Nuclear Magnetic Resonance Titration Curves of Histidine Ring Protons

A DIRECT ASSIGNMENT OF THE RESONANCES OF THE ACTIVE SITE HISTIDINE RESIDUES OF RIBONUCLEASE

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One of the four titrating histidine ring C-2 proton resonances of bovine pancreatic ribonuclease has been assigned to histidine residue 12. This was accomplished by a direct comparison of the rate of tritium incorporation into position C-2 of histidine 19 of S-peptide (residues 1 to 90) derived from ribonuclease S, with the rates of deuterium exchange of the four histidine C-2 proton resonances of ribonuclease S under the same experimental conditions. The same assignment was obtained by a comparison of the NMR titration curves of ribonuclease S, the noncovalent complex of S-peptide and S-protein (residues 21 to 124) with the results for the recombined complex in which position C-2 of histidine 12 was fully deuterated. The second active site histidine resonance was assigned to histidine residue 119 by consideration of the NMR titration results for carboxymethylated histidines and 1-carboxymethylhistidine 119 ribonuclease. This assignment is a reversal of that originally reported, and has important implications for the interpretation of NMR titration data of ribonuclease.

Interpretations of the NMR titration curves of the histidine ring C-2 proton resonances of ribonuclease have relied upon the assignments of Meadows et al. (1). Ambiguities were noted in their assignment of the resonances of the two active site histidine residues 12 and 119 (2) by King and Bradbury (3) and by Cohen et al. (4). Subsequently, Bradbury and Chapman (5) claimed to have confirmed the original assignments of these 2 residues. However, we have arrived at the opposite assignment on the basis of tritium and deuterium exchange studies with ribonuclease S and NMR titration studies with normal and deuterated histidine 12 in ribonuclease S' (6). During the course of this work two other groups (7, 8) have arrived at the same conclusion as ourselves regarding this assignment, although on the basis of less direct evidence.

EXPERIMENTAL PROCEDURE

Ribonuclease S, S-peptide, and S-protein were obtained from Sigma Chemical Co. Carboxymethylhistidines were obtained from Calbiochem and 1-carboxymethylhistidine 119 ribonuclease was purchased from Miles Laboratories, and a sample was also kindly provided by Dr. J. Beilin, Roswell Park Memorial Institute, Buffalo, N. Y. Protein samples were desalted on Sephadex G-25 in ammonium bicarbonate (0.1 M) and lyophilized several times from D2O before NMR spectroscopy. Preparation of ribonuclease S was carried out by mixing equimolar amounts (1.4 × 10^{-4} M) of S-peptide and S-protein in 0.15 M sodium phosphate/D2O at 24°. Samples were assayed by the method of Crook et al. (9) using a Gilford 240 spectrophotometer at 286 nm. Tritium exchange was carried out by the addition of 30 μl of tritiated water (100 mCi/ml) to 0.4-ml samples of ribonuclease S (1.21 mM) in 0.1 M NaCl in D2O at pH 8.8 in sealed tubes. The solutions were kept at 30° and daily one was placed in a freezer at -20°. After 30 days, the samples were thawed, 50% trichloroacetic acid (0.86 ml) was added (10), and after standing for 1 hour at room temperature, the samples were centrifuged and the supernatant containing S-peptide was removed. The precipitate was reconstituted in water (0.4 ml) and reprecipitated with 20% trichloroacetic acid. The second supernatant was combined with the first. The samples were then lyophilized, and lyophilized three times from water (1 ml). After the samples were dissolved in water (0.25 ml), 10 ml of Aquasol (NEN Inc.) was added, and radioactivity was measured in a Nuclear Chicago Mark 1 liquid scintillation counter. Three samples of S-peptide (0.91 nmol by amino acid analysis) were exchanged for 5 days in the same volume of the same solution, but at 37.5°, to determine the maximum level of tritium incorporation. In these cases, acetic acid was added and samples were lyophilized directly. The mean value of cpm obtained (149,500 cpm) was corrected to the expected value for the ribonuclease S experiment (197,300 cpm) and used to convert to a molar scale of tritium incorporated into S-peptide as a function of time.

NMR spectra were recorded at 220 MHz on a Varian HR 220 spectrometer equipped with a pulse Fourier transform accessory. An acquisition time of 0.8 s and a pulse delay time of 0.2 s were generally used, and 100 to 800 π/2 pulses (45 μs) were accumulated, giving 2 to 4 K real points. Deuterium exchange was carried out using a solution of ribonuclease S (2.17 mM) in 0.1 M NaCl/D2O at pH 8.8 in a sealed NMR tube which was kept in a water bath at 30°. Daily spectra were recorded using identical spectrometer settings. Since the line widths of the four resolved histidine C-2 signals remained essentially constant, the peak intensity was used as a direct monitor of proton exchange for deuterium. It was not possible to obtain significant data after 500 hours.

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Assignment of RNase NMR Resonance

due to the reduction in the signal to noise ratio for the most rapidly exchanging peaks. Chemical shift values are reported in parts per million downfield from external tetramethylsilane at an ambient probe temperature of 20 ± 1°C. Direct pH meter readings in D₂O are reported using an Ingold microcombination electrode.

RESULTS

Deuterium Exchange—The exchange of the four histidine C-2 protons of ribonuclease S for deuterium was monitored by NMR at 30°C. The results are shown in Fig. 1A and the rates and half-times of exchange derived by a linear least squares fit to the semilog data are shown in Table I. The numbering system used for these resonances is the same as that of Meadows et al. (1).

Tritium Exchange—The results for the incorporation of tritium into histidine 12 position C-2 of S-peptide derived from ribonuclease S as a function of time at 30°C are shown in Fig. 1B. No other group in the peptide is expected to show exchange at high pH which is not rapidly reversible at low pH. The rate and half-time of exchange for histidine 12 position C-2 of ribonuclease S is given in Table I. From the comparison with the results for deuterium exchange it is clear that resonance H-3 corresponds to histidine 12.

Identity of Ribonuclease S' and Ribonuclease S—Ribonuclease S-peptide (5 mg) and S-protein (25 mg) were combined under the same conditions used for preparations for NMR experiments (24°C, 5 ml of 0.15 M sodium phosphate/D₂O). Following column chromatography on sulfopropyl (SP) Sephadex in phosphate buffer, and desalting on Sephadex G-25 in ammonium bicarbonate (0.1 M), fully active ribonuclease S' was obtained in 41% yield. This gave identical NMR spectra to those which we have reported previously for ribonuclease S (4).

NMR Titration Curves of Ribonuclease S-Peptide and S-Protein—These were obtained in sodium acetate (0.2 M) and phosphate (0.15 M) solutions. Details are reported in a separate publication (11).

Histidine 12 C-2-deuterated Ribonuclease S—The histidine 12 C-2 proton of S-peptide was exchanged for deuterium in D₂O at pH 9 at 37.5°C for 5 days, followed by the addition of S-protein in equimolar amount to the solution at pH 5.5 at 24°C. Aliquots of this sample gave 60% of the enzyme activity of a sample of ribonuclease A. An identical control preparation of normal ribonuclease S' was carried out. The NMR spectra and titration data obtained are shown in Figs. 2 and 3. The NMR titration curves of ribonuclease S in 0.15 M sodium phosphate have been published elsewhere (12). It is clear from the comparison that resonance H-3 is the one which is missing in the experiment with deuterated material.

![Fig. 1. A, concentration of protons in four imidazole positions C-2 in ribonuclease S derived from intensity measurements as a function of time. B, concentration of tritium incorporated into histidine 12 position C-2 of S-peptide derived from ribonuclease S as a function of time at 30°C are shown in Fig. 1B.](http://www.jbc.org/)

| Table I |
| Rate constants for exchange of histidine C-2 protons of ribonuclease S at 30°C in 0.1 M NaCl/D₂O |

<table>
<thead>
<tr>
<th>Method</th>
<th>Exchanged</th>
<th>k x 10⁻³</th>
<th>t½</th>
<th>Assignment histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tritium</td>
<td>Residue 12</td>
<td>1.38 ± 0.1</td>
<td>20.9</td>
<td>histidine 12</td>
</tr>
<tr>
<td>Deuterium</td>
<td>Resonance H-1</td>
<td>5.1 ± 0.3</td>
<td>5.7</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Resonance H-2</td>
<td>3.3 ± 0.2</td>
<td>8.8</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Resonance H-3</td>
<td>1.1 ± 0.2</td>
<td>26.0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Resonance H-4</td>
<td>0.05 ± 0.01</td>
<td>642</td>
<td>48</td>
</tr>
</tbody>
</table>

![Fig. 2. Histidine C-2 proton NMR spectra at 220 MHz and various pH values. The top spectrum in each pair is for the mixture of ribonuclease S-peptide and S-protein in equimolar amounts in 0.15 M sodium phosphate/D₂O. The bottom spectrum of each pair is the same except for the deuterium substitution of histidine 12 position C-2 of S-peptide. Resonance H-3, which is missing in the latter case, is shown by an arrow and is assigned to histidine residue 12.](http://www.jbc.org/)
Assignment of RNase NMR Resonance

Assignment—We have obtained a direct assignment of one of the histidine C-2 proton resonances of ribonuclease by utilizing the slow alkali-catalyzed exchange at this position (1, 10). Measurement of the rates of deuterium exchange of the four histidine C-2 proton resonances of ribonuclease S, and the rate of tritium incorporation into histidine 12 position C-2 of the S-peptide of ribonuclease S, allowed a direct quantitative comparison of these exchange rates under the same experimental conditions (Table I). No significant isotope effect was anticipated (15). NMR titration studies were also carried out on ribonuclease S' using S-peptide fully deuterated in histidine 12 position C-2. Both of these approaches gave the same result, namely that resonance H-3 corresponds to histidine residue 12.

Two histidine C-2 proton resonances are reported to be affected by carboxymethylation of either histidine 12 or 119 (1), the 2 active site histidine residues (2). We have confirmed that this is so for the 1-carboxymethylhistidine 119 ribonuclease derivative. We have also shown that these effects are not a direct result of carboxymethylation, but must be due to secondary structural effects in the protein. Thus, the two affected resonances H-2 and H-3 must correspond to the active site histidine residues. Since we have now assigned resonance H-3 to histidine 12, resonance H-2 must correspond to histidine 119. These assignments are opposite to the original assignments of Meadows et al. (1).

We have shown that ribonuclease S', the product of recombination of S-peptide and S-protein, gives the same NMR results as ribonuclease S. This was considered necessary in view of reports of minor differences between ribonuclease S and S' (16, 17). Previously we have also shown that the active site histidine resonances H-2 and H-3 are the same in ribonuclease S as in A (4). Consequently, these assignments can be applied to ribonuclease A. The rates of deuterium exchange for ribonuclease S at 30° are in the order of H-1 > H-2 > H-3 > H-4 and the rates of tritium exchange obtained by Ohe et al. (18) for ribonuclease A at 37.5° were in the order His 105 > His 119 > His 12 > His 48. This is consistent with the relative solvent accessibility of the environments of these residues in the x-ray structure of ribonuclease S (19). Also, of the 2 active histidine residues, 12 is the more accessible to alkylation (2) and to solvent, as indicated by the entropies of ionization of the imidazole groups (20).

Comparison with Other Assignments—Ribonuclease S was chosen for the deuterium exchange experiment since at high pH and 20° all four resonances are resolved. Consequently, the intensities of the four peaks could be monitored directly by NMR. By contrast, the same experiment with ribonuclease A requires the readjustment of the pH to lower values to observe the resolved resonances. This difference is of particular importance since opposite results have been reported for exchange of ribonuclease A depending on the lower value used (5, 7, 8). The absence of a proton resonance for the S-peptide derived by Bradbury and Chapman (5) from deuterium-exchanged ribonuclease A would also not appear to be a sufficiently clear criterion on which to base an assignment, particularly since this resonance is usually very broad (1) probably due to aggregation (21).

There are several differences in our approach to the assignments of the histidine resonances compared to the recent reports of Markley (7) and Patel et al. (8). Markley has compared the order of the rates of deuterium exchange of the histi-
dines in ribonuclease A obtained by NMR (7) with the order of the tritium incorporation following peptide cleavage of Ohe et al. (18) carried out under different conditions. We felt it was important to carry out the two exchange experiments under the same conditions to confirm this correlation, and to obtain quantitatively similar rate constants. It should also be noted that the values of the ionization constants obtained for the active site histidine residues from tritium exchange as a function of pH (18) are opposite in relative magnitude to those from NMR titration experiments (22).

Patel et al. (8) repeated the ribonuclease S' recombination experiment of Meadows et al. (1) but showed only two pair of spectra for normal and deuterated S-peptide at a single low pH value, one pair in the presence of 3'-CMP. They did not show that the NMR titration curves of ribonuclease S and S' are identical nor did they report NMR titration curves of S-protein, a control which they judged to be necessary. Other work of these authors is only inferentially related to the question of the histidine C-2 proton assignments (23, 24). However, they are clearly correct in attributing the slowly exchanging NH resonance corresponding to the C-2-H resonance to histidine 12 (23).

We are attempting in collaboration with Dr. R. Jones and his associates to observe directly the exchange of the histidine C-2 positions for tritium by tritium NMR (26).

Implications of Reassignment—The reassignment reported here has important implications for the interpretation of the NMR titration curves of ribonuclease. Notably, the assignment of histidine 12 to the titration curve of resonance H-3 results in an apparent anomaly, namely, that this curve shows a significant inflection at low pH in addition to the main inflection due to the imidazole ionization. This inflection corresponds to a pK_a value of about 4.5, which must be attributed to a carboxyl group in the protein (4, 22). However, there is no carboxyl group within 9 Å of this histidine residue in the structure determined by x-ray crystallography (19). We believe the low pH inflection for histidine 12 arises from the ionization of a distant carboxyl group, in other words from a pH-dependent conformational change (12).

The interpretation of the selective and significant effects found in the presence of mononucleotide inhibitors (27–30), and on thermal denaturation of ribonuclease (31, 32), would likewise require re-evaluation. However, the results obtained in the presence of a bound dinucleoside phosphate substrate analog do not appear to be affected by this reassignment since no significant effects on the active site histidine NMR titration curves were observed (33). Consequently, our interpretation of the lack of direct interaction of these histidine residues with substrate remains unchanged.

Addendum—Bradbury and Teh (34) have reversed Bradbury's and Chapman's previous assignment (5) and are now in agreement with the assignment presented here.

REFERENCES

Nuclear magnetic resonance titration curves of histidine ring protons. A direct assignment of the resonances of the active site histidine residues of ribonuclease.
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