PROTON MAGNETIC RESONANCE STUDIES ON RIBONUCLEASE T_1 [1]

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Summary: The chemical shifts of the C-2 protons of the histidine residues of ribonuclease T_1 (RNase T_1) have been studied as a function of pH in ^2H_2O. The results are interpreted in terms of interactions of the histidine residues with carboxylate anions of acidic amino acid residues. The presence of histidines in the active site of the enzyme is indicated by changes, which occur in their C-2-PMR-absorption region on the addition of guanosine, 2'-GMP, and 3'-GMP. One of the three histidines interacts with the phosphate group of 3'-GMP. The differences in the PMR-spectra of the RNase T_1-3'-GMP and RNase T_1-2'-GMP complexes suggest that these nucleotides are bound to the enzyme differently.

It has been shown in previous investigations that the proton magnetic resonance (PMR) signals of the C-2 protons of histidine residues in proteins usually shift downfield by about 1 ppm upon protonation of the imidazole ring (1-4). Hence approximate pK-values of histidine residues in proteins can be derived from the pH-dependence of the chemical shift of this C-2 PMR signal. Also, influences of the immediate environment of the histidine residues can be detected, which may be reflected, in shifts of their pK-values or in changes of the chemical shifts of the C-2 protons of their protonated or deprotonated imidazole rings. In case of pancreatic ribonuclease A (RNase A) the C-2 PMR signals could be inadvertently assigned to the corresponding histidine residues of this protein (3). The study of influences of inhibitors on these C-2 PMR signals provided an important contribution to the understanding of the structure of the active site of RNase A (4, 5).

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Based on chemical modification studies it has been reported that two histidines are essential for the activity of ribonuclease T_1 (RNase T_1) (6). Furthermore, glutamic acid 58 plays an important role in the action of this enzyme (7). The presence of these amino acid residues in the active site was also indicated by kinetic investigations (8, 9). Therefore, we investigated the pH - dependence of the chemical shifts of the C-2 protons of the histidine residues in RNase T_1 in the presence and absence of inhibitors. We wish to report these results in this communication.

Experimental

RNase T_1 (Lot No. 8R020, lyophilized and salt free) was purchased from Sankyo Co., Ltd., Tokyo, Japan, and was used without further purification. The enzyme solutions (0.005 M) were prepared by mixing an appropriate amount of RNase T_1 with a solution of 0.2 M NaCl/^2H_2O. Aliquot amounts of 2'-GMP and 3'-GMP (Sigma Chem. Co., Grade I) and guanosine (Zellstoff-Fabrik Waldhof, Mannheim, Germany) were added to the corresponding enzyme solution. 'pH' - and PMR - measurements (Varian Associates HA-100 spectrometer in connection with C 1024 CAT) were carried out as described previously (4). pH meter readings are not corrected for deuterium isotope effects.

Results and Discussion

In Fig. 1, the chemical shifts of the C-2 protons of the three histidines of RNase T_1 are plotted against 'pH'. Compared to free histidine or imidazole the histidine residues of RNase T_1 behave differently. At low pH the chemical shift of about 900 Hz[3] of a C-2 proton of a protonated imidazole ring is

[3] The chemical shifts are given in Hz relative to the external standard tetramethyl silane (TMS).

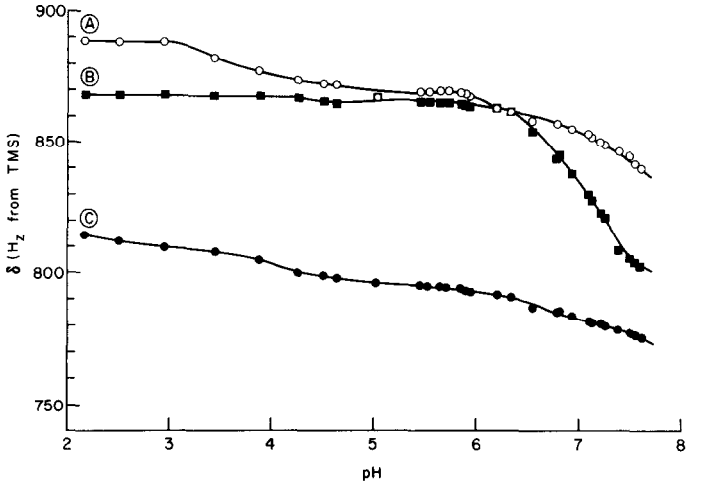

Fig. 1. Chemical shift of the imidazole C-2 protons of the three histidine residues of ribonuclease T_1 as a function of 'pH' in a 0.2 M NaCl ^2H_2O solution. Concentration of ribonuclease T_1 is 0.005 M.

not found. Only one histidine (A) approaches this value below pH 3. This suggests that with respect to free histidine the environment of this histidine is only normalized at acid pH - values. Above 'pH' 3 the C-2 PMR signal of histidine A shifts upfield to the position of 870 Hz, where it maintains in the pH - range of 4.8 - 5.9. Above 'pH' 5.9 the C-2 PMR signal shifts further upfield following the deprotonation of the imidazole ring. The pK-value of this histidine should be around 8. Histidine B reveals a C-2 PMR signal, which is positioned throughout the pH - range of 2 - 6.3 at 865-870 Hz Above 'pH' 6.4 it shifts upfield. The pK - value of this histidine residue is about 7.1. In a very anomalous manner the C-2 PMR signal of histidine C shifts gradually from 815 Hz at 'pH' 2 to 775 Hz at 'pH' 7.5. Therefore, a pK - value for this histidine is not derived. Studies with model compounds such as imidazolyl-4(5)-acetic acid (10) or β-imidazolyl-4(5)-propanoic acid (11), showed that the interaction of carboxylate anions with the adjacent imidazole ring shifts the pK - value of imidazole up to 7.5 - 7.6 compared

to the pK - value of 6.2 of imidazole itself. For histidine A and B a similar shift in pK to higher values is found. The upfield shift of the C-2 PMR signal of histidine A in the pH range of 3.0 - 5 reveals a pK - value of about 3.8, which is characteristic for a carboxylate anion. Therefore, it is reasonable to conclude that histidine A is interacting with a carboxylate anion of an acid amino acid residue of the protein. Further support for such an interaction is obtained by the observation that at pH - values below 6 a similar chemical shift of about 870 Hz is found for the C-2 proton of His 48 of RNase A, which is close to Asp 14 according to the tertiary structure of this enzyme (12), and which was assumed to interact with Asp 14 (4).

For the C-2 PMR signal of histidine B a chemical shift of 865-870 Hz is found, too. Compared to histidine A this chemical shift is maintained in a larger pH - range. It is most likely that this histidine interacts with a carboxylate anion even stronger than histidine A.

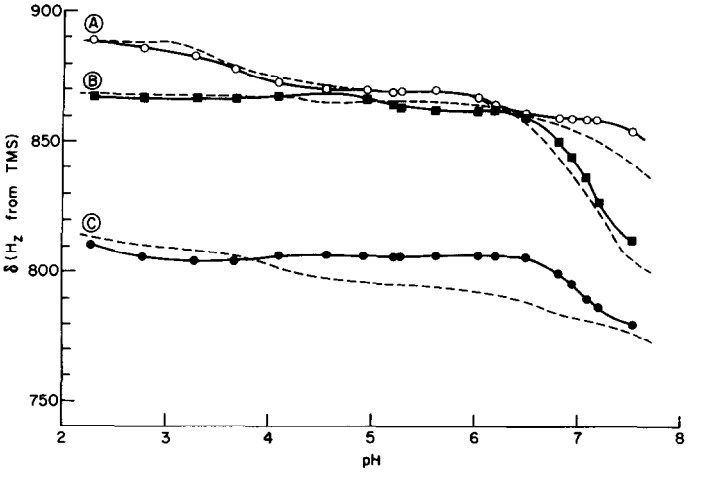

Fig. 2. 'pH' dependence of the chemical shifts of the three histidine C-2 protons of ribonuclease T_1 in the presence of guanosine in a 0.2 M NaCl ^2H_2O solution. Concentration of ribonuclease T_1 is 0.005 M and of guanosine is 0.01 M (saturated solution). Dashed lines show titration curves of histidine peaks in ribonuclease T_1 alone.

The abnormal chemical shift of the C-2 proton of histidine C and its pH-dependence is difficult to understand. Either a cluster of negative charges of the immediate environment or ring currents of adjacent aromatic amino acids or both may account for this shift. On the addition of guanosine to a solution of RNase T_1, only the pH-dependence of the chemical shift of the C-2 proton of histidine C has changed (Fig. 2). In the pH - range of 4 - 6.5 the C-2 PMR signal of this histidine is positioned at 806 Hz and shifts upfield at pH 6. This suggests that histidine C is located near the binding site of RNase T_1. However, it cannot yet be decided whether this histidine is directly bound to guanosine or not.

3'-GMP is a strong inhibitor of RNase T_1-reaction (13), and there are accordingly remarkable changes in the absorption region of the histidine C-2 protons on the addition of 3'-GMP to an RNase T_1 solution (Fig. 3). The C-2-H chemical shift of histidine B is only slightly affected. These slight changes may be due to a change in conformation of the enzyme following the binding of the inhibitor. The C-2 PMR signal of histidine A shifts downfield for about 20 - 30 Hz. In addition, this signal is extremely broadened in the pH - range of 4.5 - 6.5. The shift of the C-2 PMR signal of histidine C is nearly the same as found in the RNase T_1-guanosine complex. It shifts upfield at a slightly higher pH - value.

Since 3'-GMP differs from guanosine mainly in the additional phosphate group, one would expect that the downfield shift of the C-2 PMR signal of histidine A is caused by an interaction of this histidine with the phosphate group. The exchange broadening indicates that this histidine is tightly bound in the 3'-GMP - RNase T_1 complex and that the average lifetime of this complex is of the order of 0.01 sec. A downfield shift of the C-2 PMR signal of His 119 of RNase A was also observed on the addition of 3'-CMP, 2'-CMP,

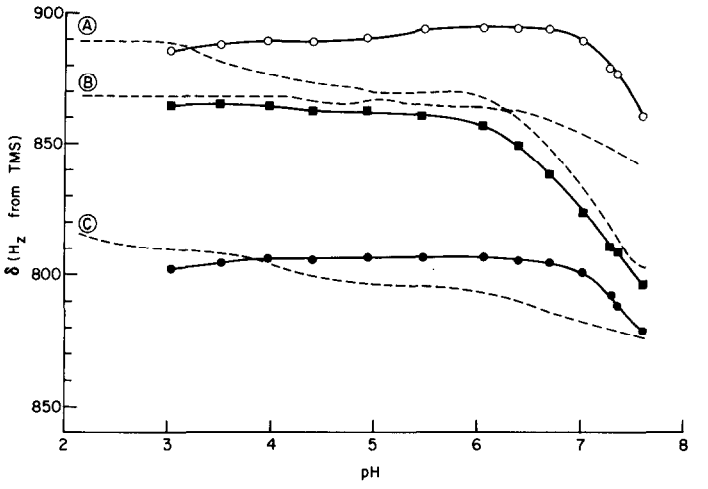

Fig. 3. 'pH' dependence of the chemical shifts of the three histidine C-2 protons of ribonuclease T_1 in the presence of 3'-GMP in a 0.2 M NaCl ^2H_2O solution. Concentration of ribonuclease T_1 is 0.005 M and of 3'-GMP is 0.02 M. Dashed lines show titration curves of histidine peaks in ribonuclease T_1 alone.

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or $Na_4P_2O_7$, due to an interaction of the phosphate moiety with this histidine (4, 5). In the presence of 2'-GMP similar changes occur in the C-2-H absorption region of RNase T_1 as with 3'-GMP (Fig. 4). However, the extent of the shift of the C-2 PMR signals is different. Histidine B again is slightly affected. The C-2 PMR signal of histidine A shifts downfield, but does not exceed the position of 880 Hz downfield from TMS. The signal is not markedly broadened. The histidine C C-2-H signal maintains a position of 795 Hz throughout the pH-range of 2.5 - 7.5, which is different from the chemical shift of this histidineC-2 proton in the RNase T_1-guanosine and RNase T_1 - 3'-GMP complexes. There are also differences in the aromatic absorption region near 750 Hz. Hence, we would conclude that 2'-GMP is bound to RNase T_1 in a different manner than 3'-GMP. The C-8 PMR signal of the guanine base is in all three complexes very broad and can hardly be detected. The chemical shift is about 850 Hz in agreement with the one of the free compounds.

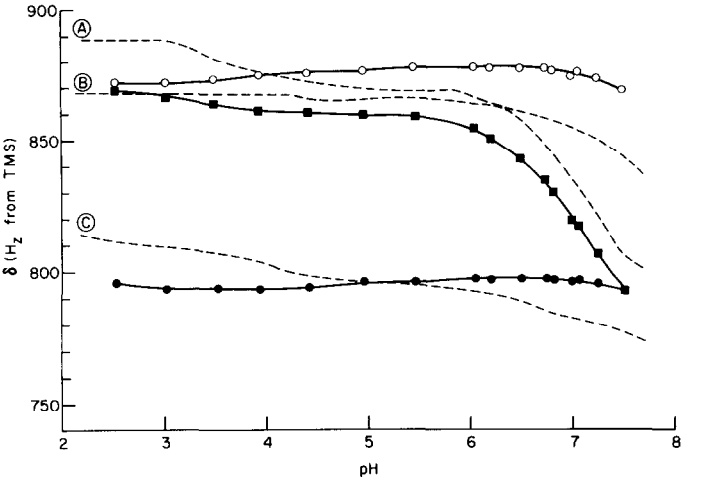

Fig. 4. 'pH' dependence of the chemical shifts of the three histidine C-2 protons of ribonuclease T_1 in the presence of 2'-GMP in a 0.2 M NaCl ^2H_2O solution. Concentration of ribonuclease T_1 is 0.005 M and of 2'-GMP is 0.02 M. Dashed lines show titration curves of histidine peaks in ribonuclease T_1 alone.

The reported PMR-data provide direct evidence for the presence of the amino acids, which have been found to be essential for the activity of RNase T_1 (6, 7), in the active center of this enzyme.

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