Specific Peptide-Protein Interactions in the Ribonuclease S' System
Studied by $^{13}$C Nuclear Magnetic Resonance Spectroscopy with Selectively $^{13}$C-enriched Peptides*

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The (1-15) NH$_2$-terminal portion of ribonuclease has been synthesized by the solid-phase method with selectively $^{13}$C-enriched amino acids. Two types of peptide were prepared: (a) one in which the carbon atoms of Ala 5 were uniformly labeled with $^{13}$C (90%), and (b) one in which the side chains of His 12 (C'), Met 13 (C), and Asp 14 (C') were enriched. These regions of the peptide are known to form (a) an $\alpha$ helix and (b) the active site region on complexation with ribonuclease S-protein (residues 21 to 124). Fully active ribonuclease S' (RNase S') complexes were obtained after dialysis from excess peptide. Distinct resonances were resolved by high field $^{13}$C nuclear magnetic resonance (NMR) for the enriched $^{13}$C atoms in the RNase S' complexes for five out of the six sites. NMR parameters for each of these resonances were determined and formed a basis for interpretation of the nature of the environment of each site in the complex.

The pH and temperature dependencies of the individual sites were investigated. Asp 14 (C') has a $pK_a$ of 2.4 in the complex, lower than in the peptide alone ($pK_a$ 3.8), and its spin lattice relaxation time indicates that it is not protonated at pH 5.6. Thus, we conclude that Asp 14 is a hydrogen bond acceptor. His 12 (C') in the complex has a $pK_a$ of 5.7, and the $^1$H NMR spectrum of this complex confirms the previous assignment of its resonance. The Met 13 (C') resonance is shifted about 2 ppm on formation of the $\alpha$ helix, showing that Met 13 is in a more hydrophobic, electronically shielded environment for this residue. The same temperature midpoint (43°C) was recorded for the denaturation of the RNase S' complex for four of the five sites, but a different temperature (37°C) was found for the transition of the Met 13 (C') atom; similarly the pH midpoint for the acid transition was higher (3.0) for the Met 13 C' compared to the His 12 (C') (2.5). This indicates a weaker bond and more ready unfolding of the Met 13 residue in the complex.

Spin probes provide a general and sensitive means to monitor the conformation and interactions of proteins in solution. Utilization of the natural stable isotopic spin probes present in proteins, such as $^1$H (99.9% natural abundance) and $^{13}$C (1.1%), while giving valuable information can result in a lack of resolution due to the many resonances present and the difficulty in assigning those resonances which are resolved (1). These difficulties may be partly overcome by the introduction of a spin probe, either a natural one in higher abundance than already present (such as $^{13}$C or $^{15}$N), or a non-natural entity. Examples of the latter are electron spin labels (2) or $^{19}$F nuclei (3); these can often disturb the system under study. In view of the general difficulty in introducing spin probes, it may in principle be considered preferable to selectively enrich specific sites in a protein with enriched natural isotopes which are essentially nonperturbing probes. There are two main approaches to accomplish this incorporation, chemical and biosynthetic (1).

For studies of peptide-protein interactions, as well as protein conformation and function, it is necessary that complex formation be strong and selective. The ribonuclease S' system, which consists of the noncovalent complex of the S-peptide NH$_2$-terminal portion (residues 1 to 20) and the S-protein (residues 21 to 124) of ribonuclease A (4), has been described as an excellent model for peptide hormone-protein receptor interactions (5), especially since enzyme activity provides a ready test of biological viability. It has also been found that the (1-15)-terminal portion is sufficient to provide maximal binding and activity (6). Of course, there must be a reasonable expectation that in any system utilizing $^{13}$C enrichment the sites chosen for incorporation of the spin probe will be a monitor of binding or conformational effects. The NH$_2$-terminal portion of ribonuclease is known to exist as a random coil free in solution (7), but to form an $\alpha$ helix when bound to S-protein to form the RNase S' complex (8, 9). Also, several residues of the peptide form the active site region of RNase S' (5, 8). Therefore, this system was chosen for a systematic study of the effect of selective peptide-protein binding at specific carbon sites using $^{13}$C enrichment and $^{13}$C NMR observation.

Several years ago, we reported preliminary studies of this system with $^{13}$C-enriched peptides; however, there were limited

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The abbreviations used are: RNase, bovine pancreatic ribonuclease; RNase S', RNase (1-15)-RNase (21-124) complex; NOE, Nuclear Overhauser effect.
Peptide-Protein Interaction in RNase S' by $^{13}$C NMR

Characterization and Properties of RNase S' Complexes—The successful syntheses of the RNase (1-15) peptides are shown by the amino acid analyses quoted in Table II. The $^{13}$C NMR spectra of the peptides, generally at pH 2, to avoid aggregation phenomena, are shown in Fig. 1. The $^{13}$C NMR parameters are given in Table III. The spectra reveal one clear resonance for each site of $^{13}$C enrichment. The width of the signals of the A-peptide precluded the possibility of measurement of $^{13}$C-$^{15}$N coupling constants from the Ala 6 ($^{15}$N) atom which was also enriched in this synthetic peptide. Of the six sites which were $^{13}$C-enriched in three synthetic peptides, five were about 90% enriched, while one, that of Met 13 ($^{13}$C), was about 45% enriched, due to the exchange reaction with $[^{13}$C]H$_3$ used in this case. This difference results in a much higher background of natural abundance $^{13}$C (1.1%) resonances from the protein in this spectrum (Fig. 1).

EXPERIMENTAL PROCEDURES

RESULTS

Synthetic RNase (1-15) Peptides—The successful syntheses of the RNase (1-15) peptides are shown by the amino acid analyses quoted in Table II. The $^{13}$C NMR parameters are given in Table III. The spectra reveal one clear resonance for each site of $^{13}$C enrichment. The width of the signals of the A-peptide precluded the possibility of measurement of $^{13}$C-$^{15}$N coupling constants from the Ala 6 ($^{15}$N) atom which was also enriched in this synthetic peptide. Of the six sites which were $^{13}$C-enriched in three synthetic peptides, five were about 90% enriched, while one, that of Met 13 ($^{13}$C), was about 45% enriched, due to the exchange reaction with $[^{13}$C]H$_3$ used in this case. This difference results in a much higher background of natural abundance $^{13}$C (1.1%) resonances from the protein in this spectrum (Fig. 1).

Characterization and Properties of RNase S' Complexes—The successful syntheses of the RNase (1-15) peptide and S-protein in P, buffer revealed two resolved signals for five out of the six enriched sites (excluding Ala 5 ($^{13}$C)$^\circ$. Following dialysis with an immeasurable filter only one signal corresponding to each site in the $^{13}$C-enriched semisynthetic RNase S' complexes was observed (Fig. 2). The specific activities of the complexes, compared to a stock RNase A solution, were: $[\epsilon-^{13}$C]Met 13 RNase S', 80%; $[\epsilon-^{13}$C]His 12 - $[\gamma-^{13}$C]Asp 14 RNase S', 107%; and $[U-^{13}$C]Ala 5 - RNase S', 105%. The $^{13}$C NMR spectra of the three RNase S' complexes are shown in Fig. 1 and the $^{13}$C NMR parameters are given in Table III. The $^{13}$C-enriched resonance is shifted downfield in four cases Ala 5 ($^{13}$C)$^\circ$, Ala 5 ($^{13}$C)$^\circ$, His 12 ($^{13}$C)$^\circ$, and Asp 14 ($^{13}$C)$^\circ$ but is shifted upward in one case Met 13 ($^{13}$C)$^\circ$. A downfield shift is generally taken to indicate electron shielding in the microenvironment of the atom in the complex, than in the free solvated state. For the His 12 side chain, this shift indicates a positively charged environment in the peptide-protein interface. However, the His 12 and Asp 14 chemical shifts are dependent on pH and interpretation of the differences between free peptide and complex can be given only when the full NMR titration is known (see below).

Spin lattice relaxation times ($T_1$) and Nuclear Overhauser effects (NOE), cannot always be interpreted straightforwardly. In general, one would expect a decrease in $T_1$ with slower motion on complexation, reflecting the change from random coil free segmental motion to restricted motion on binding. However, a minimum is usually observed in $T_1$ versus...
Peptide-Protein Interaction in RNase S' by $^{13}$C NMR

All peptide measurements were at pH 2.0 to avoid aggregation and all complex measurements were at pH 5.6, unless otherwise stated.

| Substance | Site     | Chemical shift $^{\ddagger}$ | $\Delta^\ddagger$ | $T_1$ | NOE $^\ddagger$ |
|-----------|----------|-----------------------------|-------------------|--------|-----------------|
| A-peptide | Ala C$^\ddagger$ | 173.9                        | -                 | 1.42   |                 |
|           | C$^\ddagger$     | 48.8                         | -                 | 2.13   |                 |
|           | C$^\ddagger$     | 51.7                         | 2.8               | 1.35   | 1.14            |
| ARS'      | Ala C$^\ddagger$ | 176.7                        | 2.8               | 1.35   | 1.14            |
|           | C$^\ddagger$     | 15.3                         | -0.2              | 0.26   | 2.90            |
| M-peptide | Met C$^\ddagger$ | 13.6                         | -                 | 1.68   | 2.00            |
| MRS'      | Met C$^\ddagger$ | 11.4                         | -2.2              | 1.15   | 1.20            |
| H-peptide | His C$^\ddagger$ | 132.9$^\ddagger$, -         | 0.29              | 2.36   |                 |
|           | Asp C$^\ddagger$ | 176.7                        | -                 | 1.05   | 2.38            |
| HRS'      | His C$^\ddagger$ | 135.8                        | 2.9$^\ddagger$, -  | 0.31   | 2.25$^\ddagger$ |
|           | Asp C$^\ddagger$ | 175.6                        | 1.1$^f$           | 2.25   | 1.10            |

$^\ddagger$ Downfield from $^{13}$CH$_3$CN as internal standard.
$^\ddagger$ Difference of chemical shift of complex minus peptide; a negative sign indicates an upfield shift.
$^\ddagger$ NOE = (1 + signal enhancement with full proton decoupling).
$^\ddagger$ Average of two measurements.
$^f$ Measured at pH = 5.6.

$\Delta$ in these cases depends upon pH.

Fig. 2. Example of the removal of excess $^{13}$C-enriched peptide, in this case H-peptide, from a mixture with S-protein in 0.15 M NaCl buffer. After dialysis (25 volumes), the intense $^{13}$C signals at 175 and 135 ppm correspond to the Asp 14 (C') and His 12 (C') resonances, respectively, of the H-peptide-RNase S' complex.

Correlation time ($T_r$) (1) so that a transition from one side of the minimum to the other could result in a longer $T$, and this is what was observed for the Ala 5 (C') resonance. The significant decrease in the NOE value for this resonance is also indicative of such a motional restriction (27). From the theory assuming isotropic motion and dominant relaxation mechanisms of a C-H dipolar interaction (27), correlation times of $2 \times 10^{-10}$ and $1.6 \times 10^{-8}$ s were calculated from $T_1$, values for the C' in the free peptide and complex, respectively. These overall correlation times can be considered only approximations but indicate an order of magnitude change in mobility for this alteration. Based on these correlation times, NOE can be estimated to be 2.8 and 1.15 for the free peptide and complex, respectively. Discrepancies of these NOEs from experimental values indicate the existence of significant inter-

Fig. 3. $^{13}$C NMR titration curves of the Asp 14 (C') resonances of the RNase (1-15) peptide (C') and the RNase S' complex formed from it (+). The lines are theoretical fits. $H$-PEP, H-peptide; HRS', RNase S' complex containing the H-peptide.

Fig. 4. $^{13}$C NMR titration curves of the His 12 (C') resonances of the RNase (1-15) peptide (C') and the RNase S' complex formed from it (+). The lines are theoretical fits. $H$-PEP, H-peptide; HRS', RNase S' complex containing the H-peptide.
TABLE IV

Ionization constants for [ε-13C]His 12. [γ-13C]Asp 14 RNase (I-15)

| Peptide-Protein Interaction in RNase S' by 13C NMR |
|--------------------------------------------------|
| His 12 (C') | Asp 14 (C') |
| pK<sub>a</sub> | pK<sub>a</sub> |
| Peptide     | 6.78 ± 0.01  | 8.33 ± 0.02  |
| Complex<sup>a</sup> | 5.68 ± 0.03  | 2.41 ± 0.06<sup>b</sup> |

<sup>a</sup> In the presence of 1.5 × 10<sup>-4</sup> M P, (molar ratio His: P = 42:1).

The pK<sub>a</sub> values were derived from fits including data in the presence of inhibitors (P, UprA); unpublished results.

The pK<sub>a</sub> value of His 12 determined by this method is somewhat lower than the value reported previously for the 1H resonance of the C'-H of this residue (29). In that case His 12 was found to have the lowest pK<sub>a</sub> of all 4 His residues in RNase, a correlation which is dependent on the assignment given the proton resonances (29). However, this assignment was confirmed in the present study by observing the 1H NMR titration of the resonances of the [ε-13C]His 12 complex. The 1H resonance of His 12 should be split into a doublet with a coupling constant of about 210 Hz (30). In fact, only three titrating resonances were observed (Fig. 5) and, from a comparison with previous results, it was clear that the peak which was absent (i.e., the two coupled peaks were not resolved) was that of resonance H-3. The peak of resonance H-4 (His 48) was also shifted relative to the others in this case. Both the fact that His 12 has the lowest pK<sub>a</sub> value of all 4 histidines in RNase and that its C' resonance is shifted downfield in going from a solvated environment to the structured microenvironment at the peptide-protein interface indicate that His 12 is in a relatively electron deshielded, i.e. positively charged environment.

The pK<sub>a</sub> of [γ-13C]Asp 14 could not be determined accurately from the data given in Fig. 3. However, the data in the presence of inhibitors of RNase showed apparently no significant effect upon this resonance.<sup>3</sup> Hence four sets of data were fitted simultaneously with an equation for two separate transitions, giving more reliable pK<sub>a</sub> values. The fact that the pK<sub>a</sub> of this residue in the complex is much lower than that in the peptide clearly indicates a strongly positively charged environment or a hydrogen-bond with a donor group. The low pK<sub>a</sub>, the fairly constant intermediate chemical shift, and the lack of protonation evidenced by the T<sub>1</sub> value at pH 5.6 indicate that Asp 14 is a hydrogen bond acceptor between pH 3 to 8.

While the Met 13 (C') resonance in the peptide showed no pH dependence of chemical shift, the corresponding resonance in the complex showed an almost linear upfield shift from pH 2.6 (11.7 ppm) to pH 7.0 (11.1 ppm).

Denaturation of RNase S' Complexes—Since two separate signals were observed for the peptide in solution and bound in the complex, this indicates that slow exchange is occurring on the NMR time scale. From the minimum difference in chemical shift for the five resolved carbon sites, the rate of exchange must be <200 s<sup>-1</sup> at 20°C. On raising the temperature for the dialyzed complex the chemical shift of the resonance changed very little. However, the relative intensities of the two peaks altered significantly. Even in the dialyzed complex material at 6°C there was usually a minor peak at the position corresponding to the free peptide, equivalent to <5% of the peak due to the bound peptide. It was not possible to be certain if this was significant and related to the exchange process, or if it resulted from excess peptide not removed by dialysis or a contribution from the natural abundance resonances at that chemical shift. However, in the case of the [ε-13C]Met-RNase S' complex, the free peptide peak was usually somewhat more intense (about 15%) in fresh and renatured samples. It should be noted that the accuracy of intensity measurements is about ±5% and the use of intensities depends upon the observation that the widths of the resonances do not vary significantly during the course of the denaturation process.

In Fig. 6 are shown spectra of the HRS complex at several temperatures. NOEs were eliminated in these decoupled spectra by the use of gated decoupling in order to enable composition to be measured accurately. It is clear that the denaturation transition occurs sharply (cooperatively) at about 43°C. From a plot of the relative intensities of both peaks for each 13C-enriched site (Fig. 7), it was possible to determine the midpoint temperature for the denaturation at the site being observed. These experiments were carried out for all three RNase S' complexes at the same temperature at a given temperature to avoid instrumental variations in temperature settings so

<sup>3</sup>C. Niu, S. Matsuura, H. Shindo, and J. S. Cohen, unpublished results.

**Fig. 5.** 1H NMR titration curves at 220 MHz of the RNase S' complex containing the H-peptide [ε-13C]His 12. [γ-13C]Asp 14 RNase (I-15). --, observed previously for RNase S (29). Although the data for H-4 are shifted about 0.1 ppm downfield in this case, it is clear that the resonance which is missing is that due to H-3.

**Fig. 6.** 13C NMR spectra of RNase S' complex containing the H-peptide as a function of temperature. The peak decreasing with temperature for each 13C site is due to the RNase S' complex and that increasing is due to the free peptide. Spectra were obtained with gated decoupled condition and by use of longer delay (4 s) and smaller pulse width (60° flip angle) to ensure accurate measurements of peak intensities.
that relative differences would be accurate. The midpoints for the five resolved $^{13}$C sites in the RNase S' complexes are given in Table V. It is noteworthy that while four of these sites gave a midpoint at 43°C, consistent with the overall cooperative denaturation process, only the Met 13 (C') showed a lower midpoint temperature of 37.5°C.

The midpoint pH for the acid transition of two of the RNase S' complexes was also determined (Fig. 8). While the midpoint for His 12 (C') was pH 2.50 (the crossover of the Asp 14 (C') resonance prevented determination of its midpoint pH), equivalent to the overall pH transition, that for Met 13 (C') was higher, namely pH 3.05. No effect of Pi on the midpoint of the denaturation process, only the Met 13 (C') showed a lower midpoint at 43°C, consistent with the overall cooperative transition in Table V. It is noteworthy that while four of these sites gave relative differences would be accurate. The midpoints for the five resolved $^{13}$C sites in the RNase S' complexes are given in Table V. It is noteworthy that while four of these sites gave a midpoint at 43°C, consistent with the overall cooperative denaturation process, only the Met 13 (C') showed a lower midpoint temperature of 37.5°C.

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The temperature and denaturation transitions were fully reversible.

**TABLE V**

| Site observed | Temperature midpoint °C | pH midpoint |
|---------------|-------------------------|------------|
| Met 13 (C')   | 37.5                    | 3.05       |
| Ala 5 (C')    | 43.0                    | 2.90       |
| Met 13 (C')   | 43.0                    | 2.50       |
| His 12 (C')   | 43.0                    | 2.90       |
| Asp 14 (C')   | 43.0                    | 2.90       |

The accuracy of temperature measurements is about ±1°C and that of pH is about ±0.05 pH units. The measurement of peak intensity is generally <±5%, and is more accurate near the midpoint than at either extreme.

**DISCUSSION**

**Preparation of Synthetic Peptides and Semisynthetic Complexes**—Although the methods which we used for solid phase synthesis of the RNase (1–15) peptides were basically routine (31), some modifications were made in the published procedures (15, 16). The “premixed” symmetrical anhydride was used to couple the protected amino acid to the resin in order to minimize racemization (19). It is known that the t-butoxycarbonyl group will not be completely cleaved until the concentration of trifluoroacetic acid reaches 40 to 50% (32). However, it is also known that a 50% trifluoroacetic acid solution results in a small amount of cleavage of the ester bond linking the peptide to the resin (33). In order to compensate these two contradictory reactions, t-butoxycarbonyl groups were removed by treating twice with 35% TFA in methylene chloride for a relatively short time (10 min).

The coupling of synthetic (1–15) peptides with RNase S-protein to form the semisynthetic RNase S' complexes was carried out according to published procedures (10). However, the purification procedure was simplified. The use of an immersible membrane filter provided a simple one-step procedure, which was much quicker than the usual membrane ultrafiltration, and resulted in less loss of material than enzymatic or column chromatography procedures. Of course, to be able to use this procedure effectively required the addition of excess synthetic (1–15) peptide, so that any non-binding or weakly binding incorrect product would be removed by dialysis with the excess peptide. Any strongly bound peptide with an incorrect sequence would be retained by this procedure. However, the high values of specific activity determined by the standard ribonuclease assay procedure was ample evidence that no significant proportion of the retained semisynthetic RNase S' complexes were not native material.

**Properties of $^{13}$C Resonances of Semisynthetic RNase S' Complexes**—The sites chosen for $^{13}$C enrichment in the RNase (1–15) peptides which we have synthesized are shown schematically in Fig. 9. Other approaches which have been used to monitor this peptide-protein interaction include synthesis of large numbers of NH2-terminal peptide analogs (5, 31, 34–38) including use of nonnatural amino acids (39, 40), substitution with non-native isotopes, such as $^{18}$F (10, 41, 42), use of $^3$H labeling (43, 44), and a $^1$H NMR study (45). Apart from the two last-mentioned, all of these methods arrive at conclusions on the basis of altering the system under study. In principle, and usually in practice, it is preferable to utilize a synthetic peptide to provide the most direct evidence of the interaction with the protein.
nondisturbing method. Proton NMR is such a method. However, the \(^1H\) spectrum of the S-peptide is quite complex, and the results of following changes upon the addition of aliquots of S-protein when both components are known to aggregate at high concentrations in solution (7, 46) cannot be considered reliable. The elegant \(^3H\)-labeling experiments of Schrader and Baldwin (43, 44) provide information on complex hydrogen exchange kinetics.

There are several ways in which stable isotopes may be incorporated into a peptide or protein (1). While \(^13C\) NMR studies at natural abundance do give valuable results (47, 48), it would not be possible to attain the degree of specificity or the amount of spectroscopic information as found in the present work if one were working at much lower enrichment levels. For example, the \(^13C\) NMR spectrum of ribonuclease at natural abundance is shown in Fig. 10, together with the resonance assignments determined in the present work.

The process of isotope substitution for methyl groups of methionine is fairly stringent chemically and hence is also limited to certain cases (23, 49). This process is also nonselективive and hence less specific than peptide synthesis, but fortunately the RNase (1-15) peptide contains only 1 Met residue at position 13.

The earlier studies reported by one of us showed the feasibility of the approach utilizing \(^13C\) enrichment and \(^15N\) NMR observation (10, 11). For example, it was found that a downfield shift of 1.3 ppm was observed for the Phe 8 carbonyl \(^13C\) atom on forming the \([\U{2013}^{13C}\text{Phe}]_8\)-RNase S' complex (10). This chemical shift change correlated with results of studies of homopolypeptides on formation of an \(\alpha\)-helix (50, 51) and with the results for RNase S' complex with (1-15) peptide containing [\(^13C\)Gly 6 (45\% \(^{13C}\)) substituted for Ala at position 6, which gave a downfield shift of 2.3 ppm (52). In the present work, with much better spectra for the \([13C\text{-enriched RNase S'] complex, a downfield shift of 2.9 ppm was found for \([13C\text{-}]\text{Ala 5. Consequently we may now generalize these five results to conclude that a carbonyl carbon involved in an \(\alpha\)-helix experiences a downfield shift of about 1 to 3 ppm. What the contributions of hydrogen bonding and orbital distortion are to this value are unclear. It is nevertheless difficult to make clear predictions about chemical shift changes as a result of the complex nature of the physical basis of this parameter. Also, pragmatically, a downfield shift may derive from other origins, such as that observed for the \([\U{2013}^{13C}\text{His}]_12\), which is almost certainly due to a reduction of electronic shielding for this residue in the protein cleft microenvironment compared to its solvated solution environment (8). Such an origin for this downfield shift in this case is also indicated by the fact that the pK\(_a\) value for His 12 is quite low (~5.7), the lowest of the 4 histidines present in RNase. The reason for this was not apparent with the earlier erroneous assignment of the proton resonance of this residue. But, the assignment has been corrected (26), and we now definitively confirm it by the proton NMR titration curve of the \([\U{2013}^{13C}\text{His}]_12\)-RNase S' complex (Fig. 5). Thus, the low pK\(_a\) and downfield shift of the His 12 (C') resonance can be attributed to the positively charged nature of the active site cleft, particularly due to close proximity to the side chain of Lys 41 (8).

Thus, the origin of downfield shifts, or the electronic deshielding giving rise to them, can be manifold. However, usually the insertion of a nucleus into a hydrophobic environ-
this residue by \(^1\)H NMR titrations (8), it would seem most likely that the origin of the pK\(_a\) 6.1 inflection in the Asp 14 (C') titration curve is His 48. On the other hand, the pK\(_a\) of Asp 14 is much lower than the pK\(_a\) of about 4, which is seen in the \(^1\)H NMR titration curves of 3 histidine residues of RNase, including His 48 and 12 (29). This difference would indicate that Asp 14, notwithstanding its close proximity to His 48 in the structure of RNase S' determined by x-ray crystallography, is not the origin of these low pK\(_a\) inflections, as had previously been assumed (57, 58). Thus, Asp 14 cannot be the origin of the conformational transition with pK\(_a\) ~ 4 which affects 3 histidine residues (29), and for which there is other evidence (59–62). Indeed, our results show that Asp 14 is an H-bond acceptor, presumably from Tyr 25 (8), throughout the pH range studied. Our use of \(^13\)C enrichment allowed us to test and to eliminate the most likely candidate for a carbonyl pK\(_a\) namely Asp 14, which we had suggested as the origin of this conformational effect. Thus, the origin of this effect must be sought elsewhere in the molecule, and we are continuing our studies on this problem.

The pK\(_a\) of His 12 determined from these \(^13\)C experiments (5,7) is slightly lower than determined previously by \(^1\)H NMR (about 5.9). This difference we attributed to our more efficient dialysis method which removed essentially all P, from the complex mixture down to a level of 10^{-10} M. This pK\(_a\) value and the \(^1\)H NMR titration of the enriched [\(^{13}\)C]His 12-RNase S' complex (Fig. 5) also confirm our assignment of the active site \(^1\)H resonances. The observation of a second low pK\(_a\) inflection in the \(^13\)C NMR titration curves of His 12 in the presence of RNase inhibitors will be described elsewhere.\(^3\)

The almost linear pH dependence of the Met 13 (C') resonance in the RNase S' complex of 0.6 ppm might be attributed to the effects of small overlapping pK\(_a\) transitions. These transitions could include the titrations of Asp 14 (pK\(_a\) 2.4) and His 12 (5.7) and the conformational transition (about 4).

Denaturation of RNase S' Complexes—The unfolding process of the RNase S' complexes was monitored at the \(^13\)C-enriched sites both as a function of decreasing pH and increasing temperature. In effect, we are observing a composite of the overall unfolding of the complex, except for the Met 13 (C') atom. This site gave a lower temperature midpoint and a higher pH midpoint for unfolding (Table V) than the other sites. It is a remarkable demonstration of the degree of specificity obtainable by this method that the side chains of His 12 and Asp 14 show the same temperature midpoints for unfolding, but the side chain of the Met 13 residue between them unfolds about 6°C lower. The origin of this difference is not clear, but it may reflect the weaker nature of the hydrophobic binding of this side chain, which might be a more general phenomenon. It is noteworthy that the side chains of His 12 and Asp 14 show the same temperature midpoints as the peptide backbone at Ala 5, indicating the extensive nature of the overall cooperative unfolding of the peptide in the RNase S' complex.

CONCLUSION

Detailed information was obtained at individual atomic sites on the nature of the peptide-protein interface in the ribonuclease S' system using the combination of stable isotope enrichment and NMR observation.

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Peptide-Protein Interaction in RNase S' by $^{13}$C NMR

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Peptide-Protein Interaction in RNase S' by 13C NMR

To elucidate the role of 13C NMR in the study of protein interactions, particularly in the context of RNase S', this article presents a detailed experimental procedure.

**Materials and Methods**

The study involved the use of 13C NMR spectroscopy to analyze the interaction of a specific peptide-protein complex. The experimental setup included the preparation of a 13C-enriched peptide and its complexation with RNase S'.

**Results**

Upon analyzing the 13C NMR spectra, significant changes in the chemical shifts were observed, indicating strong interactions between the peptide and the enzyme. These changes were further confirmed by comparative studies at different temperatures and pH levels.

**Conclusion**

The data obtained from this study provide valuable insights into the mechanism of action of RNase S' and its interactions with various peptide substrates. The findings are crucial for advancing the understanding of protein-protein interactions and their implications in biological processes.

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**Table 1**

| Step | Operation and reagents | Mixing time (min) |
|------|------------------------|------------------|
| 1.   | **C** (5 times)         |                  |
| 2.   | 1, 075, 920 ± 21 1813 925 | 10               |
| 3.   | **C** (5 times)         |                  |
| 4.   | 1, 075, 920 ± 21 1813 925 | 10               |
| 5.   | **C** (5 times)         |                  |
| 6.   | 1, 075, 920 ± 21 1813 925 | 10               |
| 7.   | **C** (5 times)         |                  |
| 8.   | 1, 075, 920 ± 21 1813 925 | 10               |
| 9.   | **C** (5 times)         |                  |
| 10.  | **C** (5 times)         |                  |
| 11.  | 1, 075, 920 ± 21 1813 925 | 10               |
| 12.  | **C** (5 times)         |                  |
| 13.  | 1, 075, 920 ± 21 1813 925 | 10               |
| 14.  | 1, 075, 920 ± 21 1813 925 | 10               |

**Table 2**

| Amino Acid | Expected Mass (Da) | Masses (Da) | Mass Error (Da) |
|------------|--------------------|-------------|-----------------|
| Lysine     | 1401.05            | 1410.49     | 9.39            |
| Histidine  | 1584.19            | 1593.52     | 9.35            |
| Arginine   | 1706.23            | 1715.67     | 9.44            |
| Aspartic Acid | 133.08          | 133.11      | 0.23            |
| Threonine  | 117.07             | 117.10      | 0.23            |
| Serine     | 113.08             | 113.11      | 0.23            |
| Glutamic Acid | 159.15           | 159.18      | 0.23            |
| Alanine    | 115.08             | 115.11      | 0.23            |
| Phenylalanine | 165.14           | 165.17      | 0.23            |
Specific peptide-protein interactions in the ribonuclease S' system studied by 13C nuclear magnetic resonance spectroscopy with selectively 13C-enriched peptides.

C Niu, S Matsuura, H Shindo and J S Cohen

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