# *ASSIGNMENT OF THE HISTIDINE PEAKS IN THE NUCLEAR MAGNETIC RESONANCE SPECTRUM OF RIBONUCLEASE**

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The four histidine residues in bovine pancreatic ribonuclease A (RNase A) occur at positions 12, 48, 105, and 119 in the amino acid sequence.[1] Two of them, residues 12 and 119, are known to be in the active site of the enzyme.[2-4] The C-2 protons of these four histidines were shown to give rise to distinct NMR absorptions that were initially observed as three peaks in the 60-Mc spectrum of RNase A.[5] Subsequent experiments at 100 Mc showed four resolvable peaks,[6] the chemical shift for each of them being pH-dependent; it was also noted that two of the four peaks shift position in the presence of mononucleotide inhibitors.

In the present paper we establish that the two imidazole $C_2$-H peaks of RNase A that are affected by inhibitor binding do indeed correspond to the two active-site histidines. From studies of carboxyalkylhistidine RNase derivatives and the subtilisin-cleaved enzyme RNase S,[7] it has been possible to assign each of the four observed peaks to the appropriate histidine residue.

*Materials and Methods.*—RNase A (lyophilized and phosphate-free) was obtained from Worthington Biochemical Corporation and used without further treatment. RNase S from Sigma Chemical Corporation was used directly in NMR studies and also further separated into S-peptide and S-protein by Dr. Sandor Varga of Merck and Co.

The carboxymethyl RNase derivatives were prepared according to the procedure of Crestfield, Stein, and Moore[2] by the reaction of RNase A with iodoacetic acid (Eastman Organic Chemicals). 1-CM-His-119 RNase and 3-CM-His-12 RNase were separated on an IRC-50 column, and the pooled fractions of each derivative were desalted on a column of AG 501-X8 (D) mixed-bed resin (BioRad Labs.) and lyophilized. Amino acid analyses of the two derivatives agreed well with those previously found.[2]

Carboxypropyl ribonuclease was prepared by reaction of RNase A with DL-2-bromobutyric acid (Eastman) under conditions described by Heinrikson, Crestfield, Stein, and Moore.[8] The 3-CP-His-12 RNase was separated and rechromatographed, being twice desalted and lyophilized as described above. The amino acid analysis of this derivative was also in good agreement with theory.

The solvent for all NMR studies was 0.2 *M* acetate buffer, pH 5.25, made from 99.85% $D_2O$ (BioRad), $CD_3COOD$, and NaOD (Merck of Canada). The concentration of RNase A or its derivatives was approximately 0.01 *M* (12%). The pH was adjusted with 1.0 *M* DCl or NaOD and read with a Radiometer model 26 pH meter with microelectrodes. Uncorrected meter readings are given in all cases. Samples of RNase S, S-peptide, and S-protein were centrifuged in a Clay-Adams microchemistry centrifuge before each spectrum was taken.

NMR spectra were obtained with a Varian HA 100 spectrometer with a low impedance magnet and power supply and a Varian V4343 variable temperature controller. Probe temperature was 32°C unless otherwise indicated. Sweep rate was 1 cps/sec, and spectra were averaged over 20–100 sweeps on a Varian C1024 computer of average transients. Wilmad precision-bore NMR cells were used with coaxial inserts containing the external standard HMS.

*Results and Discussion.*—(1) *RNase A histidine titration curves:* The titration curves for the histidine peaks of RNase A have been reported previously.[6] In the present series of experiments, done on a new spectrometer system, a slight shift of the curves was found. This may be attributed to a difference in the ambient probe temperature (32°C, as compared to 28°C) and a change in calibration. The new RNase A titration curves are shown for reference in Figure 1. The corresponding pK values are: peak 1, 6.7; peak 2, 6.2; peak 3, 5.8; peak 4, 6.4. In addition, a peak attributable to a $C_4$-H of histidine has been observed. Its pK is 6.7, and therefore it can be identified with the same residue as peak 1.

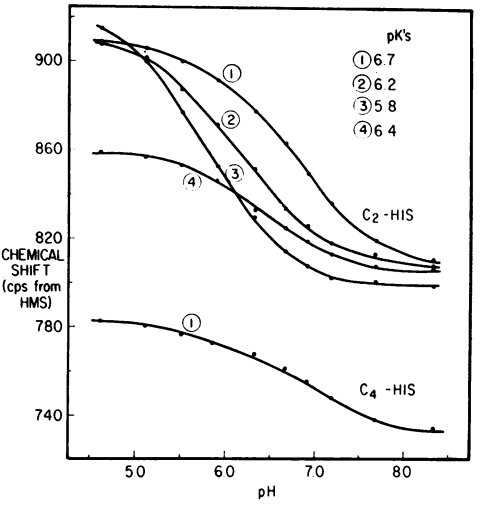


FIG. 1.—Titration curves of histidine $C_2$-H peaks and one histidine $C_4$-H peak of RNase A. Reported pK's are for 32°C in 0.2 *M* deuteroacetate buffer.

(2) *Carboxyalkyl derivatives of RNase A:* The variation of chemical shift of the histidine peaks of 1-CM-His-119 RNase as a function of pH is shown in Figure 2. It is apparent that the positions of $C_2$-H peak 4 and $C_2$-H and $C_4$-H peaks 1 are unaffected at all pH values by the carboxymethyl substitution on histidine-119. However, the histidine residues of peaks 2 and 3 have both been increased in pK to values of 6.7 and 6.9.

The same type of behavior is observed in the carboxymethyl derivative of histidine-12. The titration curves of 3-CM-His-12 RNase (Fig. 3) again show that peaks 1 and 4 are unchanged and that histidine residues 2 and 3 have been increased in pK. The pK's of the affected peaks are 6.7 and 7.6. Essentially the same results are obtained with the carboxypropyl histidine-12 derivative.

It is clear from these findings that the two histidine residues at the active site of RNase A are indeed near each other in the folded structure of the protein chain, since substitution on either of these two histidines changes the magnetic environment of both. The observed increase in pK of the two imidazole rings must be due to the local electrostatic interaction arising from the negative charge on the carboxyalkyl substituent, rather than a more widespread conformational change, since neither the pK's nor the chemical shifts of the other two histidine residues are altered.

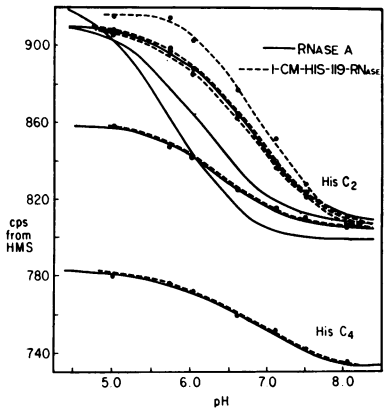


FIG. 2.—Titration curves of histidine peaks of 1 CM-His-119-RNase, with RNase A titration curves shown for reference. Peaks 2 and 3 (as numbered in Fig. 1) have been shifted to pK's of 6.7 and 6.9.

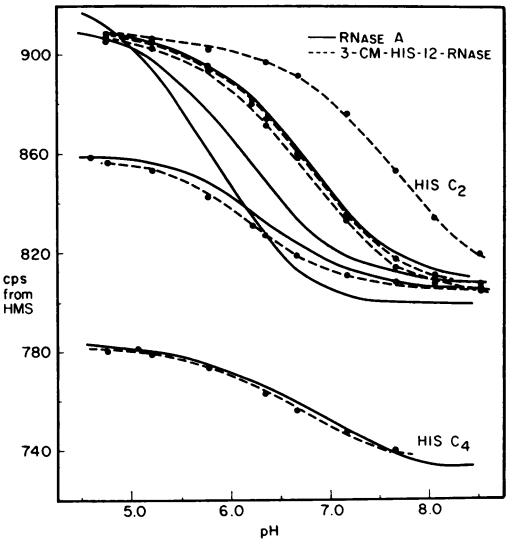


FIG. 3.—Titration curves of histidine peaks of 3-CM-His-12-RNase. Peaks 2 and 3 have been shifted to pK's of 6.7 and 7.6.

Unfortunately, it is not possible to decide for any of these three derivatives which of peaks 2 and 3 has shifted to either of the new positions. Peaks 2 and 3 can now be associated with histidine-12 and histidine-119 and peaks 1 and 4 with histidine-48 and histidine-105, but experiments of this kind do not enable us to distinguish between the two active-site histidines. A substitution is required that is subtle enough to affect only one of the two neighboring histidine residues, but that is still detectable in the NMR spectrum. Chemical substitutions clearly do not satisfy this criterion because the perturbation of molecular structure is too large. The obvious alternative is isotopic substitution of D for H on one of the active-site histidine residues.

(3) *Deuterium exchange of His $C_2$-H:* In the course of the above studies it was observed that solutions of RNase A or its derivatives that were kept in $D_2O$ over periods of several weeks gradually lost intensity in the imidazole $C_2$-H region of the spectrum. This apparent exchange of the imidazole $C_2$ proton for deuterium was studied in the model compounds histidine and L-histidyl-L-histidine as a function of temperature and pH by following the disappearance of the $C_2$-H peak in the NMR spectrum. The average time required for loss of $^1/_2$ peak intensity of the $C_2$-H peak in the model compounds (as compared with the $C_4$-H peak, which did not exchange) was as follows:

| *pH* | *Temperature (°C)* | *Exchange time (days)* |
|---|---|---|
| 7 | 26 | >10 |
| 7 | 37 | 4 |
| 9 | 26 | >10 |
| 9 | 37 | 2 |

This result suggested that a deuterium exchange of the histidine $C_2$ protons of RNase would be possible under relatively mild conditions. In order to obtain fractions of the peptide chain that contain only one of the two active-site histidine residues, and that would therefore enable us to carry out a selective deuterium exchange, we turned to the modified enzyme, ribonuclease S.

(4) *RNase S histidine titration curves:* The titration curves of ribonuclease S, which is identical in chemical structure to RNase A except for the cleavage of the 20-21 peptide bond,[7] are shown in Figure 4. From the positions of both the $C_4$-H and $C_2$-H resonances at all pH's, it can be seen that the histidine residue corresponding to peak 1 is in the same magnetic environment in RNase S and RNase A. The two active-site histidines (peaks 2 and 3) are each higher in pK by 0.5 pH units in RNase S than in RNase A, but their respective chemical shift values in the protonated and unprotonated forms are the same in the two enzymes. Peak 4 has shifted downfield by 20–30 cps at all pH values in RNase S, but its pK value is unchanged.

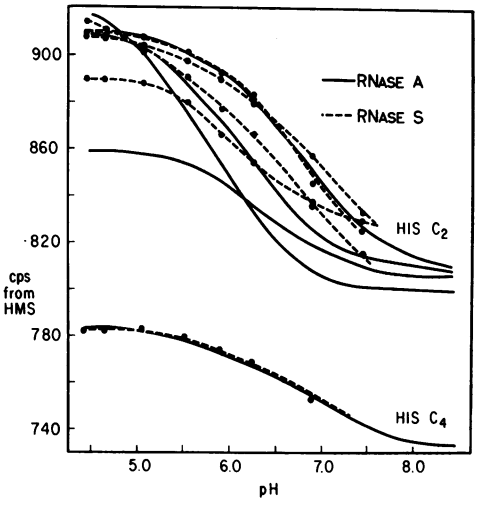


FIG. 4.—Titration curves of histidine peaks of RNase S at 32°C in 0.2 *M* deuteroacetate buffer.

It was previously suggested[6] that peak 4 could be assigned to histidine-48, the "buried" histidine of RNase A,[4] because of its anomalous chemical shift in the acid pH region. The greater line-width of this peak also indicates a low mobility compared to that of the other three histidine side chains. The shift of position of this peak in RNase S toward more normal values for histidine $C_2$-H resonances is a further argument for its association with histidine-48, since Wyckoff *et al.* have reported that this side chain is less buried in RNase S than in RNase A.[4]

The increase in pK's of the active-site histidines might also be expected in RNase S, since the breaking of the 20-21 bond could create a somewhat greater flexibility in the cleft region, exposing the two histidines more to the solvent and causing their pK's to approach that of free histidine.

(5) *Deuterium exchange of S-peptide:* It was our intention to expose the separated S-peptide fragment of RNase S (residues 1–20) to the same exchange

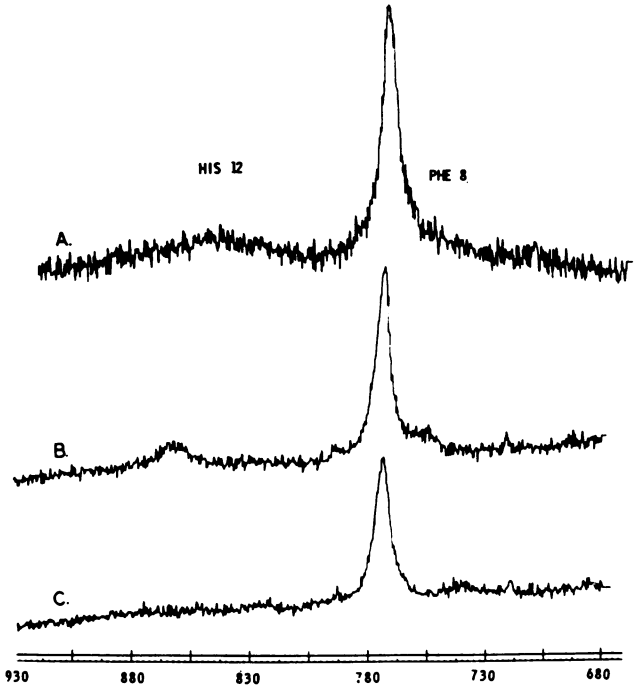


FIG. 5.—(*A*) Spectrum of aromatic region of S-pep at 32°C in 0.2 *M* deutero-acetate buffer. $C_4$-H peak of His-12 is buried under Phe-8 peak.
(*B*) Spectrum of same solution of S-pep at 10°C.
(*C*) Spectrum of same solution of S-pep at 10°C after it had been maintained at 40°C for 5 days.

conditions described in section 3 for histidine and L-histidyl-L-histidine, in order to deuterate the $C_2$ position of histidine-12 selectively, and to follow the exchange by NMR. However, the spectrum of the unexchanged S-pep (Fig. 5*A*) showed such a broad histidine-12 $C_2$-H peak (~35 cps) that it would have been difficult to monitor its disappearance. This anomalous peak-width was not affected by the addition of EDTA and therefore was not caused by trace paramagnetic metal ion binding to the imidazole ring. The broadening was specific to the histidine-12 peaks, since the phenylalanine-8 aromatic peak and the aliphatic region of the spectrum showed normal line widths. The histidine-12 $C_2$-H peak-width was temperature-dependent, however, and, as shown in Figure 5*B*, at 10°C it was sharp enough to be measurable with reasonable accuracy. Therefore, the S-pep solution was kept in a constant-temperature water bath at 40°C, and the exchange of its histidine-12 $C_2$-H was monitored daily by means of its NMR peak area, measured at 10°C.

After maintaining the S-pep solution at 40°C for five days at pH 7, the $C_2$ proton had exchanged enough to be undetectable by NMR (Fig. 5*C*). An equivalent amount of S-protein was added to the solution, and the resulting spectrum of reconstituted RNase S′ contained only three histidine peaks as shown in Figure 6. The titration curves of these peaks (Fig. 7) indicate that the

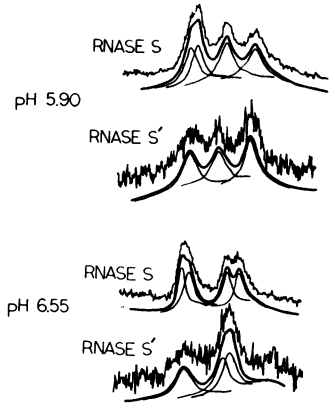


FIG. 6.—Histidine $C_2$-H peaks of RNase S and RNase S' (with His-12 $C_2$-H deuterated) at 2 pH's. Fitted curves, obtained with a Dupont 310 curve resolver. assuming equal areas for all peaks, are shown under each spectrum.

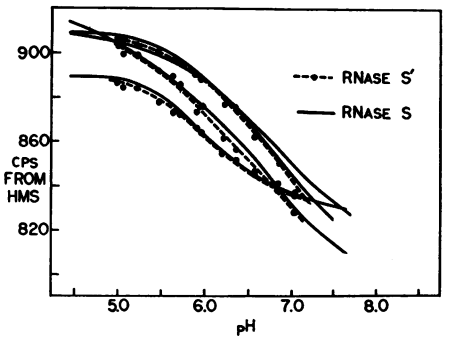


FIG. 7.—Titration curves of histidine peaks of RNase S, with histidine-12 deuterated in the $C_2$-H position. Curves of RNase S are shown for reference.

missing His-12 $C_2$ proton is in the region of the spectrum where peaks 1 and 2 overlap at nearly all pH's. Since we have evidence that peak 1 can be assigned to His-105, the exchanged proton must be that of peak 2, and therefore a final assignment of all four RNase A histidine peaks can be made.

| *Peak* | *pK at 32°C in 0.2 M deuteroacetate buffer* | *Histidine* |
|---|---|---|
| 1 | 6.7 | 105 |
| 2 | 6.2 | 12 |
| 3 | 5.8 | 119 |
| 4 | 6.4 | 48 |

*Summary.*—The NMR spectrum of RNase contains four histidine $C_2$-H peaks, distinguishable by their pK values as follows: (1) 6.7, (2) 6.2, (3) 5.8, (4) 6.4; the last may be assigned to His-48 on the basis of its anomalous chemical shift and line width. Carboxymethylation of *either* His-12 *or* His-119 shifts *both* peaks (2) and (3) but not (1) or (4); exchange of the His-12 $C_2$ proton for deuterium eliminates peak (2). The assignment is therefore: (1) 105, (2) 12, (3) 119, (4) 48.

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Abbreviations: RNase A, bovine pancreatic ribonuclease A; NMR, nuclear magnetic resonance; 1-CM-His-119 RNase, 1-carboxymethylhistidine-119-ribonuclease; 3-CM-His-12 RNase, 3-carboxymethylhistidine-12-ribonuclease; HMS, hexamethyldisiloxane; S-pep, S-peptide; EDTA, ethylenediaminetetraacetate.

* This is the third in a series on nuclear magnetic resonance studies of the structure and binding sites of enzymes (Merck), the 27th in a series on structural studies of ribonuclease (Cornell), and part of a dissertation submitted by D. H. M. to Harvard University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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