Tautomeric States of the Histidine Residues of Bovine Pancreatic Ribonuclease A

APPLICATION OF CARBON 13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY*

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Natural abundance 13C NMR (at 67.9 MHz) is used to study the nonprotonated aromatic carbons of bovine pancreatic ribonuclease A. The C' resonances of the 4 histidines are identified and assigned to specific residues. The effect of pH on the chemical shifts of these resonances yields information about the tautomeric state of the imidazole form of each histidine residue. Only His-119 exists predominantly or entirely in the "common" N'-H (N'-H) tautomeric state. His-12 and His-48 exist mainly or entirely in the N'-H (N'-H) tautomeric state. His-105 exhibits about 50 to 90% of the N'-H tautomer. These results, when taken together with known crystallographic coordinates for ribonuclease A, strongly suggest that H1 of His-105 is hydrogen bonded to the COOH-terminal carboxylate group, that H3 of His-48 is hydrogen bonded to the carboxylate group of Asp-14 or (less likely) to the hydroxyl oxygen of Thr-17, and that H6 of His-12 is probably hydrogen bonded to the hydroxyl oxygen of Thr-45.

Deprotonation of the imidazolium form of a histidine residue can yield either the N'-H (N'-H) tautomer or the N'-H (N'-H) tautomer of the imidazole form of the residue. It is now well established that the N'-H tautomers are predominant (70 to 90%) not only for L-histidine (1-3) and N'-acetyl-L-histidine (1), but also for His-Gly (1), bacitracin (1), and thyrotropin-releasing factor (4). These results suggest that the N'-H tautomer should be considered "normal" for nonprotonated L-histidine residues in peptides and proteins. Therefore, the detection of a histidine residue predominantly in the N'-H tautomeric state may provide useful structural information. As far as we know, only two reports of such histidines have been published. Ugurbil et al. (5) used natural abundance 13C NMR to show that one of the two tautomeric histidines of Pseudomonas aeruginosa azurin exists predominantly in the N'-H tautomeric state. Bacchovchin and Roberts (6) used 13N NMR to show that the histidine (N'-enriched) of the "catalytic triad" in α-lytic protease exists in the N'-H tautomeric state. In contrast, 13C NMR studies of horse myoglobin have detected 8 tautomeric histidine residues, all of which appear to be predominantly (or entirely) in the N'-H tautomeric state (7); the single histidine of hen egg white lysozyme also behaves "normally" (8).

In this report, we use natural abundance 13C NMR spectroscopy to show that three of the four histidines of bovine pancreatic ribonuclease A have the N'-H tautomeric structure, and we discuss some structural implications of this finding.

EXPERIMENTAL PROCEDURES

"Experimental Procedures" are described in the adjacent miniprint supplement.1

RESULTS AND DISCUSSION

Although many 13C NMR studies of bovine pancreatic ribonuclease A have been published (see miniprint supplement), as far as we know the titration behavior of the histidine residues has not been examined. Fig. 1 shows the region of aromatic carbons (and C' of arginine residues) in the natural abundance 13C NMR spectrum of bovine pancreatic ribonuclease A in H2O (pH 4.13, 0.2 M acetate), obtained at 67.9 MHz under conditions of noise-modulated off-resonance proton decoupling (4-11). The convolution-difference method was used to eliminate the broad methine aromatic carbon bands (9-11). Therefore, only the narrow resonances of nonprotonated carbons (10, 11) are observed. In the case of ribonuclease A, which lacks tryptophan residues, the 4 arginines, 6 tyrosines, 3 phenylalanines, and 4 histidines (12) contribute a total of 23 nonprotonated side chain carbons, all of which yield observable resonances in Fig. 1.

In Fig. 2 we show the effect of pH on the chemical shifts of the nonprotonated aromatic carbons and C' of arginines of ribonuclease A. Best-fit (single pK) titration curves are shown for all resonances which exhibit significant chemical shift changes in the pH range 4 to 8. For each of these resonances, the best-fit values of the pK, the low pH chemical shift (δL), and the difference between δL and the limiting chemical shift at high pH (Δm) are given in Table II of the miniprint supplement. On the basis of their large values of Δm (4.0 and -5.4 ppm, respectively), Peaks 14 and 20 can be confidently assigned to histidine residues. However, some nonprotonating amino acid residues have fairly large Δm values (Table II), presumably caused by the ionization of nearby histidines, which interfere with the identification of the other 2 histidine

1 Some of the data (including Tables I and II and Fig. 3) are presented as a miniprint supplement immediately following this paper. For the convenience of those who prefer to obtain the supplementary material in the form of 14 pages of full size photocopies, it is available as JBC Document No. 78M-2355. Orders should specify the title, authors, and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $2.10 per set of photocopies.

2 The abbreviations used are: δL, best-fit limiting chemical shift at low pH; δm, δL-δH; Δm, difference between δL and the best-fit, limiting chemical shift at high pH.

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residues on the basis of $\Delta_{AB}$ values.\textsuperscript{1} We used the selective paramagnetic broadening caused by the binding of Cu\textsuperscript{2+} (13, 14) and Mn\textsuperscript{2+} (15) to show, without invoking the pH titration data, that Peak 16 (with $\Delta_{AB} = 2.1$ ppm) and Peak 19 (with $\Delta_{AB} = 0.5$ ppm) arise from His-12 and His-105, respectively. The specific assignments of Peaks 14 and 20 to His-48 and His-119, respectively, follow from a combination of the titration data (Table II) and the effects of Cu\textsuperscript{2+} and Mn\textsuperscript{2+}.\textsuperscript{1}

Our pK values of 5.7, 6.0, 6.2, and 6.6 for His-12, His-48, and His-105, respectively, follow from a combination of the titration data (Table II) and the effects of Cu\textsuperscript{2+} and Mn\textsuperscript{2+}.\textsuperscript{1} We used chemical shift changes caused by inhibitor binding and by deuterium isotope effects as supporting evidence for our assignments.\textsuperscript{1} Our pK values of 5.7, 6.0, 6.2, and 6.6 for His-12, His-48, and His-105, respectively, follow from a combination of the titration data (Table II) and the effects of Cu\textsuperscript{2+} and Mn\textsuperscript{2+}.\textsuperscript{1}

In summary, our results indicate that three of the four histidines of bovine ribonuclease A exhibit major proportions of the N\textsuperscript{2+}-H tautomeric structure ($\Delta_{AB} = 2.1$ ppm, and $\Delta_{AB} = 0.5$ ppm, respectively). The imidazole forms of His-12 and His-48 are most certainly mainly or entirely the N\textsuperscript{2+}-H tautomers (Table II). The imidazole forms of His-12 and His-48 are most certainly mainly or entirely the N\textsuperscript{2+}-H tautomers (Table II). Our results on tautomeric states of the four histidines of bovine ribonuclease A exhibit major proportions of the N\textsuperscript{2+}-H tautomeric structure ($\Delta_{AB} = 2.1$ ppm, and $\Delta_{AB} = 0.5$ ppm, respectively). The imidazole forms of His-12 and His-48 are most certainly mainly or entirely the N\textsuperscript{2+}-H tautomers (Table II). The imidazole forms of His-12 and His-48 are most certainly mainly or entirely the N\textsuperscript{2+}-H tautomers (Table II).

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**REFERENCES**

Tautomeric States of Histidine Residues of Ribonuclease


Additional references are found on p. 6204.
TAUROMERIC STATES OF THE HISTIDINE RESIDUES OF RIBONUCLEASE A

**METHODS AND RESULTS**

The use of NMR spectroscopy to examine the tautomeric states of histidine residues was initiated in 1969 with a protein, NADPH dehydrogenase, obtained from Neurospora crassa. A detailed study of this enzyme soon showed that there were three distinct tautomeric states of histidine residues in the protein, designated as A, B, and C. The A state is characterized by a rapid exchange of the histidine nitrogen with water, while the B state is characterized by a rapid exchange of the histidine nitrogen with solvent. The C state is characterized by a slow exchange of the histidine nitrogen with solvent.

**DISCUSSION**

These results suggest that the tautomeric states of histidine residues in proteins are important in determining the protein's structure and function. The tautomeric states of histidine residues are likely to be influenced by factors such as the protein's environment, including pH, temperature, and solvent composition. These results also suggest that NMR spectroscopy is a useful tool for studying the tautomeric states of histidine residues in proteins.
In Table 1 we show the change in the chemical shifts of the ε proton of histidine residues in the ribonuclease enzyme. The chemical shifts were determined in the presence and absence of NaCl. The ε proton of histidine residues is strongly affected by the presence of NaCl, as shown in the table. The chemical shifts of the ε proton of histidine residues in the absence of NaCl are given in the first column, and the chemical shifts in the presence of NaCl are given in the second column. The chemical shifts were determined by 1H NMR spectroscopy. The table shows that the chemical shift of the ε proton of histidine residues is significantly affected by the presence of NaCl, with a significant increase in the chemical shift of the ε proton of histidine residues in the presence of NaCl. The data in Table 1 are consistent with the hypothesis that the ε proton of histidine residues is strongly affected by the presence of NaCl.

In Figure 2, we show the effect of NaCl on the chemical shifts of the ε proton of histidine residues in the ribonuclease enzyme. The chemical shifts were determined in the presence and absence of NaCl. The ε proton of histidine residues is strongly affected by the presence of NaCl, as shown in the figure. The chemical shifts of the ε proton of histidine residues in the absence of NaCl are given in the first column, and the chemical shifts in the presence of NaCl are given in the second column. The chemical shifts were determined by 1H NMR spectroscopy. The figure shows that the chemical shift of the ε proton of histidine residues is significantly affected by the presence of NaCl, with a significant increase in the chemical shift of the ε proton of histidine residues in the presence of NaCl. The data in Figure 2 are consistent with the hypothesis that the ε proton of histidine residues is strongly affected by the presence of NaCl.