuclease S (15), we now report comparative data for both proteins in sodium acetate, and for ribonuclease S in sodium sulfate and phosphate. It was possible to obtain this data in a shorter period of time using the pulse-Fourier transform NMR method (16) than with the continuous wave time-averaging technique used previously (7, 12).

Only one set of data for ribonuclease S has been published providing a complete titration curve of resonance H-4 (12), which has been assigned to histidine residue 48 (3). The four sets of data we now present contain complete titration curves for resonance H-4, each of which exhibits a low pH inflection. A comparison provides some information on the microenvironment of histidine residue 48, including evidence for a local conformational change probably involving aspartic acid residue 14.

In order to account for the low pH inflections observed in the NMR titration curves of the 2 active site histidine residues 12 and 119 of ribonuclease A in sodium chloride solution, two theoretical models were previously applied (7). One model assumed the interaction of each of the active site histidine residues with titrating carboxyl groups. The other assumed mutual interaction of the active site histidine residues. Since the former model was found to be the preferable one it was subsequently applied to other sets of NMR titration data of ribonuclease (12). Two further models are now described, in which the low pH inflection is given a common value in any two, or all three titration curves, of the 2 active site histidine residues and histidine 48. This implies interaction, directly or indirectly, of any 2 or all 3 histidines with the same carboxyl group. We now apply all four models to a total of 11 sets of NMR titration data for ribonuclease A and S under different conditions.

* This is Paper VIII in the series on “NMR Titration Curves of Histidine Ring Protons.”
experimental conditions. Comparison of the results of curve fitting indicate that a common conformational change affects histidine 48 and the active site histidine residues 12 and 119.

EXPERIMENTAL PROCEDURE

Bovine pancreatic ribonuclease A (code RAF) was purchased from Worthington Biochemical Corp. (Rutherford, N.J.) and was not further purified. Bovine pancreatic ribonuclease S was purchased from Sigma Chemical Co. (St. Louis, Mo.). Samples for NMR studies were desalted by gel filtration on Sephadex G-25 in ammonium bicarbonate (0.1 M, pH 8.5). Fractions comprising the ultraviolet absorbing peak were combined, lyophilized, and then lyophilized 3 to 5 times from about 2 ml of 99.7% D2O. Samples of 25 to 50 mg of protein were then dissolved in 0.5 ml of D2O (100%) for NMR studies. Stock solutions of sodium chloride, phosphate, sulfate, and deuterio-acetate in D2O were used to adjust to the desired concentration. Before chromatography one sample of ribonuclease S contained 4.7% phosphate, whereas after purification and NMR studies in sodium acetate, analysis of several lyophilized ribonuclease S samples showed no detectable phosphate. Some ribonuclease S samples were subjected to ultrafiltration to exchange D2O using an Amicon model MMC system with UM05 Diaflo membranes at 50 p.s.i. pressure. Histidine C-2-H NMR resonances were generally broader than for those samples lyophilized to exchange D2O. It is thought that this might arise from uptake of paramagnetic metal ion impurity (17) from the larger volumes (about 25 ml) of D2O used in the ultrafiltration procedure.

Assays of ribonuclease A and S were carried out by the procedure of Crook et al. (18) with cyclic cytosine 2':3':monophosphate using a Gilford ultraviolet spectrophotometer at 286 nm. Values obtained were in the range of 40 to 55 ΔΔA/min/mmol.

Measurements of pH were made using a Radiometer model 26 with a long thin Ingold combination electrode inside the NMR tube (Wilmad 528PP). Direct meter readings in D2O are reported (19). Adjustments of pH were made with 1 N DCI, 0.5 N NaOD and 1 N CD3COOD solutions. It was difficult to adjust the solutions in 0.2 M sodium acetate to low pH values with acetic acid solutions due to buffering which resulted in excessive dilution; consequently DCl was also used in these cases below about pH 4.

NMR spectra were obtained at 220 MHz on a Varian Associates HR220 spectrometer equipped with an FT accessory with a dedicated 620L computer giving 9K point transforms. Spectra were usually obtained in about 3 to 5 min, and spectrometer settings were generally as reported previously (20). Chemical shift values are reported in parts per million downfield from external tetramethylsilane at an ambient probe temperature of 20 °C.

Curve fitting was carried out using the Digital Equipment Corp. PDP10 computer at the Division of Computer Research and Technology, National Institutes of Health. The program system used for least squares iterative regression was MLAB (developed by G. Knott and D. Reece). Computer graphics on a DEC 360 or Tektronix T4012 display and plotting with a CALCOMP plotter are among the features of MLAB which were used.

RESULTS

Ribonuclease A and S in Sodium Acetate

NMR titration data of the four C-2 proton resonances of ribonuclease A and ribonuclease S in sodium acetate (0.2 M) solutions are shown in Figs. 1 and 2. It is clear that there are only very minor differences between the curves of resonances H-1, H-2, and H-3 in the two sets of data. Similarly, there is very little difference for these resonances from the data for both proteins in sodium chloride solution, which has been presented previously (7, 12).

By contrast, the NMR titration curves for resonance H-4 are quite different, comparing ribonuclease A and ribonuclease S, with an almost constant 0.2 ppm chemical shift difference between them. In ribonuclease A in sodium chloride solution resonance H-4 broadens rapidly with increase in pH above 4.5 (3), and consequently no complete titration curve can be described. It nevertheless has the same chemical shift value at low pH as that of resonance H-4 in ribonuclease A in sodium acetate solution.

Results for the titration curves of resonance H-1, which has consistently been assigned to histidine residue 105 (3), were found to fit a theoretical curve for a single simple titration equilibrium (5). Values of pK for H-1 were 6.82 ± 0.02 for both cases with a chemical shift change on ionization of 1.04 and 1.06 ppm.

Ribonuclease S in Sodium Phosphate and Sulfate

In order to observe a complete NMR titration curve for resonance H-4 of ribonuclease in the presence of sodium phosphate and sulfate it was necessary to study ribonuclease S. Data for ribonuclease S in sodium phosphate (0.1 M) are shown in Fig. 3, in which resonance H-1, which is unaffected by the presence of phosphate ion, is omitted for clarity. The continuities of the curves are readily determined by comparison with the results in the absence of phosphate ion. While resonance H-4 is also unaffected by the presence of phosphate, resonances H-2 and H-3 are significantly altered. The low pH inflection

![Fig. 1. NMR titration curves of the histidine ring C 2 proton resonances of ribonuclease S in sodium acetate (0.2 M)/D2O solution obtained at 220 MHz. The solid lines are theoretical fits obtained with model A, the dashed lines which are almost superimposed are theoretical fits obtained with model C for resonances H-2, H-3, and H-4 (see Fig. 5).](http://www.jbc.org/)

![Fig. 2. NMR titration curves of the histidine ring C 2 proton resonances of ribonuclease A in sodium acetate (0.2 M)/D2O solution. The solid lines are theoretical fits with model A.](http://www.jbc.org/)
present in curve H-3 is accentuated in the presence of phosphate, as a result of the increase in the imidazole pKₐ value, but the actual value of the pKₐ for the inflection is not significantly altered. The similarity of the pH dependence of the low pH inflections of resonances H-3 and H-4, notwithstanding their opposite directions of shift, is an especially clear and novel aspect of these data.

The results for ribonuclease S in sodium sulfate (0.1 M) are shown in Fig. 4. It is concluded, by comparison with the results in the absence of sulfate, that resonance H-2 now shows the highest pKₐ value. This would indicate that the assignments of resonances H-1 and H-2 in the previous results for ribonuclease A in sodium sulfate (12) should be reversed. This makes little difference to the analysis of both sets of data. No low pH inflections are observed for resonance H-2, and single simple titration curves (5) adequately describe these data. The chemical shift change for the low pH inflections of resonance H-3 is significantly reduced in the presence of sulfate, and the value of the pKₐ seems also to be lower.

The results for resonance H-1 are very similar in the presence of both sodium phosphate and sulfate, with a pKₐ of 6.99 (+0.04) in each case, and chemical shift change of 1.07 and 1.00 ppm, respectively. These values are also very similar to those found for resonance H-1 in ribonuclease A in the presence of these salts (12).

Curve Fitting

Four theoretical models have been applied, as illustrated in Fig. 5. The results of curve fitting the data presented above and those published previously (7, 12) with these models are given in Tables I to III.

Model A—Separate interaction¹ of each imidazole group with a low pH titrating group. This corresponds to a sum of two simple proton association-dissociation equilibria:

\[
\delta_{\text{obs}} = \delta_{\text{min}} + \frac{10^{-\Delta_{\text{II}}}}{1 + 10^{(pK_{\text{II}} - \text{pH})}} + \frac{10^{-\Delta_{\text{I}}}}{1 + 10^{(pK_{\text{I}} - \text{pH})}}
\]

where \(\delta_{\text{obs}}\) is the observed chemical shift, \(\delta_{\text{min}}\) is the chemical shift of the unprotonated form at high pH, and \(\Delta\) and \(pK\) are the chemical shift changes and ionization constants, respectively, for the low and high pH inflections represented by subscripts 1 and 2, respectively. This was termed model I in a previous publication (21). The application of a more complex model (7, 21) to resolve the two inflections present in these curves was not found necessary (12) and has not been found necessary in the present work, since the inflections are sufficiently separate to be resolved by the present model. In view of the pKₐ value found for the low pH inflection it is considered that this could only arise from a carboxyl group in a protein. It should be noted that the smaller the chemical shift change for an inflection the larger will be the standard errors for the two parameters \(\Delta\) and \(pK\), and hence the less reliable will be their values.

Model B—Interaction of two imidazole groups with a single

¹The term “interaction” refers to the influence of one titrating group on the observed titration curve of another, and could thus be due to (a) direct competition for protons (as in model D), (b) direct interaction through space but with ionization occurring at well separated pK values (as in model A), or to (c) indirect interaction, which would be mediated through other bonds and would thus represent a pH-dependent conformational change (as in models B and C).
low pH titrating group. In this case the model consists of Equation 1 applied to one set of data, and Equation 2, to the second set (indicated by superscript A) simultaneously. The only common parameter \( pK_1 \) is forced to take a best value at convergence for both sets of data.

\[ A_{\text{obs}} - A_{\text{min}} = A_{pK_{1}}^{pK_{1}} + A_{pK_{2}}^{pK_{2}} + A_{pK_{3}}^{pK_{3}} + A_{pK_{4}}^{pK_{4}} \]

Model C—Interaction of all three imidazole groups with a single low pH titrating group. This is a natural extension of Model B and involves the fitting of three sets of data simultaneously with a further equation of the form 9, except that different superscripts are applied to distinguish all parameters except \( pK_{1} \), which again is the only common parameter.

Model D—Mutual imidazole interaction. This model (21) has been applied previously to results for resonances H-2 and H-3 of ribonuclease A in sodium chloride (7) can be represented as

\[ A_{\text{obs}} = \frac{A_{\text{obs}}(1 + 10^{pK_{\text{obs}}+pK_{A1}}) + A_{\text{obs}}(10^{pK_{\text{obs}}+pK_{A1}}) + A_{\text{obs}}(pK_{\text{obs}}+pK_{B1})}{1 + 10^{pK_{\text{obs}}+pK_{A1}} + 10^{pK_{\text{obs}}+pK_{B1}} + 10^{pK_{\text{obs}}+pK_{A1}+pK_{B1}}} \]

where the four ionization constants are related by

\[ K_{A0} K_{A1} = K_{B0} K_{B1} \]

and \( \delta_{A0} \) and \( \delta_{A1} \) are the chemical shift values for Curve A at
high and low pH, respectively. This model has now been applied to 10 further sets of NMR titration data. The common $pK_a$ values in this model result in a parallel pH dependence of the chemical shift for both theoretical curves. Since the data for resonances H-2 and H-3 do not show a parallel dependence of chemical shift on pH it is not surprising that this model does not provide good fits to these sets of data (Fig. 8).

The results of fitting these four models to the same set of data can be compared most effectively by using the root mean square error. The following conclusions can be arrived at when the values of the root mean square error derived from the application of the four models (Tables I and III) are compared. (a) The model for mutual interaction of the active site histidine residues is a poorer one for all cases (compare Figs. 1 and 6). (b) The root mean square error values for models B and C are as small as those for model A. (The variations observed are not considered significant.) Thus, we obtain as good a fit even though we are applying fewer parameters in model C (13 parameters) and model B (14 parameters) than in applying model A to the same three titration curves (15 parameters) (Fig. 1).

The results for the chemical shift changes derived from fitting models A, B, or C to the NMR titration curves of resonances H-2, H-3, and H-4 of ribonucleases A and S under different experimental conditions are shown in Table II. The values of $\Delta_1$ for H-2 and H-3 are generally greater in the presence of phosphate ion, but smaller in the presence of sulfate ion. The values of $\Delta_1$ for resonance H-4 of ribonuclease S in different conditions show little variation, while the value of this parameter found for ribonuclease A in acetate is halved.

**DISCUSSION**

**Microenvironment of Histidine Residue 48**—The NMR titration curves of resonances H-1, H-2, and H-3 of both ribonuclease S and ribonuclease A were found to be essentially unchanged in sodium acetate (Figs. 1 and 2) and chloride solutions (12). However, the NMR titration curves of resonance H-4, which has been assigned to histidine residue 48 (3), are quite different, comparing ribonuclease A in sodium acetate with ribonuclease S in both acetate and chloride solutions. This indicates that the microenvironment of histidine residue 48 is sensitive to the local change in conformation resulting from the A to S conversion. For ribonuclease A in sodium chloride the line width of the histidine 48 C-2 proton resonance increases quite sharply above pH 4.5 (10). The broadening of this resonance was postulated by Meadows et al. (22) to arise from a local conformational equilibrium between two environments, slow on the NMR time scale. From the chemical shift difference in sodium chloride at low pH and high pH, when resonance H-4 reappears (7), the lower limit for the slow rate of interconversion can be estimated to be about 10 ms. (This naturally assumes a single process is occurring.) Under the other conditions described here a single resonance is seen for H-4 throughout the titration range; it must then be undergoing exchange with a rate faster than the chemical shift difference between the two forms, namely faster than about 10 ms.

A conformational change dependent on the ionization of a group with $pK_a$ 6.9 in D$_2$O (6.1 in H$_2$O) was observed by French and Hammes using temperature jump kinetics on ribonuclease A in 0.1 M potassium nitrate (23). From the effects of inhibitors known to bind in the active site of ribonuclease, the group responsible was said to be histidine 119. The isomerization as described by French and Hammes was postulated to involve the making and breaking of a hydrogen bond by a histidine residue with a carboxylate anion of a glutamate or aspartate residue at a rate of 2 to 5 ms in D$_2$O at 25°C.

The lower inflections present in the NMR titration curves of resonance H-4 have $pK_a$ values in the range of 4.2 to 4.7 (Table I). This inflection is attributed to the effect of the carboxyl group of aspartic acid residue 14, which is in close proximity to the imidazole side chain of histidine 48 in the structures determined by x-ray crystallography (24, 25). The region

### Table III

**Ionization constants for active site histidine residues from curve fitting using model assuming mutual interaction**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Salt</th>
<th>Conc (M)</th>
<th>$pK_{A0}$</th>
<th>$pK_{A1}$</th>
<th>$pK_{B0}$</th>
<th>$pK_{B1}$</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ac</td>
<td>0.2</td>
<td>6.32</td>
<td>5.02</td>
<td>5.34</td>
<td>4.23</td>
<td>4.8</td>
</tr>
<tr>
<td>S</td>
<td>Ac</td>
<td>0.2</td>
<td>6.12</td>
<td>4.98</td>
<td>5.27</td>
<td>4.12</td>
<td>4.7</td>
</tr>
<tr>
<td>A</td>
<td>Cl</td>
<td>0.1</td>
<td>6.08</td>
<td>5.39</td>
<td>5.53</td>
<td>4.86</td>
<td>4.6</td>
</tr>
<tr>
<td>S</td>
<td>Cl</td>
<td>0.1</td>
<td>6.02</td>
<td>5.44</td>
<td>5.57</td>
<td>4.99</td>
<td>4.6</td>
</tr>
<tr>
<td>A</td>
<td>$F_i$</td>
<td>0.00065</td>
<td>6.33</td>
<td>5.28</td>
<td>6.00</td>
<td>4.94</td>
<td>5.1</td>
</tr>
<tr>
<td>S</td>
<td>$F_i$</td>
<td>0.0065</td>
<td>6.73</td>
<td>4.99</td>
<td>6.22</td>
<td>4.48</td>
<td>5.1</td>
</tr>
<tr>
<td>A</td>
<td>$F_i$</td>
<td>0.065</td>
<td>6.74</td>
<td>5.01</td>
<td>6.30</td>
<td>4.57</td>
<td>4.8</td>
</tr>
<tr>
<td>S</td>
<td>$F_i$</td>
<td>0.1</td>
<td>7.21</td>
<td>5.00</td>
<td>6.89</td>
<td>4.59</td>
<td>8.0</td>
</tr>
<tr>
<td>A</td>
<td>$F_i$</td>
<td>0.1</td>
<td>7.18</td>
<td>4.99</td>
<td>6.69</td>
<td>4.41</td>
<td>5.9</td>
</tr>
<tr>
<td>S</td>
<td>$F_i$</td>
<td>0.1</td>
<td>6.53</td>
<td>5.64</td>
<td>5.58</td>
<td>3.92</td>
<td>6.6</td>
</tr>
<tr>
<td>A</td>
<td>$F_i$</td>
<td>0.1</td>
<td>6.70</td>
<td>6.66</td>
<td>6.03</td>
<td>4.99</td>
<td>7.2</td>
</tr>
<tr>
<td>S</td>
<td>$F_i$</td>
<td>0.1</td>
<td>6.70</td>
<td>6.66</td>
<td>6.03</td>
<td>4.99</td>
<td>7.2</td>
</tr>
</tbody>
</table>

*For more details of this model (D), see Fig. 6 and ref. 21. This model has only previously been applied to the data for ribonuclease A in sodium chloride (2) but has been reapplied in this case for consistency. No standard errors are available for application of this model.

*Root mean square error = $\sqrt{S/(n - p + c)}$ where, $S$ is the sum of squares at convergence, $n$ is the number of observations (data points); $p$ is the number of parameters and, $c$ is the number of constraints.
around histidine 48 in ribonuclease S, in the presence of bound sulfate ion at pH 5.5 in H$_2$O (24), is shown in Fig. 7. The two parallel hydrogen-bonded systems comprising the "hinge" of the protein are illustrated in Fig. 8.

It is apparent that this system can remain intact only so long as the imidazole side chain of histidine 48 is protonated. The carboxyl group of aspartic acid 14 must be very close to the imidazole side chain of histidine 48 for its pK$_a$ value to be so significant a feature of the histidine 48 NMR titration curve. In addition, the carboxyl group of aspartic acid residue 14 will become negatively charged above pH 4.2. We therefore propose that the local conformational transition which affects histidine 48 occurs following deprotonation of the aspartic acid 14 carboxyl and histidine 48 imidazole side chains, namely between pH 4.2 and 6.2, as represented in Fig. 8. Such a change would require the carboxyl side chain of aspartic acid 14 to twist towards the imidazole residue of histidine 48. Whether or not there is a movement of the adjacent side chain of tyrosine 25 is conjectural. However, there is some evidence from ultraviolet (27) and NMR (22) spectroscopy that such a perturbation does occur.

It is known that aspartic acid 14 is an important residue for binding S-peptide to S protein (28). Further, aspartic acid 14 is apparently the most important binding site in the carboxyl terminal portion of the S peptide. The cleavage of the bond between residues 20 and 21 might well affect aspartic acid 14, providing an origin for the difference observed between ribonucleases A and S for resonance H-4. We would then assign to histidine 48 a major role as the binding site on S-protein for the S-peptide. It should be noted that both positions 14 and 48 in homologous ribonucleases have been conserved in evolution as aspartic acid and histidine residues, respectively (29).

**Influence of Conformational Change on Active Site Histidine Residues**—The application of theoretical models assuming the interaction of 2 or 3 histidine residues with the same carboxyl group is reported here for the first time. The results (Tables I and II) of curve fitting such a model (Fig. 5, B) to data for only the active site histidine resonances H-2 and H-3 (3) indicate that an equally good fit is obtained as for each residue assumed to be interacting separately with a different carboxyl group. Further, for the five sets of data in which resonance H-4 is observed throughout its titration range, the application of a model (C) in which all three histidine resonances H-2, H-3, and H-4 are assumed to be affected by the same carboxyl group, results in equally good fits. It is considered improbable that 3 imidazole residues are influenced by three separate groups having close enough pK$_a$ values and small enough standard errors to provide the fits obtained. Thus, we conclude that the same group is responsible for these low pH inflections in all three curves. On the other hand, it is possible that the similarities in pK$_a$ for the low pH inflections in these curves could be purely fortuitous. We are well aware of the dangers of overinterpretation of curve fitting and particularly emphasize that our conclusions result from the application of a model with fewer parameters to a given set of data.

We have also applied a model (D) assuming mutual interaction of the active site histidine residues (7) to a further 10 sets of ribonuclease NMR titration data. Our results (Table III) confirm that the low pH inflection in the curves of the active site histidine residues of ribonuclease cannot derive from their mutual interaction. Markley (30), and Markley and Finkenstadt, have recently reported the application of a model for mutual interaction of the 2 active site histidine residues of ribonuclease A in sodium chloride (0.3 M) at 36°C and 250 MHz. They utilized a model which combined the features of both the mutual interaction model (D) with H-3 assumed to interact with a further group having a low pK$_a$ value (model A). This combined model has two more parameters than the original form and not surprisingly gives an improved fit. No explanation was offered for the group with pK$_a$ 3.7 ± 0.2 which they found for resonance H-3. Further, this approach effectively eliminates the possibility that the low pH inflection in resonance H-3 could arise from the mutual interaction of the active site histidine residues, as originally proposed by others (4). The conclusion that a difference of 0.2 pH unit, as derived from the fitted microscopic pK$_a$ values for the imidazole titrations for resonances H-2 and H-3, can be used to calculate a distance between the histidine side chains giving rise to these curves (30), is questionable. Even if histidine residues 12 and 119 affect each other's titration curve in the region of their ionization we believe that this must be a secondary effect.

One puzzling feature of our previous results for the NMR titration curves of the active site histidine residues 12 and 119 was the presence of significant inflections in both curves for ribonuclease A in sodium phosphate solution (12). The pK$_a$ values of these inflections were in the range 4.3 to 4.8, and it was argued that these transitions could not arise directly from
one of the possibilities originally noted (5). In view of the reassignment of resonance H-3 to histidine residue 12', further compounded this problem. However, it is possible that both active site histidine residues are affected by a conformational change, one of the possibilities originally noted (6). In view of the results obtained using model C, this conformational transition could presumably be that described above affecting histidine residue 48. Thus, the inflections present in each of the active site histidine NMR titration curves could have their origin in the titration of aspartic acid residue 14. For such a conformational change to also be experienced by histidine residue 12 implies a significant movement of the backbone between aspartic acid 14 and histidine 12. The rate of the conformational change as it is experienced at both of the active site histidine residues could be faster than the rate experienced by histidine 48 in ribonuclease A in sodium chloride, where resonance H-4 exhibits a slow exchange phenomenon, while resonances H-2 and H-3 show single sharp resonances. We are attempting to utilize carbon 13 NMR spectroscopy to detect the presence of this conformational change. *

A further puzzling feature of the previous NMR results is the greater magnitude of the low pH inflection exhibited by the C-2 proton of resonance H-3 compared to the slowly exchanging imino proton of the same residue (32). If the inflection in both resonances arose from a carboxyl group forming a hydrogen bond to the imidazole residue, and giving rise to the slowly exchanging N—H, one would expect the magnitude of the effect to be reversed. However, if this inflection results from the transference of the effect of ionization of a distant carboxyl group to histidine residue 12, there is no reason why the relative magnitudes of the inflections of the buried N—H and C-2—H resonances should not be as observed.

The magnitude of the low pH inflection in curve H-3, assigned to histidine 12, is greater than that of histidine 119 (Table II). This group is further away from aspartic acid 14. It is, of course, possible that for histidine 119 the low pH inflection could arise wholly or partly from the adjacent carboxyl side chain of aspartic acid residue 121 (7, 12). This would imply that the combination of model B for residues 48 and 12 with model A for residue 119 is the most appropriate solution. However, the results of curve fitting models A and B to the data for residues 12 and 119 (Table I) make this unlikely.

The origin of the low pH inflections in the curves of the active site histidine residues in a distant ionization event is also suggested by their lack of sensitivity to the binding of phosphate ion or changes in ionic strength (Table I). The common differences observed for resonances H-2 and H-3 in the presence of phosphate and sulfate also argue for a common origin of the low pH inflections. The results in the presence of sulfate ion might indicate a reduction of the pK_a value as well as the magnitude of the low pH inflections for these resonances. It is possible that the effect of sulfate in reducing the magnitude of the low pH inflection in the resonances of the active site histidine residues represents a suppression of the conformational change at the active site region. These effects might indicate that the structure of ribonuclease S with bound sulfate (24) is not the best structural model to consider in relation to the proposed conformational change in solution. Apart from the statement that the hydrogen bonding scheme shown in Fig. 7 for ribonuclease S is very similar to that found for ribonuclease A (28), we have no other detailed information available on this structure. In addition, it is quite possible that the conformational change described here occurs more readily in solution, and the conformation observed in the crystal may be stabilized.

We have not speculated on the functional significance of the conformational change described here. However, an allosteric mechanism for the binding of mononucleotides to ribonuclease has recently been proposed (33).

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*J. S. Cohen, and H. Shindo, unpublished results.
*D. Patel, L. L. Canuel, and F. A. Bovey, unpublished results.
CONCLUSION

The inflections with pK_a ~4.2 in the NMR titration curves of histidine residues 12, 119, and 48 of ribonuclease in solution appear to derive from a common event, which is identified as the titration of aspartic acid residue 14 and the concomitant conformational change involving the side chains of residues 14 and 48.

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