Nuclear Magnetic Resonance Titration Curves of Histidine Ring Protons

RIBONUCLEASE S-PEPTIDE AND S-PROTEIN*

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The histidine C-2 proton NMR titration curves of ribonuclease S-peptide (residues 1 to 20) and S-protein (residues 21 to 124) are reported. Although S-protein contains 3 histidine residues, four discrete resonances are observed to titrate. One of these arises from the equivalent histidine residues of unfolded S-protein. The variation in area of the four resonances indicate that there is a reversible pH-dependent equilibrium between the folded and unfolded forms of S-protein, with some unfolded material being present at most pH values.

Two of the resonances of the folded S-protein can be assigned to 2 of the histidine residues, 48 and 105, from the close similarity of their titration curves to those in ribonuclease. These similarities indicate a homology of portions of the folded conformation of S-protein to that of ribonuclease in solution. These results indicate that the complete amino acid sequence is not required to produce a folded conformation similar to the native globular protein, and they appear to eliminate the possibility that proteins fold from their NH$_2$ terminus during protein synthesis. The low pH inflection present in the titration curve assigned to histidine residue 48 in ribonuclease is absent from this curve in S-protein. This is consistent with our previous conclusion that this inflection arises from the interaction of histidine 48 with aspartic acid residue 14, which is also absent in S-protein.

The third titrating resonance of native S-protein is assigned to the remaining histidine residue at position 119. The properties of this resonance are not identical with either of the titration curves of the active site histidine residues 12 and 119 of ribonuclease. The resonance assigned to histidine 119 is the only one significantly affected on the addition of sodium phosphate to S-protein, indicating that some degree of phosphate binding occurs. In both the absence and presence of phosphate this curve also lacks the low pH inflection observed in the histidine 119 NMR titration curve in ribonuclease. This difference presumably arises from a conformational difference between ribonuclease and the folded S-protein involving a carboxyl group.

Conformational properties of proteins have traditionally been studied using physicochemical techniques which detect an aggregate property of the molecule. Titration curves obtained from histidine ring C-2 proton resonances in NMR spectra of proteins are sensitive nondisturbing probes of local conformation (1, 2). Notwithstanding the many studies of the individual histidine resonances in ribonuclease (3), the NMR titration curves of ribonuclease S-protein (residues 21 to 124) and S-peptide (residues 1 to 20) (4) have not previously been reported. Meadows et al. (5) pointed out that the histidine 12 C-2 proton resonance of S-peptide is very broad, and attributed this to an exchange phenomenon. It is known that NH$_2$ terminal peptides of ribonuclease aggregate (6). Utilizing the pulse-Fourier transform NMR method at 220 MHz, we were able to obtain spectra at lower concentrations of S-peptide, providing a complete NMR titration curve.

Similarly, avoiding aggregation of S-protein (7) by using lower concentrations, we were able to obtain complete NMR titration curves for four titrating resonances. By comparison with ribonuclease, three of these could be attributed to the 3 histidine residues present in S-protein and could be specifically assigned to these residues in the folded or "native" conformation. The fourth resonance represents unfolded or "denatured" S-protein. From the variations in the relative areas of the peaks the pH-dependence of the equilibrium between these conformations could be investigated. These results have important implications for considerations of the relationship between primary sequence and structure. In addition, these results tend to support our previous conclusions regarding the origin of the
low pH inflections observed in the NMR titration curves of ribonuclease, but absent in those of S-protein.

**EXPERIMENTAL PROCEDURE**

Bovine pancreatic ribonuclease S, S-protein, and S-peptide were purchased from Sigma Chemical Co. (St. Louis, Mo.). Samples for NMR studies were lyophilized three to five times from D2O. Samples were also desalted by gel filtration on Sephadex G-25 in ammonium bicarbonate (0.1 M, pH 8.5) and in 50% acetic acid. A solution of fully active ribonuclease S (25 mg) was treated with 20% trichloroacetic acid following the procedure of Doscher (8). After centrifugation the precipitate was redissolved in 50% acetic acid and chromatographed in the same solvent on Sephadex G-25. Samples comprising S-protein were combined and lyophilized. Samples of 5 mg of S-peptide and 8 to 10 mg of S-protein were dissolved in 0.5 ml of D2O (100%) for NMR studies. Stock solutions of sodium phosphate and deutero-acetate in D2O were used to adjust to the desired concentration. Analysis of several lyophilized S-protein samples showed variable levels of phosphate. Only those with very low levels were used directly; others were desalted.

Assay of S-protein by the method of Crock et al. (9) with cyclic 2'-3'-CMP using a Gilford ultraviolet spectrophotometer at 290 nm gave no activity, but a mixture of S-protein with S-peptide (1.3 molar ratio) gave 54% of the activity of a ribonuclease A sample. The S-protein sample derived from ribonuclease S by trichloroacetic acid treatment gave an 4% relative activity.

Measurements of pH were made using a Radiometer model 26 with a long, thin Ingold combination electrode inside the NMR tube (Wilmad 5003P). Direct meter readings in D2O were used to adjust to the desired concentration. Analysis of several lyophilized S-protein samples showed variable levels of phosphate. Only those with very low levels were used directly; others were desalted.

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NMR spectra were obtained at 220 MHz on a Varian Associates HR-220 spectrometer equipped with an FT accessory with a dedicated 620L computer giving 2 or 4 K real point transforms. Usually 100 transients were accumulated and spectrometer settings were generally as reported previously. Chemical shift values are reported in parts per million downfield from external tetramethylsilane at an ambient probe temperature of 20 ± 1°C.

Computer curve fitting was carried out using the MIAL system on a Digital Equipment Corp. PDP10 at the Computer Center, National Institutes of Health. Spectra were digitized by tracing on a CALMA digitizer.

**RESULTS**

S-Peptide—NMR spectra of S-peptide (5 mM) contained a single titrating histidine C-2 proton resonance. The peak broadened distinctly at intermediate pH values, but was observable throughout the titration range. The NMR titration curves in sodium acetate (0.2 M) and phosphate (0.15 M) gave pKₐ values, derived from curve fitting using an equation for a simple proton association-dissociation equilibrium (10), of 6.86 ± 0.01 and 7.03 ± 0.03, respectively.

S-Protein NMR Titration Curves—Four titrating histidine C-2 proton resonances were observed in the NMR spectra of S-protein at concentrations in the range of 0.75 to 0.9 mM. At pH values above 6 some samples began to precipitate, and a single sharp resonance of the equivalent histidine residues remained. Other samples showed discrete resonances up to pH 8. At pH values below 3 one resonance, H-U, became predominant, and this was attributed to the acid denaturation process, leading to unfolding of the S-protein (11). Both the acid and alkaline transitions were reversible, although the latter was much less reproducible, partly due to precipitation.

The NMR titration data for S-protein with the results of curve fitting with the equation for a simple proton association-dissociation equilibrium are shown in Fig. 1. In this figure, the resonances are designated in a common nomenclature with the results for ribonuclease A and S (3, 5). The titration curves of resonance H-1 are found to be almost superimposable in every case. This resonance has been assigned to histidine residue 105 (5). Resonance H-4, assigned to histidine residue 48 (5), occupies a similarly upfield shifted position in S-protein as in ribonuclease S (3, 5), but this lacks the low pH inflection usually present in the curve of resonance H-4. The third resonance of native S-protein can be assigned to the remaining histidine residue 119. From the assignment for ribonuclease itself we refer to this as resonance H-2 (12). However, its titration curve is somewhat different in S-protein compared to ribonuclease S or A (3).

Due to the variations observed between samples, and the broadness of some of the peaks, notably that of H-4, which in some cases broadened beyond detection at pH > 5, we repeated these titration studies on samples of S-protein prepared under several different experimental conditions (Table 1). The results, following column chromatography of S-protein on G-25 in ammonium bicarbonate (pH 8.8, 0.1 M), were closely similar to those for untreated S-protein. However, the standard errors of the parameters obtained were greater due to generally broader lines and less data obtainable with this sample. Another sample of S-protein was derived from enzymatically active ribonuclease S by precipitation twice with 20% trichloroacetic acid (8). This sample gave results consistent with those obtained with other samples (Table 1), although resonance H-4 was observed only at low pH values.

Finally, two samples which had been successfully submitted to NMR analysis were lyophilized and redissolved in sodium phosphate at two concentrations. The results indicated a small increase in the pKₐ value of resonances H-1, H-4, and H-U, similar to those observed for S-peptide, but a larger increase in that of resonance H-2 (Table 1). This indicates that phosphate ion is binding to S-protein and affecting mainly this histidine residue (2). This curve of resonance H-2 shows no low pH inflection either in the absence or presence of phosphate, contrary to the results for ribonuclease A and S (2, 3).

S-Protein Area Measurements—As the pH is raised above pH 3, the area of resonance H-U decreases and those of the discrete resonances of the folded form increase (Fig. 2). This is the phenomenon expected for a slow rate of exchange between the two forms in equilibrium in solution. The lower limit of the rate of exchange can be calculated from the reciprocal of the difference in chemical shift between the resonances of the

![Fig. 1. NMR titration curves of the histidine ring C-2 proton resonances of S-protein. The resonances are identified by comparison with the results for ribonuclease (3). The lines are theoretical fits using an equation for a simple proton dissociation equilibrium.](https://example.com/image-url)
**Conformation of S-Protein**

**Table I**

Properties of histidine NMR titration curves of ribonuclease S-protein

Parameters obtained using the equation for a simple protonation equilibrium (12) where \( \delta \) is the chemical shift at high pH, \( \Delta \) is the change of chemical shift on protonation, and \( pK_a \) is the apparent ionization constant. Standard errors are less than \( \pm 0.1 \) unit except for the sample chromatographed in ammonium bicarbonate, where the values are higher.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Salt (M)</th>
<th>( pK_a )</th>
<th>( \Delta )</th>
<th>( \delta )</th>
<th>( pK_a )</th>
<th>( \Delta )</th>
<th>( \delta )</th>
<th>( pK_a )</th>
<th>( \Delta )</th>
<th>( \delta )</th>
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<tr>
<td>None</td>
<td>NaAc (0.2)</td>
<td>6.72</td>
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<td>7.59</td>
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<td>0.83</td>
<td>7.37</td>
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<td>Sephadex G-25/ammonium bicarbonate (0.1 M)</td>
<td>NaAc (0.2)</td>
<td>6.65</td>
<td>0.86</td>
<td>7.74</td>
<td>6.88</td>
<td>0.72</td>
<td>7.88</td>
<td>0.86</td>
<td>0.79</td>
<td>7.35</td>
</tr>
<tr>
<td>Trichloroacetic acid precipitate/sephadex G-25/acetic acid (50%)</td>
<td>NaAc (0.2)</td>
<td>6.81</td>
<td>1.00</td>
<td>7.58</td>
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<td>n.f.</td>
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<td>0.88</td>
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<td>Trichloroacetic acid precipitate/sephadex G-25/acetic acid (50%)</td>
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<td>1.02</td>
<td>7.57</td>
<td>7.19</td>
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<tr>
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<td>7.48</td>
<td>7.35</td>
<td>1.04</td>
<td>7.45</td>
<td>5.96</td>
<td>0.82</td>
<td>7.30</td>
</tr>
</tbody>
</table>

* This peak was observed in every case, but was broad. Where insufficient data was obtained, n.f. = not fitted.

a H-U is the resonance of the unfolded form; for assignments see text.

**Fig. 2. Examples of curve fitted spectra of S-protein using a sum of Lorentzian peaks. Asterisks represent the digitized spectra, solid lines the best fit, and dashed lines the individual components.**

Folded and unfolded forms at pH < 5 to be slower than 30 ms. However, unlike the transition to a native globular protein (13) the resonance of the unfolded form remains present in the spectra of S-protein throughout the titration range. Its area reaches a minimum at ~ pH 6 and increases above this value.

These results were essentially reversible whether pH was raised or lowered up to about pH 7. The areas of the peaks in the pH range 3 to 5.5 were determined by curve fitting the spectra using a sum of Lorentzian peaks (13). The results are shown in Fig. 3 normalized to a total area of 3. It was not possible to adequately curve fit spectra at pH values greater than 4.5 due to the broadening of the peaks and the increase in the steepness of the base-line due to the presence of aromatic resonances. From the area measurements it is possible to estimate the proportion of folded and unfolded S-protein conformers present in solution at a given pH value (see "Discussion"). It should be noted that the presence of such discrete resonances indicates that each histidine residue can exist only in two possible environments, one folded and the other unfolded.

**Fig. 3. Areas of histidine C-2 proton peaks of S-protein, determined as shown in Fig. 2, as a function of pH. The areas are normalized to a total value of 3 since S-protein contains 3 histidine residues. Vertical bars represent the standard error in the area determination. The symbols are: □, resonances H-1 and H-2; ○, H-U; and ●, H-4. The lines are hand-drawn.**

**Discussion**

Comparison of NMR Titration Curves of S-Protein with Those of Ribonuclease—In the original assignment of the four histidine C-2 proton resonances observed in spectra of ribonuclease Meadows et al. (5) used the stratagem of deuteration of S-peptide, followed by recombination with S-protein. They exchanged the histidine 12 C-2 proton in D_2O at high pH, and compared the NMR titration curves of the recombined ribonuclease S' with those of ribonuclease S, the cleaved but unseparated product. Apart from other limitations of this method which have been discussed elsewhere (2, 12) no titration curves were reported for S-protein. These are clearly needed to avoid confusion in the assignment since a mixture of S-peptide and S-protein does not necessarily give 100% yield of the recombined enzyme in solution, even if a high level of enzymatic activity is determined. We now report the NMR titration curves of S-protein, and have used these to clarify our reassignment of the active site histidine resonances of ribonuclease by the deuteration-recombination method (12). Similarly, the NMR titration curve of S-peptide was required for comparison with the results of recombination using non-deuterated S-peptide.

By comparison of the NMR titration curves of ribonuclease S-protein with those of ribonuclease S or A (2, 3) it is possible to correlate two of the resonances, H-1 and H-4, due to the...
characteristic positions of their curves. These resonances have been assigned to histidine residues 105 and 48, respectively, in ribonuclease (5, 12). This is a reasonable, although not rigorous, assignment for S-protein. The resonance corresponding to the 3rd histidine residue present in S-protein, histidine 119, has clearly been shifted as a result of the conversion of ribonuclease A or S to S-protein since no titration curve corresponds exactly to either resonance H-2 or H-3 in ribonuclease. From the presence of a single sharp peak at pH values below the acid denaturation transition of S-protein (Fig. 2), it is possible to distinguish the resonance of the unfolded form of S-protein from the resonance of histidine 119 in the folded form. Neither NMR titration curves of histidine residues 48 nor 119 show the characteristic low pH inflections observed in the ribonucleases (2, 3).

Conformation of S-Protein—From the above observations, and since NMR titration curves are sensitive nondisturbing probes of local microenvironment in a protein, it can be concluded that the local conformations around residues 48 and 105 in “native” S-protein are very similar to these regions in ribonuclease itself. Particularly, the retention of the very unusual chemical shift characteristics of the resonance of histidine 48 indicates that the highly structured environment of this group is essentially intact in this “hinge” region of the molecule (3). The upfield shift of this resonance indicates that the proximity of the side chain of tyrosine residue 25 is very similar in S-protein and ribonuclease. By contrast, the region around histidine 119 is clearly altered. The active site region would be expected to be significantly changed on removal of the NH₂-terminal portion of the protein. Nevertheless, this curve is affected the most on the addition of phosphate, thus indicating some residual binding capability in S-protein.

Since S-protein lacks 20 residues of the NH₂ terminus of ribonuclease, these results would appear to eliminate the possibility that proteins fold from their NH₂ termini during protein synthesis (14). Further, these results indicate that not all of the amino acid sequence is required for a good portion of the molecule to fold into a discrete conformation resembling that of the native globular conformation.

It has been suggested that the significant low pH inflection observed in the NMR titration curve of histidine residue 48 in ribonuclease arises from interaction with the adjacent carboxyl group of aspartic acid residue 14 (3). The absence of this inflection in the curve of histidine 48 in S-protein would seem to support this suggestion. A change in conformation around histidine 48 could conceivably be related to a different carboxyl group, although there is no other such group close to histidine 48 in the structures determined by x-ray crystallography (15, 16). The concomitant absence of a low pH inflection in the NMR titration curve of histidine 119 in S-protein, even in the presence of phosphate (2), is also suggestive of a common origin to these inflections, as has been argued elsewhere (3).

Denaturation of S-Protein—Globular proteins usually exhibit a sharp reversible cooperative denaturation process from a folded to an unfolded state (17). Such a transition has been described for S-protein, although the melting temperature (18) and thermodynamic parameters (19, 20) show that S-protein is more labile than ribonuclease and exhibits a shallower transition. A study of tyrosine absorption as a function of pH also indicated a broader transition for S-protein than for ribonuclease S’ (11). In fact, while ribonuclease S’ exhibited a cooperative transition steeper than a simple theoretical acid dissociation equilibrium, that of S-protein was shallower than such an equilibrium.

The relative areas of discrete resonances in NMR spectra represent the relative concentrations of the nuclei giving rise to those resonances (13). Measurement of the relative areas of the three resolved peaks at pH values less than five, where curve fitting could be satisfactorily accomplished (Fig. 2), gave a quantitative estimate of the relative proportion of these peaks (Fig. 3). Since the peak at 8.6 ppm contains two proton resonances (H 1 and H 2 resolved at higher pH values), it should level off to a value of 2, taking a normalized total area of all peaks to be 3 for the three histidine C-2 protons. In fact it levels off at a value less than 2, and resonance H-4 remains less than 1. This is attributed to the presence of unfolded material represented by resonance H-U. From the relative areas of these peaks, the amount of unfolded material at pH 4.5 can be estimated to be 30%. Of particular note is the fact that while the area of H-4 levels off at about pH 2.5, those of the other peaks do not do so until pH 4 or above. This would indicate that the portion of the S-protein molecule containing the group giving rise to H-4, namely histidine 48, is among the first to nucleate as the pH is raised. This is consistent with the results for the thermal transition of ribonuclease A obtained from NMR studies (21) and a rather detailed scheme based on several sources of evidence proposed by Burgess and Scheraga (22).

The value of ~pH 3 as the midpoint for the transitions of resonances H-1 and H-2 is much lower than the value of about 4.5 observed by absorbance studies as a function of pH (11). This indicates that several steps occur in the acid denaturation process, those involving the histidine residues generally occurring at lower pH values than those involving tyrosine residues. This may also contribute to the very shallow nature of the overall transitions of S-protein monitored by absorbance and other means (11, 16–20).

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