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Proton and Phosphorus Nuclear Magnetic Resonance Studies of Ribonuclease T_1†

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ABSTRACT: ^1H and ^{31}P nuclear magnetic resonance (NMR) studies of ribonuclease (RNase) T_1 are reported. Assignments of the C2-H proton resonances of the three histidine residues were made using a tritium labeling technique which is a combination of differential tritium exchange at the C2-H position of histidine [Matsuo, H., Ohe, M., Sakiyama, F., & Narita, K. (1972) *J. Biochem. (Tokyo) 72*, 1057; Ohe, M., Matsuo, H., Sakiyama, F., & Narita, K. (1974) *J. Biochem. (Tokyo) 75*, 1197] and ^1H NMR of a differentially deuterated protein. ^1H NMR data taken in the absence and presence of guanosine 3′-monophosphate (3′-GMP), a strong competitive inhibitor to the enzyme, were used along with ^{31}P NMR spectra of the inhibitor to provide information on the structure of the active site of the enzyme. It was concluded that histidine-40 along with a carboxyl group which is probably that of glutamic acid-58 is responsible for the catalytic action of the enzyme. The structure of the active site of RNase T_1 is in a marked contrast with that of RNase A where two histidine residues are known to act as a general acid and general base to conduct the catalytic action. Interaction involving histidine-92 and N-7 of 3′-GMP through a hydrogen bond is most likely responsible for the enzyme–inhibitor binding. A scheme of the active site and of the interaction of the enzyme with 3′-GMP is presented on the basis of the present experimental results.

Ribonuclease T_1 (RNase T_1;[1] EC 2.7.7.26) is highly specific to the 3′-phosphodiester bridge of a guanosine base in the RNA chain (Uchida & Egami, 1971; Takahashi, 1974). In RNase T_1, which is composed of 104 amino acid residues with the two disulfide bridges, the three histidine RNase occur at positions 27, 40, and 92 (Takahashi, 1971). Two of them, His-40 and His-92, along with one glutamic acid (Glu-58) and one arginine (Arg-77), have been suggested to be in or near the active site, participating in either binding or catalytic action of the enzyme (Takahashi et al., 1967; Takahashi, 1970, 1973).

It has been well established that ^1H NMR peaks of histidines give invaluable information about the structure of proteins in solution once each individual resonance can be assigned to a particular histidine residue in the amino acid sequence (Roberts & Jardetzky, 1970; Markley, 1975a). A first NMR study of RNase T_1 was reported by Ruterjans and

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[1] Abbreviations used: RNase, ribonuclease; GMP, guanosine monophosphate; CMP, cytosine monophosphate; NMR, nuclear magnetic resonance; FT, Fourier transform; Me_4Si, tetramethylsilane; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

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co-workers (1969, 1971), who observed at 100 MHz the C2-H proton peaks of the three histidine residues. They concluded that two histidine residues (which they designated as His-A and His-C) are in the active site and suggested that in the complex of the enzyme with guanosine 3′-monophosphate (3′-GMP), a strong competitive inhibitor, His-A and His-C interact with the phosphate and guanine part of the inhibitor, respectively. Walz & Terenna (1976) and Walz (1976, 1977a,b) measured ultraviolet difference spectra of RNase T_1 in the presence of inhibitors and substrate analogues and used these data along with the NMR data reported by Ruterjans and co-workers (1969, 1971) to discuss the binding of the inhibitors and substrate analogues with RNase T_1. However, assignments of the histidine C2-H proton resonances have not been made, and therefore any definite conclusion has not yet been drawn as to the role of each of these active site histidines.

It has been shown that a tritium labeling technique which is a combination of differential tritium exchange at the C2-H position of histidines (Matsuo et al., 1972; Ohe et al., 1974) and ^{1}H NMR of differentially deuterated proteins can be a general method for the assignment of the histidine NMR peaks (Markley, 1975b; Arata et al., 1976; Shindo et al., 1976). In a previous paper, we have briefly described a ^{1}H NMR study of RNase T_1 which is based on the assignment of the C2-H proton resonance of His-40 by the tritium labeling technique (Arata et al., 1976).

In the present paper, complete assignments using the tritium labeling technique of the three histidine C2-H proton peaks of RNase T_1 will be reported in detail. ^{1}H and ^{31}P NMR data will be used along with the pH profile of the binding constant of 3′-GMP to the enzyme to discuss the structure of the active site of RNase T_1.

Materials and Methods

Materials. RNase T_1 was extracted from Takadiastase and purified by the procedure of Minato et al. (1966). Phosphate contaminant was removed by passing the RNase T_1 preparations through a Sephadex G-25 column (Cohen et al., 1973). 3′-GMP was purchased from Sigma Chemical Co. (Grade I, lot no. 112C-7370).

NMR Measurements. RNase T_1 was dissolved in D_2O at pH 5, heated at 60 °C for 2 min, and then lyophilized in order to replace labile NH protons with deuterium. 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. The pH was adjusted with 1 M DCl or NaOD; reported pH values are uncorrected meter readings of D_2O solutions made with glass electrodes standardized using H_2O buffers. Proton NMR spectra were obtained at 360 MHz with a Bruker HXS-360 spectrometer in the pulse FT and correlation mode and at 100 MHz with a JEOL PS-100 spectrometer in the correlation mode (Arata & Ozawa, 1976). ^{31}P NMR spectra were observed at 40.5 MHz using a Varian XL-100 spectrometer in the pulse FT mode. Typically 100–1000 transients were accumulated for proton and 3000 transients for phosphorus NMR. Unless otherwise stated, ^{1}H chemical shifts are given in parts per million from external Me_4Si [10% (w/w) CCl_4 solution] and have not been corrected for bulk magnetic susceptibility.[2] ^{31}P chemical shifts are given in parts per million from external H_3PO_4 (85%). All NMR spectra presented in this paper were taken at 32 °C.

[2] In the present experiments, an increment of +0.44 ppm has to be added to the chemical shifts taken with a superconducting magnet (Bruker HXS-360) in order to compare them with those observed with an electromagnet (JEOL PS-100).

Differential Deuterium and Tritium Labeling of RNase T_1. RNase T_1 (3.2 mg) was incubated at 37 °C for various periods of time in 0.2 mL of buffered tritiated water (4 mCi, pH 8.73, ionic strength 0.2) by a method reported previously (Matsuo et al., 1972; Ohe et al., 1974) to afford differentially tritiated RNase T_1 preparations in which each histidine residue is specifically tritiated at the C2-H position. Histidine peptides I, II, and III were isolated from the trypsin–thermolysin digest

peptide I, Val-Ile-Thr-His^{92}-Thr-Gly
Val-Ile-Thr-His^{92}-Thr
peptide II, Val-Gly-Ser-Asn-Ser-Tyr-Pro-His^{40}-Lys
peptide III, Leu-His^{27}-Glu-Asp-Gly-Glu-Thr

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 experiments for bovine pancreatic ribonuclease (RNase A) reported previously (Ohe et al., 1974). Locations of the histidine peptides in RNase T_1 of the known amino acid sequence (Takahashi, 1971) were assigned by amino acid and N-terminal analysis. The rate of tritium exchange for individual histidines was determined from the specific radioactivity of each histidine residue (Kimura, 1977). Details of the experiments of tritium exchange for RNase T_1 will be given elsewhere.

Deuterium exchange at the C2-H position of the histidine residues was carried out in 99.8% D_2O solution under the same condition as used for the tritium exchange, and differentially deuterated RNase T_1 obtained was used for NMR measurements after desalting by passing it through a Sephadex G-25 column.

The Binding Constant of 3′-GMP to RNase T_1. The binding constant of 3′-GMP to RNase T_1 was determined by a gel filtration method (Hummel & Dreyer, 1962); the concentration of 3′-GMP was determined using an extinction coefficient of 7.75×10^3 at 380 nm.

Stability of RNase T_1. The enzyme assay was performed according to Takahashi's method (Takahashi, 1962). It was confirmed that the brief exposure to 60 °C involved in the exchange procedure for NMR measurements to remove the slowly exchanging NH protons had no effect on the enzyme activity. It was also confirmed that RNase T_1 is stable at 37 °C in the pH range 1–9 under the present experimental conditions, and the samples used for NMR measurements restore full enzymatic activity after the measurements. Above pH 9, the enzyme becomes less stable. For example, the enzyme kept at pH 9.5 for 30 min (enzyme concentration 1 mM, ionic strength 0.2, 37 °C) loses about 10% of its original activity. Therefore, results obtained above pH 9 have to be interpreted with care.

Results

Examples of ^{1}H NMR spectra observed at 360 MHz were given in our previous communication (Arata et al., 1976). Three peaks, H1, H2, and H3, which titrate with pH are observed in the low-field region. The observed chemical shifts of the three peaks are plotted in Figure 1a as a function of pH. As described below, all of these peaks lose their intensity upon incubating in D_2O at 37 °C and pH 8.37. These results indicate that the three peaks are due to the C2-H proton of the three histidine residues of RNase T_1. It is well established that under the present conditions histidine C4-H protons do not incorporate deuterium from the solvent D_2O. Another evidence has been reported by Kawano et al. (1978), who using difference spectra were able to follow the titration curves of all C2-H proton peaks of the histidines in RNase T_1; the C4-H proton resonances were at higher field as expected.

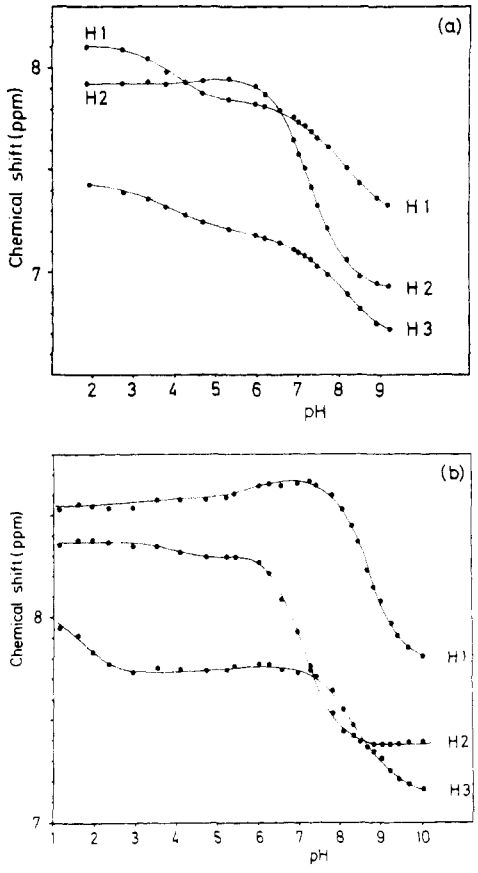

FIGURE 1: (a) Titration curves of the histidine C2-H proton peaks observed at 360 MHz of RNase T_1 (5 mM in 0.2 M NaCl/D_2O, 32 °C). (b) Titration curves of the histidine C2-H proton peaks observed at 100 MHz of RNase T_1 (5 mM) in the presence of 3′-GMP (20 mM) in 0.2 M NaCl/D_2O, 32 °C.

Differential deuterium exchange of the histidine C2-H protons was performed at 37 °C and pH 8.37 [see Figure 3 of our previous paper (Arata et al., 1976)]. Upon incubation in D_2O, all three peaks, H1, H2, and H3, lose their intensity at different rates. Of the three peaks, the peak H1 loses its intensity much more rapidly than the peaks H2 and H3. Half-time for peak H1 is approximately 20 h. However, at the pH used there is very little difference in the rate of the differential exchange for the H2 and H3 protons. The pH dependence of spectra of differentially deuterated RNase T_1 was also used to identify the crossover points in the histidine titration curves given in Figure 1a. The half-time at 37 °C and pH 8.37 for tritium exchange at the C2-H position is 1.1 ± 0.1 days for His-40, whereas those for His-27 and His-92 are quite similar and equal to 7.2 ± 0.5 days (Kimura, 1977). These results can be used along with the spectra of differentially deuterated RNase T_1 to unambiguously assign the H1 peak to His-40 (Arata et al., 1976).

As Figure 1b shows, the H1 and H3 titration curves for the native RNase T_1 are greatly influenced by the addition of 3′-GMP, which is a strong competitive inhibitor to the enzyme. The pK_a values for H1 and H3 peaks are shifted from 7.9 and ~8 in the native enzyme to 8.6 and 8.5 in the presence of 3′-GMP, respectively. The H2 titration curve is affected to a much less extent. The tritium-exchange experiments in the absence (presence) of 3′-GMP gave pK_a values (0.2 M NaCl, 32 °C) for the three histidine residues: His-27, 7.3 (7.5); His-40, 7.7 (8.5); His-92, 7.5 (8.3) (Kimura, 1977). This result clearly indicates that the two histidine residues which are strongly influenced by 3′-GMP are His-40 and His-92. From these results, the H3 peak can be assigned to His-92,

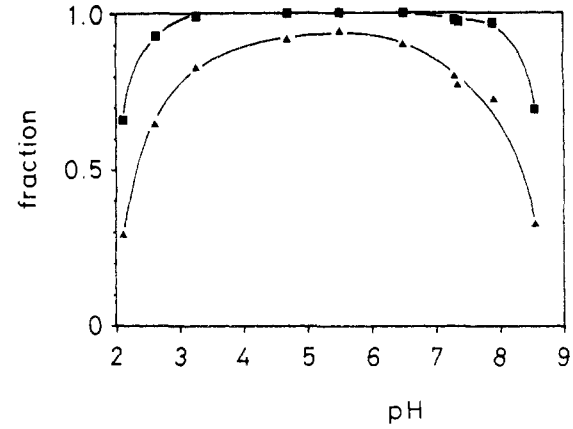

FIGURE 2: The pH dependence of the fraction of RNase T_1 saturated with 3′-GMP at RNase T_1:3′-GMP concentration ratios of 1:1 (▲—▲) and 1:4 (■—■). RNase T_1: 5 mM in 0.2 M NaCl/H_2O. Temperature: 32 °C.

and therefore the H2 peak to His-27. In summary, established assignments for the three histidine C2-H proton peaks of RNase T_1 are as follows:

H1 → His-40
H2 → His-27
H3 → His-92

The dissociation constant K_i for the RNase T_1–3′-GMP complex was determined by a gel filtration method (Hummel & Dreyer, 1962); K_i (mM) obtained at pH values indicated in the parentheses are 8.6 (2.11), 1.0 (2.60), 0.17 (3.23), 0.036 (4.68), 0.017 (5.48), 0.056 (6.47), 0.25 (7.29), 0.34 (7.36), 0.55 (7.90), >7 (8.55). On the basis of the results obtained, the degree of saturation of RNase T_1 with the inhibitor for RNase T_1:3′-GMP ratios of 1:1 and 1:4 is calculated; the results are plotted in Figure 2 as a function of pH.

Figure 1b shows titration curves for the C2-H protons of the three histidine residues in an RNase T_1 solution (5 mM) containing 20 mM 3′-GMP. Correspondence between each of the three histidine C2-H proton peaks in the presence and absence of 3′-GMP was made clear by our previous experiment (Arata et al., 1976) where the ratio of 3′-GMP to RNase T_1 is varied. As shown in Figure 1b, the His-40 peak shows very little change in chemical shift between pH 1 and 5 in the presence of the inhibitor. With an increase in pH, 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. The chemical shifts of the His-92 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. It should also be noted that the His-92 titration curve shows a large downfield shift below pH 3 with an apparent pK_a of about 2.4.

The titration curves of the C8-H proton of 3′-GMP in the presence and absence of RNase T_1 are compared in Figure 3, where the RNase T_1:3′-GMP ratio was 1:4.

Figure 4 shows the low-field region between 9 and 11 ppm of ^1H NMR spectra of RNase T_1 which is dissolved in H_2O (0.2 M NaCl). Titration curves for the peaks observed in H_2O in the absence and presence of the inhibitor are given in Figure 5. A peak marked by a star can be observed only in the presence of the inhibitor. It should be noted that the pH profile of the chemical shifts of this peak (titration curve c in Figure 5b) is quite similar in shape to that of the fraction of RNase T_1 saturated with 3′-GMP at the same enzyme:inhibitor concentration ratio (see Figure 2); the chemical shifts stay constant between pH 3 and 8 and exhibit an upfield shift at

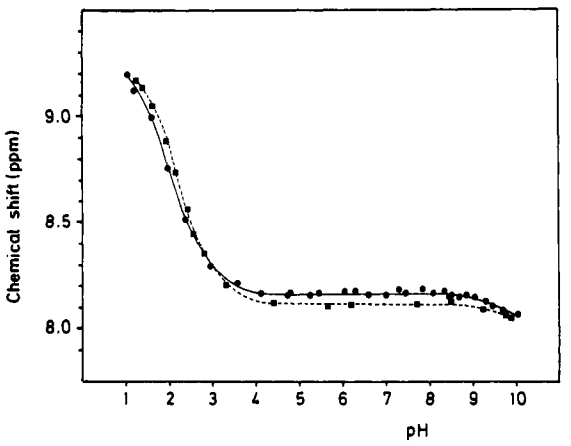

FIGURE 3: Titration curves of the C8-H proton of 3′-GMP in the absence (----) and presence (—) of RNase T_1. Concentrations of RNase T_1 and 3′-GMP are 5 and 20 mM in 0.2 M NaCl/D_2O, respectively. Temperature: 32 °C.

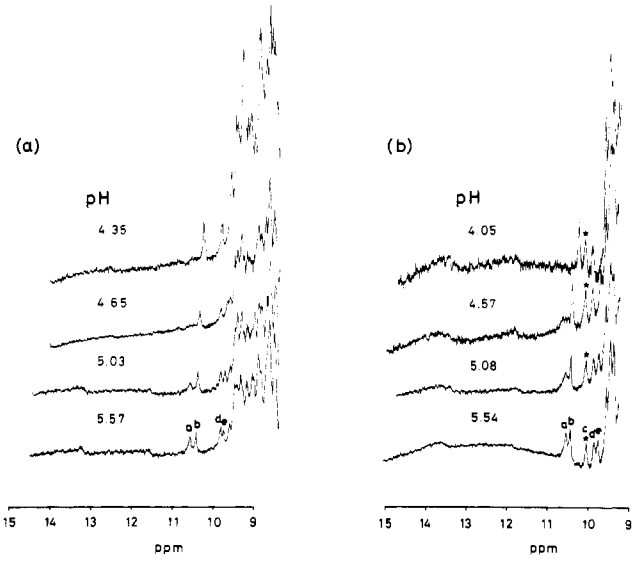

FIGURE 4: ^{1}H NMR spectra between 9 and 11 ppm of RNase T_1 in the absence (a) and presence (b) of 3′-GMP. Concentrations of RNase T_1 and 3′-GMP are 5 and 20 mM in 0.2 M NaCl/H_2O, respectively. Temperature: 32 °C. Chemical shifts are in parts per million from external DSS (5% in H_2O).

high and low pH with a concomitant loss of intensity.

In Figure 6, ^{31}P NMR spectra of 3′-GMP observed in the presence of RNase T_1 are given where the enzyme:inhibitor ratio is 1:1. The titration curves of the ^{31}P NMR peak of 3′-GMP in the absence and presence of the enzyme are given in Figure 7. The titration curve shown in Figure 7 can be used to determine a pK_a value of 6.5 for the second dissociation of the phosphate group of 3′-GMP in the enzyme–inhibitor complex (0.2 M NaCl, 32 °C). The pK_a value obtained is much larger than that of 3′-GMP in the absence of the enzyme. It should also be noted that in the presence of the enzyme there are two inflections observed at pH ~3 and ~8 in the titration curve.

Discussion

In making assignments of NMR peaks, proteins have to be more or less modified. Obviously, more reliable results can be expected when the proteins are less perturbed by this modification. It has been shown that, under the experimental conditions used in the present experiments, the isotope effect in the rate of incorporation of deuterium and tritium at the C2-H position and the effect of back-exchange in finally

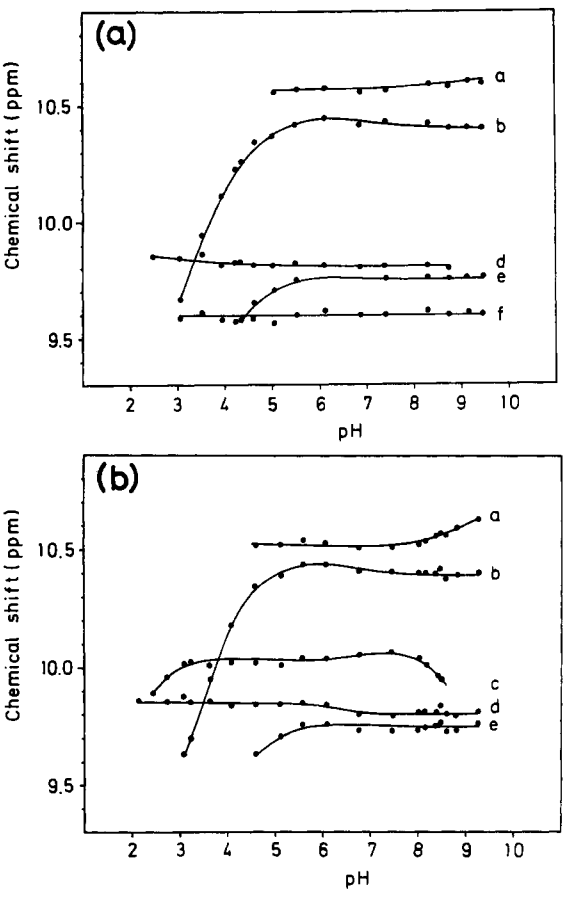

FIGURE 5: Titration curves of the low-field peaks of RNase T_1 (Figure 4) in the absence (a) and presence (b) of 3′-GMP.

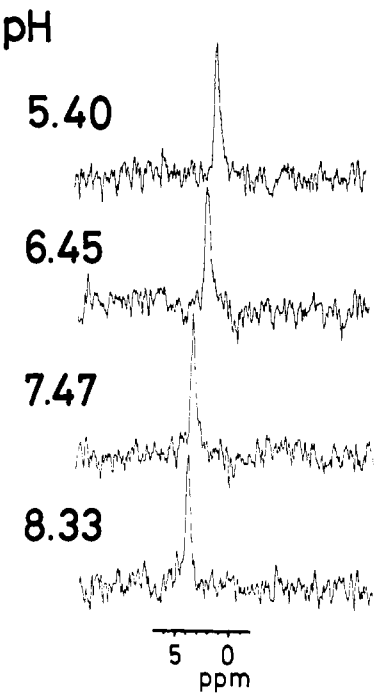

FIGURE 6: ^{31}P NMR spectra of 3′-GMP observed in the presence of RNase T_1. Concentrations of RNase T_1 and 3′-GMP are both 5 mM in 0.2 M NaCl/D_2O. Spectra were taken at 32 °C. Chemical shifts are in parts per million from external 85% H_3PO_4.

separated tritiated peptides are sufficiently small and do not affect any of the results of the assignments described in the present paper (Kangawa et al., manuscript in preparation). Quite recently, Fulling & Ruterjans (1978) have reported the

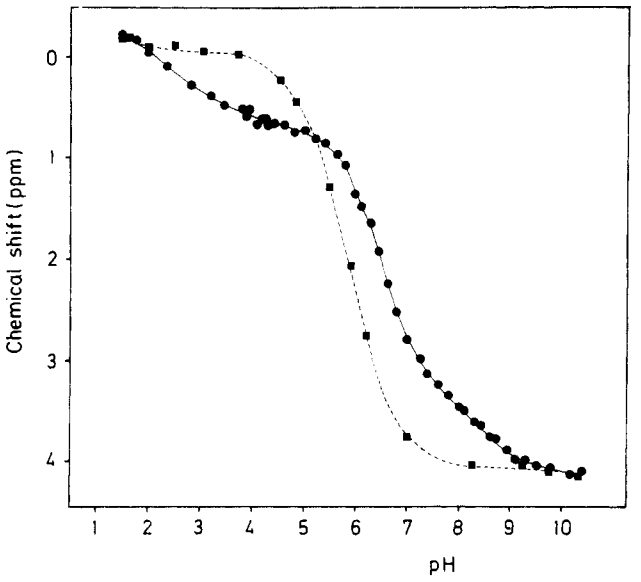

FIGURE 7: Titration curves of ^{31}P NMR signals of 3′-GMP in the absence (----) and presence (—) of RNase T_1.

assignment by photooxidation of the His-27 resonance of RNase T_1. Although the perturbation induced by the photooxidation may not be small enough, the assignment they reported is consistent with that described in the present study.

As Figure 1a shows, His-40 titrates in two steps with pK_a values 4.1 and 7.9. The titration curve of His-92 closely resembles that of His-40. These results suggest that each of these two histidine residues is interacting with at least one charged group of the enzyme. The His-27 titration curve is basically of a sigmoidal shape with a pK_a of 7.2. In the presence of 3′-GMP, His-40 and His-92 peaks are strongly influenced, but the His-27 titration curve is affected to a much less extent (see Figure 1a,b). This result is consistent with a conclusion reached by Takahashi (1973), who has demonstrated that among the three histidines His-40 and His-92 are located in the active site of the enzyme.

The inflection observed at pH 4.1 in the His-40 titration curve for the native RNase T_1 vanishes in the presence of 3′-GMP, and with an increase in pH, a small but significant downfield shift of 0.09 ppm occurs around pH 6. In the presence of the inhibitor, the His-92 titration curve becomes stationary between pH 3 and 7, but even at pH 3 the chemical shift is quite different from that observed in the absence of the inhibitor. Below pH 3, the His-92 chemical shift exhibits a further downfield shift with an apparent pK_a of 2.4. In contrast to this, His-40 as well as His-27 gives almost identical chemical shifts at pH 3 in the presence and absence of the inhibitor (see Figure 1a,b).[2]

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 a pK_a of 4.1, which is presumably one of the carboxyl groups of the enzyme. A most probable candidate for this group is Glu-58, which exhibits a unique reactivity and has been suggested to be in the active site of the enzyme (Takahashi et al., 1967). A small change in the chemical shift of the C2-H proton of His-40 between pH 6 and 7 seems to reflect the second dissociation of the phosphate group of 3′-GMP. In the enzyme–inhibitor complex, it appears that His-40 which is released from the carboxyl group mentioned above is interacting with the phosphate group of the inhibitor. The ^{31}P NMR titration curve for 3′-GMP observed in the presence of RNase T_1 gives a pK_a of 6.5 for the second dissociation of the phosphate group of the inhibitor (see Figure 7). It should be noted that in the ^{31}P NMR titration curve observed in the presence of the enzyme there are two additional inflections at pH ~3 and ~8. This observation is consistent with the above model of the enzyme–inhibitor complex in which the phosphate group is interacting with His-40 which has a pK_a of 8.6 in the complex. Above pH ~8 and below pH ~3, where extensive dissociation of the complex occurs, the phosphate group is released from the enzyme, resulting in a further shift of the ^{31}P resonance, and the ^{31}P chemical shift eventually approaches that observed in the absence of the enzyme.

It should be noted that in the presence of 3′-GMP the chemical shift of the His-40 peak shows very little change in the pH range 1–5. As indicated in Figure 2, the enzyme–inhibitor complex dissociates significantly between pH 1 and 3. This suggests that the interaction between His-40 and the inhibitor is weak, and, therefore, His-40 cannot be a major binding site for the RNase T_1–3′-GMP complex.

The above results also indicate that the positive charge at the imidazole ring of His-92 is somewhat reduced above pH 3 even if protonation occurs with pK_a = 8.5. This suggests that the imidazole group is strongly interacting with a negatively charged group in the enzyme–inhibitor complex. It should be noted that the low-field shift observed below pH 3 in the presence of the inhibitor can also be observed when 3′-GMP is replaced by guanosine (Ruterjans et al., 1969). This result suggests that the phosphate group is not primarily responsible for the interaction of 3′-GMP with His-92. It is well established that protonation at N-7 is responsible for the large shift observed for the C8-H proton signal of 3′-GMP (Jardetzky & Jardetzky, 1960); a pK_a of 2.2–2.4 has been reported for the N-7 group (Miles et al., 1963). These results suggest that in the enzyme–inhibitor complex a hydrogen bond is formed between the NH proton of 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 & Imahori (1971) and Epinatjeff & Pongs (1972) 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. In Figure 3, the chemical shifts of the C8-H proton of 3′-GMP in the absence of the enzyme are compared with those observed in the presence of the enzyme with the inhibitor:enzyme ratio 4:1. In the pH range 4–8, where the enzyme is saturated with 3′-GMP more than 95%, the C8-H proton exhibits a downfield shift of 0.05 ppm. Under the present conditions, the ratio of the free and bound inhibitor is 3:1. This means that the C8-H proton of 3′-GMP bound with RNase T_1 has an intrinsic downfield shift of 0.20 ppm in the pH range 4–8. It should be noted that a downfield shift of 1.1 ppm is observed for the free 3′-GMP when the pH is decreased from 7 to 1. The intrinsic downfield shift of 0.20 ppm observed at neutral pH for 3′-GMP bound to the enzyme is much smaller than the downfield shift of 1.1 ppm, which reflects the protonation at the N-7 position of the inhibitor. Consequently, it is quite unlikely that 3′-GMP is protonated in the enzyme–inhibitor complex at neutral pH. However, the existence of the significant difference of 0.20 ppm in chemical shift in the absence and presence of the enzyme and a further downfield shift observed for His-92 below pH 3 indicate that above pH 3 there

is interaction involving His-92 in the enzyme–inhibitor complex, and therefore cationic character of the imidazole of His-92 in the complex is somewhat reduced, presumably because of the formation of the hydrogen bond. These results strongly suggest that the interaction between His-92 and N-7 of 3′-GMP is primarily responsible for the enzyme–inhibitor binding. However, there is also a possibility that interaction between His-92 and some other group of the inhibitor is primarily responsible for the binding itself. As mentioned above, it is quite certain that N-7 of the inhibitor is also involved in the interaction of the enzyme with the inhibitor. In this case, the dissociation of the complex would also result in the breakage of the His-92 and 3′-GMP interaction, giving rise to a downfield shift observed in the His-92 titration curve. These results are consistent with a conclusion of inhibitor binding studies by Takahashi (1972), who showed that the trivalent nitrogen at the 7 position in the guanine portion is essentially required for the specific enzyme–inhibitor binding.

RNase T_1 in H_2O gives between 9 and 11 ppm signals due to exchangeable protons (Figure 4). It should be noted that a signal marked by a star can only be observed in the presence of the inhibitor. This signal broadens and disappears above pH 8.5 and below pH 2.5. It should also be noted that the pH profile of the chemical shift of this peak is quite similar to that of the degree of saturation of the enzyme with the inhibitor (see Figure 2). These results strongly suggest that this signal is due to a group which is responsible for the enzyme–inhibitor binding. From the chemical shift, this signal appears to be due to the N-1 proton of the inhibitor which in the enzyme–inhibitor complex would have to be strongly bound by a hydrogen bond with the enzyme. The N-7 proton bound with His-92 would give a signal at much lower field (Patel et al., 1975). In Figures 4 and 5, the chemical shifts and line widths of peaks a and b also exhibit an interesting pH dependence. This point will be discussed in detail elsewhere.

The ^{31}P NMR titration curve for 3′-GMP observed in the presence of RNase T_1 gives a pK_a of 6.5 for the second dissociation of the phosphate group of the inhibitor (Figure 7). This means that at pH 5.5, where 3′-GMP most strongly binds to RNase T_1 under the conditions used, the phosphate group of the inhibitor exists as the monoanion. This result is consistent with that reported by Sato & Egami (1965), who pointed out that the monoanionic form of 3′-GMP binds to RNase T_1 more strongly than the dianionic form. Epinatjeff & Pongs (1972) have reached the same conclusion from the pH dependence of ultraviolet difference spectra at 290 nm and estimated a pK_a of 6.5 for the phosphate group. A possible explanation for the decreased stability of the dianionic form is that deprotonation $PO_4H^- \rightarrow PO_4^{2-}$ would result in repulsion between the phosphate dianion and the COO^- group which is interacting with His-40 in the absence of the inhibitor.

Figure 2 shows that a sharp decrease in the binding constant occurs above pH 8 and below pH 2. As suggested previously, the decrease at high pH is most likely induced by deprotonation at His-92, which results in the breakage of the His-92 and N-7 of 3′-GMP interaction. Below pH 2.5, as shown in Figure 3, protonation occurs at N-7 of the inhibitor. This appears to be a very likely reason why the binding constant sharply decreases at low pH.

Of the two active site histidines, His-40 and His-92, His-40 incorporates tritium much faster than His-92. Relationship between the rate of incorporation of tritium at the C2-H position of a histidine residue in proteins and solvent accessibility of the amino acid residue will be discussed elsewhere in detail (Minamino, N., Matsuo, H., & Narita, K., manuscript in preparation). In view of these results, it is suggested that His-40 is exposed to the solvent, whereas His-92 is buried at the bottom of the active site. On the basis of the above experimental findings, a model for the structure of the active site of RNase T_1 is illustrated in Figure 8. In the active site, His-40 is interacting with a charged group with a pK_a of 4.1, which is most probably Glu-58 that is known to be in the active site (Takahashi, 1973); i.e., His-40 along with Glu-58 is responsible for the catalytic action of the enzyme. In the enzyme–inhibitor complex, His-40 which is released from the carboxyl group is interacting with the phosphate group of the inhibitor. His-92 acts as a binding site which recognizes with high specificity the guanine base. The His-92 and N-7 of 3′-GMP interaction is quite likely responsible primarily for the binding. Below pH 2 protonation at N-7 of 3′-GMP and above pH 8 deprotonation at His-92 imidazole ring cause dissociation of the enzyme–inhibitor complex. The pK_as of His-40 and His-92 in the complex are quite similar, suggesting that with the breakage of His-92 and N-7 of 3′-GMP interaction, His-40 is also released from the enzyme and deprotonated in a concerted manner.

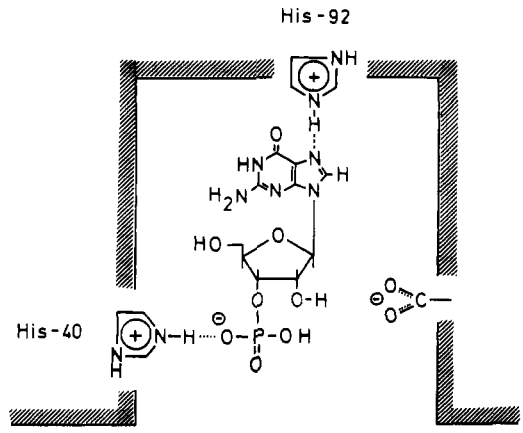

FIGURE 8: A proposed model of the RNase T_1–3′-GMP complex.

As shown in Figures 4 and 5, the enzyme–inhibitor complex in H_2O gives in the low-field region a signal which disappears as the complex dissociates at low and high pH. This signal appears to be due to the N-1 proton of 3′-GMP which is hydrogen bonded to the enzyme. These results are consistent with a conclusion reached by Takahashi (1972), who suggested on the basis of studies of binding of a variety of inhibitors to RNase T_1 that N-1 and N-7 along with 2-amino and 6-oxo groups are essential for the enzyme–inhibitor binding.

It may be interesting to compare the results obtained here with those for RNase A where the phosphate of cytosine 3′-monophosphate (3′-CMP), a typical inhibitor to the enzyme, is more stable as the dianion. Meadows & Jardetzky (1968) have shown that at low pH dissociation of the RNase A–3′-CMP complex occurs upon protonation of the phosphate group of 3′-CMP. At high pH deprotonation at His-12 has been suggested to be responsible for the dissociation. It is well established that there are two histidine residues, His-12 and His-119, in the active site of RNase A, acting as a general acid and general base for the catalytic function of the enzyme. In contrast to this, one histidine (His-40) and one glutamic acid (Glu-58) are responsible for the catalytic action of RNase T_1. This may be the reason why the monoanion of 3′-GMP binds to RNase T_1 more strongly than the dianion. Griffin et al. (1973) have studied the interaction between RNase A and a dinucleoside phosphonate inhibitor UpcA in which a methylene group replaces the 5′-oxygen of adenosine and discussed a difference in the mode of binding to RNase A

between UpcA which is monoanionic as are the substrates of RNase A and dianionic mononucleotides 2′-CMP and 3′-CMP. The significance of the results obtained in the present work in terms of RNase T_1 catalysis will be the subject of a future study.

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Melting-Profile Analysis of Thermal Stability of Thermolysin. A Formulation of Temperature-Scanning Kinetics†

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ABSTRACT: The melting-profile method consists of a continuous observation of a structural parameter while the temperature of the sample is raised at a constant rate [Fujita, S. C., & Imahori, K. (1974) in *Peptides, Polypeptides and Proteins* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) p 217, Wiley, New York, N.Y.]. An analytical solution to the melting profile was formulated for the two-state irreversible process and called temperature-scanning kinetics. The theory was tested with thermolysin with consistent results, and the thermodynamic parameters of thermal denaturation were calculated: $\Delta H^{\ddagger}$ = 80.3 kcal/mol, $\Delta S^{\ddagger}$ = 153 eu. These values agreed with the corresponding values obtained from the classical constant-temperature relaxation kinetics. The possibilities of temperature-scanning kinetics are discussed.

Melting curves are commonly used to characterize thermal stability of biological macromolecules. They are obtained by plotting some structural parameter such as ultraviolet absorbance or ellipticity of the sample as a function of temperature. The midpoint of transition is generally designated as the melting temperature or T_m to serve as a measure of thermal stability. When in such an experiment the rate of temperature rise is held at a constant value, a plot of a structural parameter against time gives essentially the same curve (as the plot against temperature), which we called the melting profile and proposed as a convenient method to characterize the thermal stability of proteins (Fujita & Imahori, 1974).

In interpreting and evaluating such a type of experiment, reversibility of the transition is an important question. When it is reversible within the time scale of the experiment, the measurements can be made at equilibrium and thermodynamic

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