PROTON MAGNETIC RESONANCE STUDIES OF RIBONUCLEASE T_1. ASSIGNMENT OF HISTIDINE-40 PEAK AND ANALYSIS OF THE ACTIVE SITE

Y.Arata, S.Kimura*, H.Matsuo#, and K.Narita*

Department of Chemistry, University of Tokyo, Hongo, Tokyo, *Institute for Protein Research, Osaka University, Suita, Osaka, and #Department of Pharmacology, Osaka University School of Medicine, Osaka, Japan

Received August 27,1976

Summary: Nuclear magnetic resonances of the C-2 protons of the three histidine residues in ribonuclease T_1 have been studied at 360 MHz as a function of pH to discuss the structure of the active site. Comparison of the order of deuterium exchange of the histidine peaks with tritium incorporation rates into individual histidines of the enzyme leads to the unambigous assignment of one of the C-2 proton peaks to histidine-40. It has been concluded that histidine-40 is in the active site, interacting with a charged group of pK 4.1, which is replaced by the phosphate group of guanosine-3'-monophosphate in the enzyme-inhibitor complex. Histidine-92 is most likely a binding site for the complex, where the existence of a hydrogen bond between N-7 of the inhibitor and the ring NH proton of the histidine is suggested on the basis of NMR data.

Ribonuclease T_1(RNase T_1)(EC 2.7.7.26) which is highly specific to the 3'-phosphodiester bridge of a guanosine base in the RNA chain has been extensively studied by Egami and coworkers(1,2). The three histidine residues in RNase T_1 occur at positions 27, 40, and 92 in the amino acid sequence(3). Two of them, His-40 and His-92 along with one glutamic acid(Glu-58) and one arginine(Arg-77) are known to be in or near the active site, participating in either binding or catalytic action of the enzyme(4,5,6). Rüterjans and others have reported nuclear magnetic resonance(NMR) studies of RNase T_1 at 100 MHz(7). However, re solution of the spectra obtained at 100 MHz does not appear to be quite sufficient to warrant a detailed structural investigation of the enzyme. More importantly, assignment of the C-2 proton peaks of the three histidine residues remains to be determined.

In the present work, NMR titration curves for the C-2 protons of the three histidines in RNase T_1 were prepared using NMR spectra measured at 360 MHz. A tritium incorporation technique developed by our group(8,9), by which tritium exchange rates at C-2 positions of individual histidines can be determined, was used along with NMR spectroscopy of histidine-deuterated RNase T_1, and it has been possible to unambigously assign one of the C-2 proton peaks to His-40 which is suggested to be in the active site(5). We will also report NMR titration of the three histidine residues in the presence of guanosine-3'-monophosphate (3'-GMP), a strong competitive inhibitor to RNase T_1. On the basis of the NMR data, the structure of the active site of RNase T_1 will be discussed.

Materials and Methods

RNase T_1 was extracted from Takadiastase and purified by the procedure of Minato and others(10). Phosphate ion contaminant was removed by passing the RNase T_1 preparation through a Sephadex G-25 column(11). 3'-GMP was purchased from Sigma Chemical Co.(Grade I, lot no. 112C-7370).

For NMR measurements, RNase T_1 was dissolved in D_2O(pH 5), heated at 60° for two minutes, and then lyophilized so as to remove labile NH protons. All solutions were made up in 99.8% D_2O to give the final concentration of 5 mM in RNase T_1 and 0.2 M in NaCl. Aliquot amounts of 3'-GMP were added into the corresponding enzyme solution. The pH was adjusted with 1 M DCl or 1 M NaOD. Reported pH values are direct meter readings. NMR spectra were obtained at 360 MHz with a Bruker HX-360 in the FT mode, and at 100 MHz with a JEOL PS-100 in the correlation mode(12). All chemical shifts are given in ppm from external tetramethylsilane(TMS)(10%(w/w) CCl_4 solution), and have not been corrected for bulk magnetic susceptibility.(*)

For tritium exchange at the C-2 position of the histidine residues, RNase T_1(3.2 mg) was incubated at 37° for various periods of time in 0.2 ml buffered tritiated water(4 mCi, pH 8.73, ionic strength 0.2). Three radioactive histidine-peptides were isolated from the trypsin-thermolysin digest of the tritiated RNase T_1 preparations by a peptide-mapping technique, and the specific radioactivity of each peptide was determined in a manner similar to a previous experiment for bovine pancreatic RNase A(9). Locations of three histidine-peptides in RNase T_1 of the known amino acid sequence(3) were assigned by amino acid and N-terminal analyses. The rate of tritium exchange for individual histidines was determined from the specific radioactivity of each histidine residue after tritiation for the times specified as reported previously(8,9).

Deuterium exchange at the C-2 position of the histidine residues was carried out in 99.8% D_2O solution under the same conditions as used for the tritium exchange to afford differentially deuterated RNase T_1, which was submitted to NMR spectroscopy after desalting by passing through a Sephadex G-25 column. It was confirmed that RNase T_1 samples used for NMR measurements restore full enzymatic activity.

Results and Discussion

Figure 1 reproduces several representative proton NMR spectra of the aromatic region of RNase T_1 measured at 360 MHz. The chemical shifts of three peaks, H1, H2, and H3 are plotted in Fig.2 as a function of pH. Tritium exchange at the ring C-2 position of the three histidines of RNase T_1 at pH 8.73 was carried out at 37° for the times specified to determine the rate of tritium exchange for the individual residues. The results clearly reveal that His-40 exchanges most rapidly among the three residues, as shown in half-times observed; $t_{1/2}$ for His-40 = 1.1 days, $t_{1/2}$ for His-27 and -92 = 7.2 days.(**)

(*) Chemical shifts measured from an external TMS solution are dependent on the concentration of the solution, because the bulk magnetic susceptibility of the reference solution is also concentration dependent. Apparent chemical shifts are also dependent on the direction of the static magnetic field with respect to the long axis of the sample tube(13). In the present experiments, an increment of +0.44 ppm was observed when an electromagnetic system(JEOL PS-100) was used instead of a superconducting magnetic spectrometer(Bruker HX-360).

(**) There is little difference in the exchange rate for His-27 and His-92.

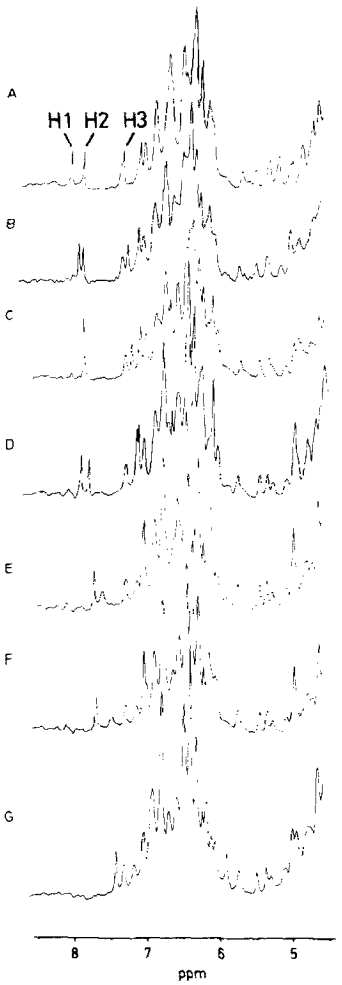

Figure 1. 360 MHz ^{1}H NMR spectra of the aromatic region of RNase T_1 (5mM in 0.2M NaCl/D_2O). (A) pH 2.75, (B) pH 3.80, (C) pH 4.26, (D) pH 5.33, (E) pH 6.91, (F) pH 7.17, (G) pH 8.49. Chemical shifts are in ppm from external tetramethylsilane (10%(w/w) CCl_4 solution).

Differential deuterium exchange of the histidine C-2 protons under the same conditions as used for tritium exchange gives spectra, which are shown in Fig.3, indicating the effect of deuterium exchange on the intensities of the C-2 proton peaks, H1, H2, and H3. Inspection of the spectra clearly shows that H1, H2, and H3 are due to the C-2 protons of the three histidine residues of RNase T_1 and the peak H1 exchanges more rapidly than two other peaks, H2 and H3. Thus, the peak H1 can be assigned to His-40 without ambiguity. The pH dependence of spectra of differentially deuterated RNase T_1 was used to identify the cross-over point in the histidine titration curves.

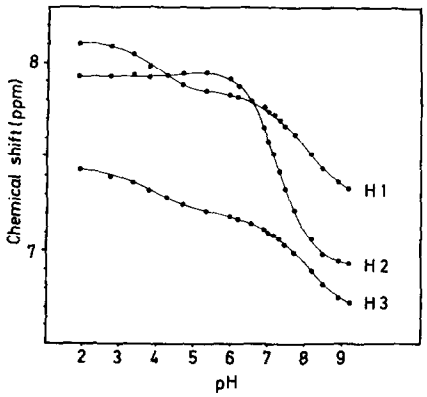

Figure 2. Titration curves of the histidine peaks of RNase T_1 (5mM in 0.2M NaCl/D_2O). Curves H1, H2, and H3 correspond to peaks H1, H2, and H3 in Fig.1.

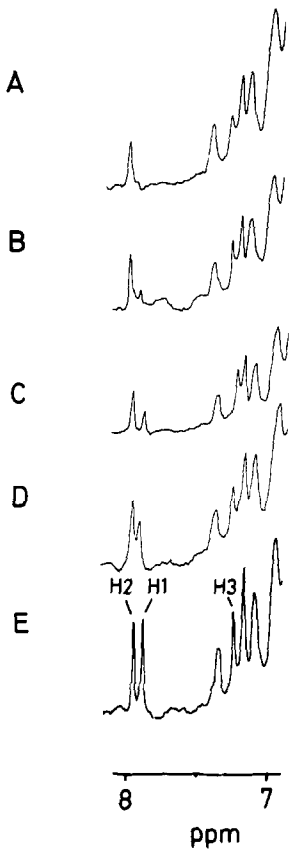

Figure 3. 360 MHz ^1H NMR spectra of the histidine C-2 protons of RNase T_1 (5mM in 0.2M NaCl/D_2O) after RNase T_1 had been deuterated at 37° in D_2O (pH 8.73) for (A) 60 hrs(4.76), (B) 40 hrs(4.87), (C) 20 hrs(4.97), and (D) 10 hrs(4.67); NMR measurement were made at pH's given in parentheses. (E) Spectrum at pH 4.70 of the same region of RNase T_1 which had not been deuterated as mentioned above.

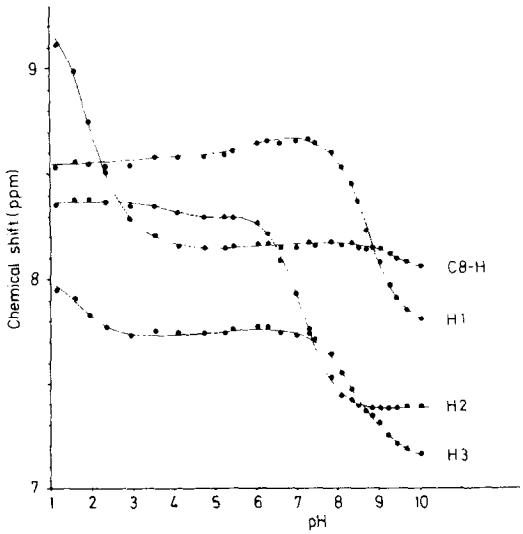

Figure 4. Titration curves of the histidine C-2 and 3'-GMP C-8 proton peaks measured at 100 MHz of RNase T_1 (5mM in 0.2M NaCl/D_2O) in the presence of 20mM of 3'-GMP.

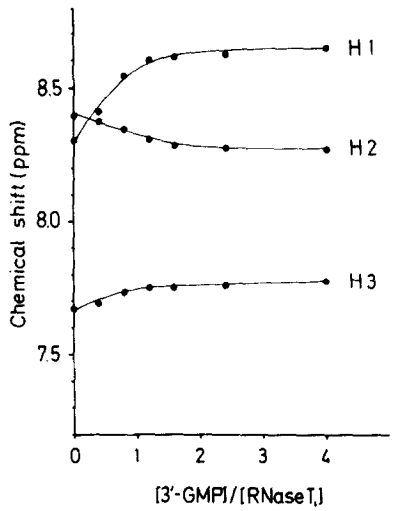

Figure 5. Chemical shifts of the histidine C-2 proton peaks of RNase T_1 (5mM in 0.2M NaCl/D_2O) measured at 100 MHz as a function of total 3'-GMP concentration at pH 6.

As Fig.2 shows, His-40 titrates in two steps. If it is assumed that two charged groups are independently responsible for the His-40 titration, a simple curve fitting procedure provides two pK values, 4.1 and 7.9. The second peak H2 gives a titration curve which is basically of a sigmoidal shape with a pK of

7.2. However, in the pH range 3.5-5.5, a small but significant shift of 0.03 ppm is clearly observed. The titration curve for the peak H3 resembles that for the peak H1 in shape, suggesting that at least two charged groups are involved in the titration of this histidine residue.

Figure 4 shows titration curves for the C-2 protons of the three histidine residues and the C-8 proton of 3'-GMP in an RNase T_1 solution(5 mM) containing 20 mM of 3'-GMP. Correspondence between each of the three histidine C-2 proton peaks in the presence and absence of 3'-GMP is clear from the experiment which is shown in Fig.5, where the ratio of 3'-GMP to RNase T_1 is varied. Among the three peaks, H2 is least influenced by the inhibitor, giving pK's of approximately 7.2 in the presence and absence of 3'-GMP. On the contrary, H1 and H3 are strongly influenced by the inhibitor. These results suggest that the peak H3 is most likely due to His-92, which is known to be in or near the active site along with His-40 giving the peak H1 (4,5,6). Unambigous assignments of H2 and H3 are going on in our laboratory. As shown in Fig.4, the H1 (His-40) peak shows very little change in chemical shift between pH 1 and 5 in the presence of 3'-GMP. However, a small but significant downfield shift of 0.09 ppm occurs around pH 6, which is followed by a large upfield shift of about 0.8 ppm with an inflection point at pH 8.6. With an increase in pH, the chemical shifts of the H3 peak stay almost constant between pH 3 and 7, followed by a large upfield shift of about 0.6 ppm with an inflection point at pH 8.5.

On the basis of the above experimental findings, it may be concluded that in the absence of the inhibitor, His-40 is interacting with a charged group with pK 4.1, which is presumably one of carboxyl groups of the enzyme. A most probable candidate for this group is Glu-58 which is known to be in the active site(4). It appears that in the enzyme-inhibitor complex, His-40 which is released from this carboxyl group is interacting with the phosphate group of the inhibitor. The titration curves for H1 and H3 histidines in the complex clearly indicate that these two histidines are both fully protonated even if the pH is increased to 7, where the binding constant of 3'-GMP is known to fall off (14). This result shows that a charged group other than these two histidines is primarily responsible for the binding of 3'-GMP to RNase T_1 at high pH. Sato and Egami first pointed out that the monoanionic form of 3'-GMP binds more strongly than the dianionic form (15). Epinatjeff and Pongs have reached the same conclusion from the pH dependence of ultraviolet difference spectra at 290 nm, and estimated a pK of 6.5 for the phosphate group (16). A possible explanation for the decreased stability for the dianionic form is that deprotonation $PO_4H^- \longrightarrow PO_4^{2-}$ would result in repulsion between the phosphate dianion and the COO^- group which is interacting with His-40 in the absence of 3'-GMP.

It should be noted that the H3 titration curve shows a large downfield shift below pH 3 with an apparent pK of about 2. As Fig.4 shows, a similar downfield shift is also observed for the C-8 proton of 3'-GMP. In a free 3'-GMP solution, protonation at N-7 is known to cause a large downfield shift of the C-8 proton; a pK of 2.2-2.4 has been reported for the N-7 group(17,18,19). At pH 3, H1 as well as H2 give almost identical chemical shifts in the presence and absence of the inhibitor. On the contrary, in the case of the H3 peak, there is still a significant difference of about 0.1 ppm at pH 3, and this difference becomes small when the pH is further decreased. These results suggest that in the enzyme-inhibitor complex, a hydrogen bond formed between the NH proton of the H3 histidine (most likely His-92) and N-7 of 3'-GMP, and that below pH 2 protonation at the N-7 position causes breakage of the hydrogen bond, resulting in dissociation of the inhibitor.

On the basis of ultraviolet difference data at 290 nm, Oshima and Imahori (20) and Epinatjeff and Pongs (16) have concluded that N-7 of 3'-GMP is protonated in the enzyme-inhibitor complex even at neutral pH, and coulombic interaction between 3'-GMP thus protonated and a negatively charged group on the enzyme is involved in the binding. The present experiment is consistent with their conclusion in that there is interaction between N-7 of 3'-GMP and the enzyme. However, the chemical shift differences between the C-8 proton of 3'-GMP (20 mM in 0.2 M NaCl/D_2O) in the presence(5 mM) and absence of RNase T_1 were confirmed to be smaller than 0.05 ppm throughout the entire pH range examined in the present experiment; in both cases a downfield shift of approximately 1.1 ppm is observed when the pH is decreased from 7 to 1. With the 3'-GMP: enzyme concentration ratio of 4 : 1 used in the present experiment, RNase T_1 is saturated with 3'-GMP in the pH range 4-6 (14). Therefore, if 3'-GMP is actually protonated in the complex, the C-8 proton of the inhibitor should give in the pH range 4-6 a chemical shift which is lower by about 0.3 ppm than that observed in the present experiment. Consequently, it is quite unlikely that 3'-GMP is protonated in the enzyme-inhibitor complex. However, a further downfield shift observed for H3 below pH 3 suggests that above pH 3, cationic character of the imidazole ring of the H3 histidine is somewhat reduced, presumably because of the formation of the hydrogen bond.

Acknowledgment

NMR measurements at 360 MHz have been made at Stanford Magnetic Resonance Laboratory. One of the authors (YA) wishes to thank Professor Oleg Jardetzky for his hospitality while YA was staying at Stanford.

References

1. Uchida,T., and Egami,F. (1971) In: The Enzymes, Vol.IV, ed. Boyer,P.D. pp.205-250, Academic Press, New York.
2. Takahashi,K. (1974) Yukigoseikagaku 32, 298-316.
3. Takahashi,K. (1971) J.Biochem.(Tokyo) 70, 946-960
4. Takahashi,K., Stein,W.H., and Moore,S. (1967) J.Biol.Chem. 242, 4682-4690.
5. Takahashi,K. (1973) J.Biochem.(Tokyo) 74, 1279-1282.
6. Takahashi,K. (1970) J.Biochem.(Tokyo) 68, 659-664.
7. RÜterjans,H., Witzel,H., and Pongs,O. (1969) Biochem.Biophys.Res.Commun. 37, 247-253; RÜterjans,H., and Pongs,O. (1971) Eur.J.Biochem. 18, 313-318.
8. Matsuo,H., Ohe,M., Sakiyama,F., and Narita,K. (1972) J.Biochem.(Tokyo) 72, 1057-1060.
9. Ohe,M., Matsuo,H., Sakiyama,F., and Narita,K. (1974) J.Biochem.(Tokyo) 75, 1197-1200.
10. Minato,S., Tagawa,T., and Nakanishi,K. (1966) J.Biochem.(Tokyo) 59, 443-448.
11. Cohen,J., Griffin,J.H., and Schechter,A.N. (1973) J.Biol.Chem. 248, 4305-4310.
12. Arata,Y., and Ozawa,H. (1976) J.Magn.Resonance 21, 67-76.
13. Live,D.H., and Chan,S.I. (1970) Anal.Chem. 42, 791-792.
14. Takahashi,K. (1972) J.Biochem.(Tokyo) 72, 1469-1481.
15. Sato,S., and Egami,F. (1965) Biochem.Z. 342, 437-448.
16. Epinatjeff,C., and Pongs,O. (1972) Eur.J.Biochem. 26, 434-441.
17. Miles,H.T., Howard,F.B., and Frazier,J. (1963) Science 142, 1458-1463.
18. Jardetzky,C.D., and Jardetzky,O. (1960) J.Am.Chem.Soc. 82, 222-229.
19. Kochetkov,N.K., and Butovskii,E.I. (1971) Organic Chemistry of Nucleic Acids, Part A, pp.149-152, Plenum Press, London.
20. Oshima,T., and Imahori,K. (1971) J.Biochem.(Tokyo) 69, 987-990.