La Mar, G. N., de Ropp, J. S., Latos-Grazynski, L., Balch, A. L., Johnson, R. B., Smith, K. M., Parish, D. W., & Cheng, R.-J. (1983a) *J. Am. Chem. Soc. 105*, 782–787.
La Mar, G. N., de Ropp, J. S., Smith, K. M., & Langry, K. C. (1983b) *J. Am. Chem. Soc. 105*, 4576–4580.
La Mar, G. N., Davis, N. L., Parish, D. W., & Smith, K. M. (1983c) *J. Mol. Biol. 168*, 887–896.
La Mar, G. N., Anderson, R. R., Chacko, V. P., & Gersonde, K. (1983d) *Eur. J. Biochem. 136*, 161–166.
Mc Donald, C. C., & Phillips, W. D. (1973) *Biochemistry 12*, 3170–3186.
Morishima, I., & Ogawa, S. (1978) *Biochemistry 17*, 4384–4388.
Morishima, I., Ogawa, S., Inubushi, T., Yonezawa, T., & Iizuka, T. (1977) *Biochemistry 16*, 5109–5115.
Morishima, I., Ogawa, S., Inubushi, T., & Iizuka, T. (1978) *Adv. Biophys. 11*, 217–245.
Morris, D. R., & Hager, L. P. (1966) *J. Biol. Chem. 241*, 1763–1768.
Morrison, M., & Schonbaum, G. R. (1976) *Annu. Rev. Biochem. 45*, 861–888.
Pickard, M. A., & Hashimoto, A. (1982) *Can. J. Microbiol. 28*, 1382–1388.
Sae, A. S. W., & Cunningham, B. A. (1979) *Phytochemistry 18*, 1785–1787.
Satterlee, J. D., & Erman, J. E. (1980) *Arch. Biochem. Biophys. 202*, 608–616.
Satterlee, J. D., & Erman, J. E. (1981a) *J. Am. Chem. Soc. 103*, 199–200.
Satterlee, J. D., & Erman, J. E. (1981b) *J. Biol. Chem. 256*, 1091–1093.
Satterlee, J. D., & Erman, J. E. (1983a) *Biochim. Biophys. Acta 743*, 149–154.
Satterlee, J. D., & Erman, J. E. (1983b) *J. Biol. Chem. 258*, 1050–1056.
Satterlee, J. D., Erman, J. E., La Mar, G. N., Smith, K. M., & Langry, K. C. (1983a) *Biochim. Biophys. Acta 743*, 246–255.
Satterlee, J. D., Erman, J. E., La Mar, G. N., Smith, K. M., & Langry, K. C. (1983b) *J. Am. Chem. Soc. 105*, 2099–2104.
Thomas, J. A., Morris, D. R., & Hager, L. P. (1970) *J. Biol. Chem. 245*, 3135–3142.
Williams, R. J. P., Wright, P. E., Mazza, G., & Ricard, J. R. (1975) *Biochim. Biophys. Acta 412*, 127–147.

Binding Modes of Inhibitors to Ribonuclease T_1 As Studied by Nuclear Magnetic Resonance†

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ABSTRACT: The binding modes of inhibitors to ribonuclease T_1 (RNase T_1) were studied by the analyses of 270-MHz proton NMR spectra. The chemical shift changes upon binding of phosphate, guanosine, 2′-GMP, 3′-GMP, 5′-GMP, and guanosine 3′,5′-bis(phosphate) were observed as high field shifted methyl proton resonances of RNase T_1. One methyl resonance was shifted upon binding of phosphate and guanosine nucleotides but not upon binding of guanosine. Four other methyl resonances were shifted upon binding of guanosine and guanosine nucleotides but not upon binding of phosphate. From the analyses of nuclear Overhauser effects for the pair of H8 and H1′ protons, together with the vicinal coupling constants for the pair of H1′ and H2′ protons, the conformation of the guanosine moiety as bound to RNase T_1 is found to be C3′-endo-syn for 2′-GMP and 3′-GMP and C3′-endo-anti for 5′-GMP and guanosine 3′,5′-bis(phosphate). These observations suggest that RNase T_1 probably has specific binding sites for the guanine base and 3′-phosphate group (P1 site) but not for the 5′-phosphate group (P0 site) or the ribose ring. The weak binding of guanosine 3′,5′-bis(phosphate) and 5′-GMP to RNase T_1 is achieved by taking the anti form about the glycosyl bond. The productive binding to RNase T_1 probably requires the syn form of the guanosine moiety of RNA substrates.

Ribonuclease T_1 (RNase T_1)[1] (EC 3.1.27.3) is an acidic protein (104 amino acid residues) isolated from Takadiastase, a commercial product of *Aspergillus oryzae* (Sato & Egami, 1957; Egami et al., 1964). RNase T_1 specifically cleaves ribonucleic acid (RNA) chains at guanylic acid residues in contrast to the pyrimidine specificity of bovine pancreatic ribonuclease A. Rigorous recognition of guanine base by RNase T_1 is a typical example of RNA–protein interactions. A number of studies have been made to eludicate this specific

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[1] Abbreviations: 2′-GMP, guanosine 2′-phosphate; 3′-GMP, guanosine 3′-phosphate; 5′-GMP, guanosine 5′-phosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; RNA, ribonucleic acid; RNase T_1, ribonuclease T_1.

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interaction and enzymatic mechanism of RNase T_1, including enzyme kinetic (Irie, 1967, 1968; Zabinski & Walz, 1976), gel filtration (Campbell & Ts'o, 1971; Takahashi, 1972), fluorescence (Pongs, 1970), ultraviolet absorption (Oshima & Imahori, 1971a; Epinatjeff & Pongs, 1972; Walz, 1976), and circular dichroism studies (Oshima & Imahori, 1971b). Chemical modification studies have suggested that the His-40, His-92, Glu-58, and Arg-77 residues lie in the active site and are essential for the enzymatic activity (Takahashi et al., 1967; Irie, 1970; Takahashi, 1970a,b, 1971). Nuclear magnetic resonance (NMR) analyses also have given important information on this problem. Rüterjan and Pongs have shown the presence of His-40 and His-92 residues in the active site (Rüterjan & Pongs, 1971), and Arata et al. have made the assignments of the C2 proton resonances of these histidine residues (Arata et al., 1976, 1979). From the pH dependence of chemical shifts of histidine C2 and C4 proton resonances of RNase T_1 and the ^{13}C resonance of γ-carboxy group of [γ-(carboxymethyl)-Glu^{58}]RNase T_1, the ionic interaction between the His-40 and Glu-58 residues has been elucidated (Inagaki et al., 1981). Models for the active site of RNase T_1 have been proposed from the analyses of NMR spectra (Rüterjan & Pongs, 1971; Arata et al., 1976, 1979; Kimura et al., 1979; Inagaki et al., 1981; Kyogoku et al., 1982).

Recently, the crystal and molecular structures of the complex of RNase T_1 and guanosine 2'-phosphate (2'-GMP) have been elucidated by X-ray analyses (Heinemann & Saenger, 1982). On the basis of such structures, the proton NMR spectra of RNase T_1 in aqueous solution may now be analyzed in detail. In addition to the ionic interactions as elucidated from the pH dependences of the chemical shifts, the analyses of NMR spectra provide information about methyl-bearing amino acid residues, which are inert to other spectroscopic methods. These aliphatic residues of RNase T_1 are possibly involved in hydrophobic interactions with inhibitors or substrates, which are essential for the rigorous recognition of guanine base by RNase T_1. In the present study, we have analyzed the dependences of chemical shifts of high field shifted methyl proton resonances of RNase T_1 on the binding of inhibitors, including guanosine, phosphate, 2'-GMP, guanosine 3'-phosphate (3'-GMP), guanosine 5'-phosphate (5'-GMP), and guanosine 3',5'-bis(phosphate). From the analyses of inhibitor binding curves and nuclear Overhauser effects (NOE), the binding modes of inhibitors to RNase T_1 will be discussed.

Experimental Procedures

Materials. RNase T_1 was prepared from Takadiastase by the use of affinity chromatography (Kanaya & Uchida, 1981). The enzymatic activity of purified RNase T_1 was about 1.6×10^4 units/mg as determined by the assay method of Takahashi (1961). Guanosine, 2'(3')-GMP, and 5'-GMP were purchased from Kyowa Hakko Co. Guanosine 3',5'-bis(phosphate) was a gift from Professor K. Takahashi of Kyoto University. 2'-GMP and 3'-GMP were isolated from 2'(3')-GMP, by the use of a Dowex 1X2 column.

Methods. The 270-MHz ^1H NMR spectra were recorded on a Bruker WH-270 spectrometer. The NMR samples of RNase T_1 were dissolved in ^2H_2O (0.2 M NaCl) at a concentration of about 1.8 mM. Labile OH and NH hydrogen atoms of RNase T_1 were completely exchanged with deuterium atoms by incubating the sample in ^2H_2O solution at pH 7.5 for about 15 min at 50 °C. The pH of the sample solutions was adjusted to pH 5.0 by the addition of 1 M ^2HCl or NaO^2H. The pH values (direct pH meter readings) were measured directly in a 5-mm NMR tube by the use of Radiometer PHM-26 pH meter. All the inhibitor solutions were incubated at 60 °C for 20 min to avoid aggregation (Chantot, 1972). Aliquots of inhibitor solutions were added successively to the sample solution. NMR spectra were recorded at 37 °C, except for NOE measurements at 30 °C. For NOE measurements, irradiation power and irradiation time were selected to minimize the spin-diffusion effects. We usually used an irradiation time of 0.5 s. Eight scans of on-resonance and off-resonance irradiation were alternatively accumulated up to total 512 scans, respectively. Negative NOE enhancements were extracted by the use of difference method (Richarz & Wüthrich, 1978).

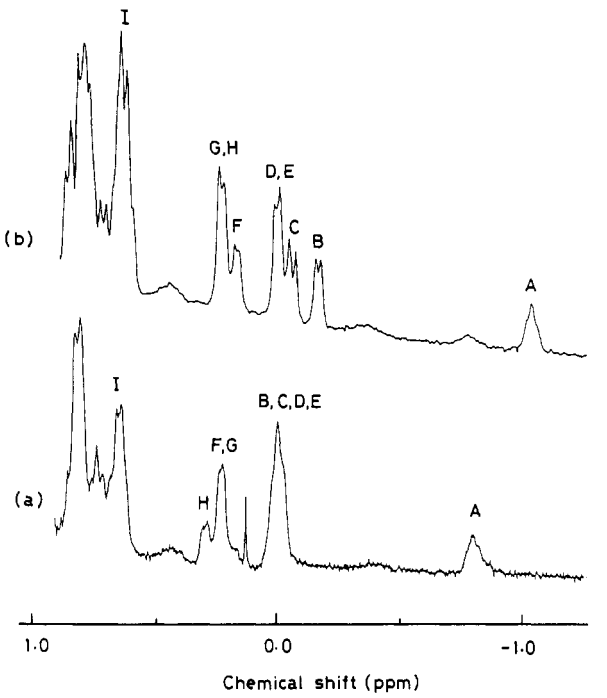

FIGURE 1: The 270-MHz proton NMR spectra (methyl region) of (a) RNase T_1 and (b) RNase T_1 with 2'-GMP (mole ratio of 1:2) in ^2H_2O solution at pH 5.0 and 37 °C.

Results

High Field Shifted Methyl Proton Resonances of RNase T_1. Figure 1a shows the 270-MHz ^1H NMR spectrum of RNase T_1 in the methyl region. There are eight methyl proton resonances (A–H) observed in the region 0.5 to −1.0 ppm. The resonances A, B, C, F, and H are shifted to high field (Figure 1b) upon binding of 2'-GMP, a competitive inhibitor, suggesting that these resonances of RNase T_1 are due to the methyl groups as located in the active site. The triplet resonance A is readily assigned to an isoleucine δ-methyl group. Doublet resonances have been assigned to specific amino acid types by the spin-decoupling experiments. Resonances B and H are simultaneously decoupled on irradiation at 1.13 ppm and are assigned to valine or leucine methyl groups. Resonance C is assigned to an alanine or threonine methyl group and resonance F to an isoleucine γ-methyl group. On the other hand, resonances D (or E) and I are not shifted to higher field upon binding of 2'-GMP. These resonances are simultaneously decoupled on irradiation at 1.43 ppm and, accordingly, are assigned to another valine or leucine residue.

NOE measurements on high field shifted methyl proton resonances have also been carried out to elucidate the spatial proximity of methyl groups. Figure 2b shows an NOE difference spectrum on irradiation at resonance A, where negative NOE enhancements are observed for resonances F and B. Since the triplet resonance A is due to an isoleucine δ-methyl

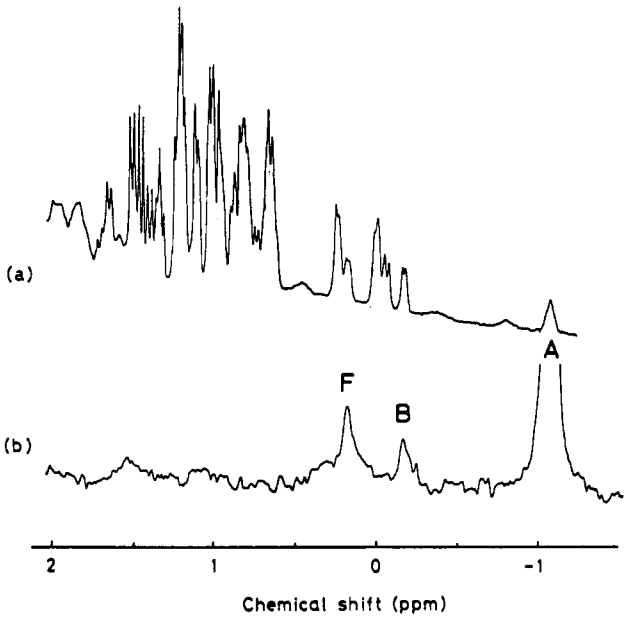

FIGURE 2: The 270-MHz proton NMR spectra (methyl region) of RNase T_1 with 2′-GMP (mole ratio of 1:2) in ^2H_2O solution at pH 5.0 and 30 °C: (a) the normal spectrum and (b) the NOE difference spectrum on irradiation at resonance A.

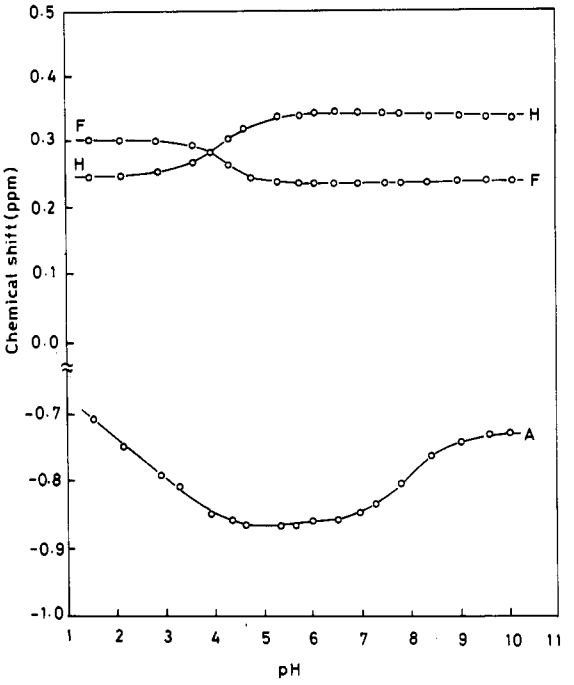

FIGURE 3: pH dependence of the chemical shifts of high field shifted methyl proton resonances of RNase T_1 at 37 °C.

group, resonance F is probably due to the same isoleucine residue. Moreover, the NOE experiment shows that the δ-methyl group of this isoleucine residue lies in close proximity to the methyl group (resonance B) of a valine or leucine residue in the active site.

pH Dependence of Chemical Shifts of High Field Shifted Methyl Proton Resonances. Figure 3 shows the pH dependence of the chemical shifts of the high field shifted methyl proton resonances in the absence of inhibitors. Resonance A shows inflection with $pK_a \simeq 7.9$ and broad inflection in the acid pH region, very similar to His-92 C2 proton resonances (Inagaki et al., 1981). Resonances F and H shows inflection with $pK_a \simeq 4.3$. However, resonance G remains unchanged. For resonances B–E, though a small but appreciable change of the envelope was observed, we could not obtain the pH dependence of the individual resonance.

Inhibitor Binding to RNase T_1. Figure 4a shows the changes in the chemical shifts of high field shifted methyl proton resonances upon binding of phosphate. Phosphate is known to inhibit the enzymatic activity of RNase T_1 (Takahashi, 1972). As the mole ratio (ρ) of phosphate to RNase T_1 is increased, resonance A is significantly shifted to higher field. By contrast, resonances B–E, G, and H are unaffected while resonance F is slightly shifted to lower field. On the other hand, upon binding of guanosine (Figure 4b), resonances B, C, F, and H are appreciably shifted to higher field while resonance A is not shifted. These results indicate that the isoleucine δ-methyl group (A) of RNase T_1 lies in the binding site for the phosphate moiety, while the methyl groups B and H (Val or Leu) and C (Ala or Thr) are involved in the binding site for the guanine moiety of the inhibitor. In comparison, the binding of adenosine 3′-phosphate to RNase T_1 induces an appreciable shift of resonance A rather than resonances B, C, F, and H (F. Inagaki et al., unpublished result). The high-field shifts of the proton resonances B, C, F, and H of RNase T_1 are thus related with the rigorous recognition of the guanine moiety.

For the high field shifted methyl proton resonances (A–H) of RNase T_1, the chemical shift changes on the binding of 2′-GMP, 3′-GMP, 5′-GMP, and guanosine 3′,5′-bis(phosphate) are shown in Figure 4c–f. In the case of 2′-GMP (Figure 4c), the binding curves for resonances A and B are discontinuous, probably because of intermediate chemical exchange. On the other hand, in the cases of 3′-GMP, 5′-GMP, and guanosine 3′,5′-bis(phosphate), the binding curves are all continuous, indicating that the chemical exchange is fast in the chemical shift time scale. From the analyses of the binding curves, the order of binding constants is obtained as 2′-GMP > 3′-GMP > guanosine 3′,5′-bis(phosphate) ≃ 5′-GMP, in agreement with the order previously found by the gel filtration studies (Campbell & Ts'o, 1971; Takahashi, 1972). However, the chemical shift changes of the proton resonances (A–C, F, and H) upon binding with the four inhibitors are remarkably similar, indicating that these inhibitors bind to the same site in RNase T_1. In addition to these resonances, a few methyl proton resonances around 0.8 ppm also show small but appreciable shifts upon binding of inhibitors. For the aromatic proton resonances of RNase T_1, the chemical shift changes on the binding of inhibitors were also studied. Figure 5 shows the titration behavior of His-40, His-92, His-27, and Trp-59 as well as H8 and H1′ proton resonances of 2′-GMP as an example. The H8 proton of guanine base shows sharp resonance below $\rho = 1.0$, but above $\rho = 1.0$, the resonance becomes broader due to exchange broadening. In addition to these resonances, a few resonances show appreciable shifts. On the basis of the crystal structure of the RNase T_1–2′-GMP complex, His-40, His-92, and Tyr-45 directly interact with the inhibitor and Tyr-42 and Trp-59 are located in the active site, while His-27 is located at the bottom of the active site. Thus, it is reasonable that the methyl and the aromatic proton resonances, which show appreciable shifts upon binding of the inhibitors, are located at the active site or near the active site.

NOE Enhancements of Bound Inhibitors. In order to elucidate the conformations of inhibitors as bound to RNase T_1, NOE measurements on the proton resonances of inhibitors have been carried out in the presence of RNase T_1. For NOE measurements, irradiation time and irradiation power were carefully adjusted to minimize the spin-diffusion effect. When the spin-diffusion effect is dominant, the NOE enhancements are not selective, and thus, the interpretation of the results is

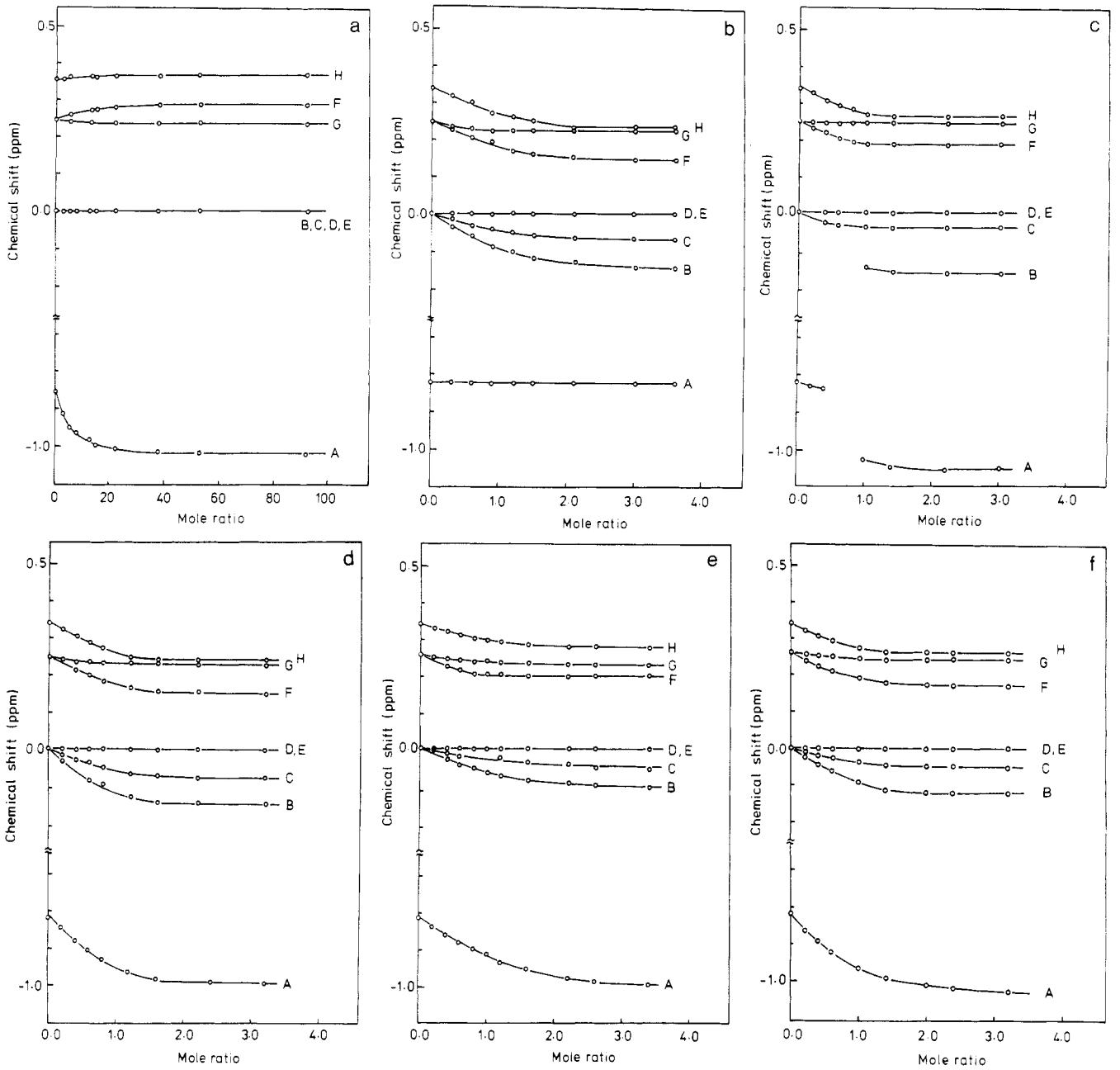

FIGURE 4: Dependences of the chemical shifts of high field shifted methyl resonances of RNase T_1 (1.8 mM in ^2H_2O) on the mole ratio of inhibitor to RNase T_1: (a) phosphate, (b) guanosine, (c) 2′-GMP, (d) 3′-GMP, (e) 5′-GMP, and (f) guanosine 3′,5′-bis(phosphate). NMR data were taken at pH 5.0 and 37 °C except for phosphate (at pH 5.5 and 23 °C).

difficult. However, under present experimental conditions, NOE enhancements are selective as shown in Figures 6–10 and are due to direct dipolar interaction. Figure 6b shows the NOE difference spectrum of an equimolar mixture (1.8 mM) of guanosine and RNase T_1 on irradiation of the H8 proton of guanosine, where more than 80% of total guanosine is found to be bound to RNase T_1 from the analysis of the binding curves (Figure 4b). The negative NOE enhancement as observed of the H1′ proton resonance of guanosine is clearly due to the inhibitor as bound to RNase T_1 rather than to the free inhibitor (the latter should give positive NOE).

It may be recalled here that, in the syn form (Figure 11), the H1′ proton is close to the H8 proton and a large NOE is expected for the H1′ proton resonance on irradiation of the H8 proton. By contrast in the anti form, the NOE will be negligibly small, since the H1′ proton is far from the H8 proton (Son et al., 1972). Therefore, the large negative NOE as observed for the pair of the H1′ and H8 protons of guanosine directly indicates that this inhibitor takes the syn form in the complex with RNase T_1.

Such NOE experiments have also been made for other inhibitors, in order to elucidate the conformation of the guanosine moiety as bound to RNase T_1. For the RNase T_1–2′-GMP complex (Figure 7b) and the RNase T_1–3′-GMP complex (Figure 8b), the irradiation of the H1′ proton causes NOE on the H8 proton resonance. Conversely, the irradiation of the H8 proton causes NOE on the H1′ proton resonance (Figure 7c). Thus, 2′-GMP and 3′-GMP are both found to take the syn form in the complex with RNase T_1 in aqueous solution. By contrast, in the cases of 5′-GMP (Figure 9) and guanosine 3′,5′-bis(phosphate) (Figure 10), the irradiation of the H8 (or H1′) proton does not cause NOE on the H1′ (or H8) proton resonance, indicating that these inhibitors as bound to RNase T_1 take the anti form (Figure 11b).

Vicinal Coupling Constants of Ribose Rings of Inhibitors. The vicinal coupling constants ($^3J_{1'2'}$) for the pair of H1′ and

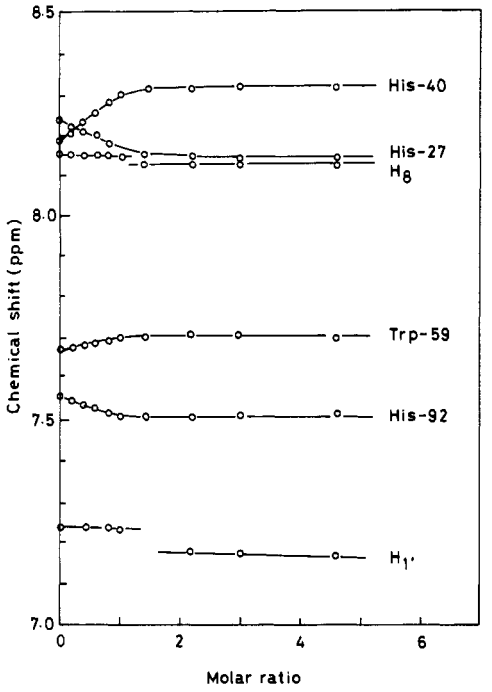

FIGURE 5: Dependence of the chemical shifts of the His and Trp proton resonance of RNase T_1 and H8 and H1′ of 2′-GMP (1.8 mM in ^2H_2O) on the mole ratio of 2′-GMP to RNase T_1 at pH 5.0 and 37 °C.

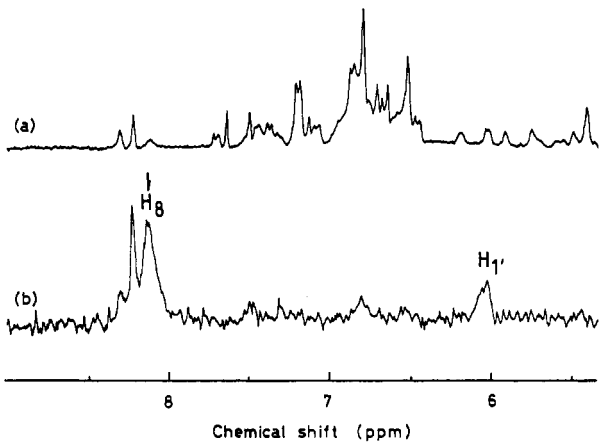

FIGURE 6: The 270 MHz proton NMR spectra (aromatic region) of RNase T_1 with guanosine (mole ratio of 1:1) in ^2H_2O solution at pH 5.0 and 30 °C: (a) the normal spectrum and (b) the NOE difference spectrum on irradiation of the H8 proton of guanine base.

H2′ protons of ribose rings are useful for studying the conformations of the ribose rings of inhibitors as bound to RNase T_1. The $^3J_{1'2'}$ values are expected to be as small as 2 Hz for the C3′-endo form and as large as 8 Hz for the C2′-endo form. For 2′-GMP, 3′-GMP, 5′-GMP, and guanosine 3′,5′-bis-(phosphate), the dependences of $^3J_{1'2'}$ values on the mole ratios (inhibitor to RNase T_1) are shown in Figure 12. As the mole ratio is decreased to zero, the $^3J_{1'2'}$ values are clearly reduced smaller than 4 Hz, indicating that the ribose rings of these inhibitors primarily take the C3′-endo form in the complex with RNase T_1 in aqueous solution. It may be noted that the C3′-endo-syn form of the guanosine moiety of the RNase T_1–2′-GMP complex in aqueous solution, as derived from the present NMR analyses, is similar to the conformation as found in the crystal (Heinemann & Saenger, 1982).

Discussion

Active Site Structure of RNase T_1. Previously, models for the active-site structure of RNase T_1 in aqueous solution have been proposed from the analyses of chemical modification and spectroscopic data. His-40 and His-92 lie in the active site; one of these histidine residues and Glu-58 serve as the general base and general acid, respectively, in the enzymatic activity of RNase T_1, and the other histidine residue is involved in the specific recognition of guanine base (Takahashi, 1970a). Trp-59 is in close proximity to Glu-58 and a histidine residue (Pongs, 1970; Walz, 1977). Arg-77 also lies in the active site and possibly binds the phosphate moiety of the inhibitor (Takahashi, 1968; Inagaki et al., 1981).

Such models for the active site structure of RNase T_1 in solution are largely found to be consistent with the molecular conformation of RNase T_1–2′-GMP complex as elucidated by the X-ray crystal analysis (Heinemann & Saenger, 1982). His-92 and Glu-58 are in fact found to be involved in the catalytic site of RNAse T_1, and Arg-77 serves as the binding site for the phosphate moiety of 2′-GMP. Trp-59 is located in the catalytic site and interacts with Glu-58 and His-40.

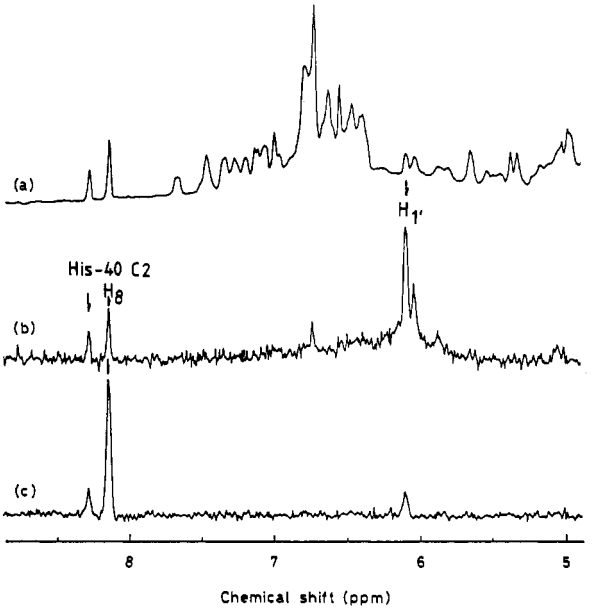

FIGURE 7: The 270-MHz proton NMR spectra (aromatic region) of RNase T_1 with 2′-GMP (mole ratio of 1:1) in ^2H_2O solution at pH 5.0 and 30 °C; the normal spectrum (a) and the NOE difference spectra on irradiation of the H1′ proton of the ribose ring (b) and the H8 proton of the guanosine base (c).

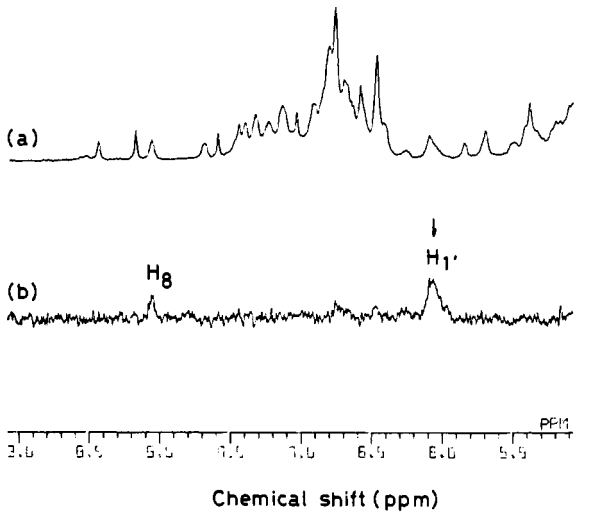

FIGURE 8: The 270-MHz proton NMR spectra (aromatic region) of RNase T_1 with 3′-GMP (mole ratio of 1:1) in ^2H_2O solution at pH 5.0 and 30 °C: the normal spectrum (a) and the NOE difference spectrum on irradiation of the H1′ proton of the ribose ring (b).

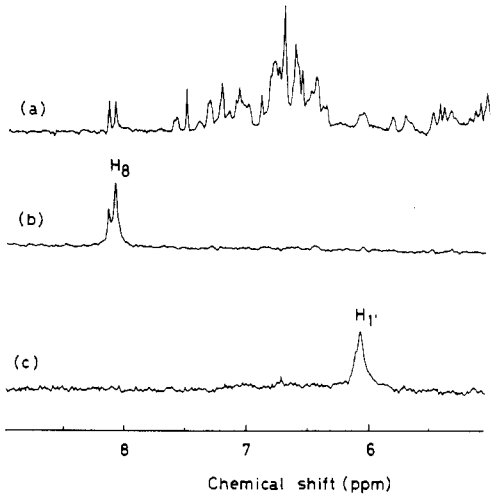

FIGURE 9: The 270-MHz proton NMR spectra (aromatic region) of RNase T_1 with 5′-GMP (mole ratio of 1:1) in ^2H_2O solution at pH 5.0 and 30 °C: the normal spectrum (a) and the NOE difference spectra on irradiation of the H8 proton of the ribose ring (b) and the H1′ proton of the guanine base (c).

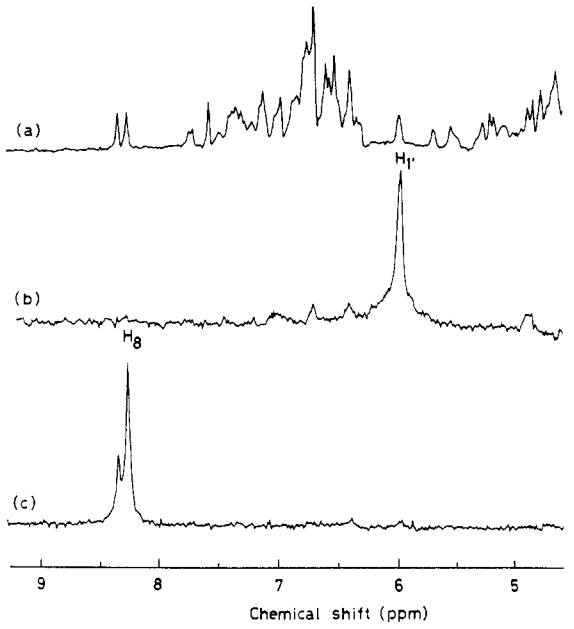

FIGURE 10: The 270-MHz proton NMR spectra (aromatic region) of RNase T_1 with guanosine 3′,5′-bis(phosphate) (mole ratio of 1:1) in ^2H_2O solution at pH 5.0 and 30 °C: the normal spectrum (a) and the NOE difference spectra on irradiation of the H1′ proton of ribose (b) and the H8 proton of the guanine base (c).

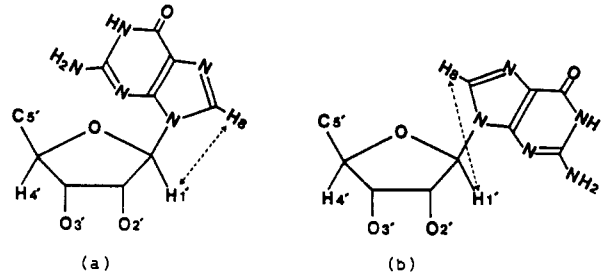

FIGURE 11: The syn (a) and anti (b) forms of the guanine moiety.

However, histidine residues are not involved in the interaction with the guanine base of 2′-GMP. Surprisingly, the rigorous recognition of the guanine base by RNase T_1 appears to be achieved by the hydrogen bonding between the guanine base and the protein main chain and by the stacking of the guanine base with the aromatic ring of Tyr-45 of RNase T_1 (Heinemann & Saenger, 1982).

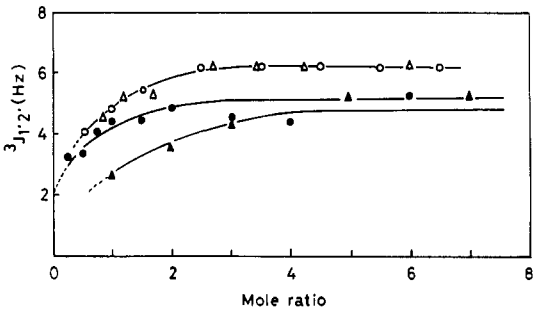

FIGURE 12: Dependences of vicinal coupling constants ($^3J_{1'2'}$) of inhibitors on the mole ratio of inhibitor to RNase T_1: (△) 2′-GMP, (○) 3′-GMP, (▲) 5′-GMP, and (●) guanosine 3′,5′-bis(phosphate).

The crystal analysis of the RNase T_1–2′-GMP complex has further shown that the ribose ring of 2′-GMP is close to His-40 of RNase T_1 (Heineman & Saenger, 1982). In the present study, we have observed a large negative NOE enhancement of the C2 proton resonance of His-40 of RNase T_1 on irradiation of the H1′ proton of 2′-GMP (Figure 7b). This directly indicates that the H1′ proton of 2′-GMP is in close proximity to the C2 proton of His-40 in the complex of RNase T_1 and 2′-GMP in solution. Therefore, the active site structure of RNase T_1 as elucidated in the crystal may be referred to in the analyses of NMR data of this enzyme in solution.

Resonance Assignments of High Field Shifted Methyl Groups in the Active Site. The pH dependence of chemical shift of the methyl proton resonance A was investigated. Inflection with $pK_a \simeq 7.9$ was observed for resonance A. This pK_a is assigned to either His-40 or His-92 (Inagaki et al., 1981). Since His-40 and His-92 are shown to be involved in the active site of RNase T_1 (Heineman & Saenger, 1982), the methyl group A also lies in the active site. Moreover, from the inhibitor binding experiments, this methyl group was shown to be located in the phosphate binding site. RNase T_1 has two isoleucine residues at positions 61 and 90. In the crystal of RNase T_1–2′-GMP complex, Ile-61 rather than Ile-90 is found in the phosphate binding site (Heineman & Saenger, 1982). Accordingly, this triplet resonance A can be assigned to the δ-methyl group of Ile-61, and subsequently, resonance F is assigned to the γ-methyl group of Ile-61. In close proximity to Ile-61, Val-78 and Val-79 are found in the RNase T_1–2′-GMP complex in the crystal. One of these valine residues is probably responsible for the methyl proton resonance B (doublet), since an appreciable NOE enhancement is observed of this resonance (Figure 2b) on irradiation at resonance A. The methyl proton resonance H (doublet) may also be the same residue, since resonances B and H are simultaneously decoupled on irradiation at 1.13 ppm. Actually, resonances H and F show inflection with $pK_a \simeq 4.3$, corresponding to the pK_a of Glu-58. Since Glu-58 is located in the catalytic site, these methyl groups (F and H) are also located in the active site. The methyl proton resonance C due to an alanine or threonine residue is also shifted on the binding of GMPs (Figure 4c–f) and guanosine (Figure 4b) but not on the binding of phosphate (Figure 4a). RNase T_1 has seven alanine residues and five threonine residues (Takahashi, 1965). Some of these residues are located in the active site or close to the active site. It is difficult to assign resonance C to the specific residue.

Binding of Inhibitors to RNase T_1. The rigorous recognition of the guanine moiety by RNase T_1 suggests a definite orientation of the guanine base relative to the active site of the

enzyme. In fact, in the present study, we have observed that the chemical shift changes of the four methyl proton resonances (B, C, F, and H) of RNase T_1 upon binding of 2′-GMP (Figure 4c) are remarkably similar to those upon binding of 3′-GMP (Figure 4d), 5′-GMP (Figure 4e), and guanosine 3′,5′-bis(phosphate) (Figure 4f). Such observations indicate that the guanine moiety of these inhibitors takes similar orientation relative to the active site of RNase T_1, such as found in the crystal of RNase T_1–2′-GMP complex. It may be remarked further that resonance A (Ile-61 δ-methyl) of RNase T_1 is similarly shifted to higher field upon binding of 2′-GMP, 3′-GMP, 5′-GMP, and guanosine 3′,5′-bis(phosphate) (Figure 4c–f). This indicates that the phosphate moiety of these inhibitors binds to the similar site of RNase T_1, although the relative disposition of the phosphate group(s) to the ribose ring is completely different among these inhibitors. Binding of various inhibitors to RNase T_1 will now be discussed in view of the overall conformations of inhibitors.

Conformations of Inhibitors Bound to RNase T_1. The overall conformations of guanine nucleotide inhibitors depend on the internal rotation about the glycosyl bond of the guanosine moiety. Previously, the analysis of the circular dichroic band around 280 nm has suggested that the guanosine moiety takes the syn form upon binding to RNase T_1 (Oshima & Imahori, 1971b). Recently, however, a similar change in the circular dichroic band upon binding to RNase T_1 has also been observed for 5′-deoxy-8,5′-cycloguanosine 3′-phosphate, where the orientation about the glycosyl bond is fixed in the anti form (Matsuda & Ueda, 1981). In this situation, the analyses of NOE enhancements for the pair of H8 and H1′ protons of the guanosine moiety provide unambiguous information on the internal rotation about the glycosyl bond of inhibitors.

From such NOE analyses in the present study, we have found two groups in guanine nucleotide inhibitors of RNase T_1. The guanosine moiety of the inhibitor as bound to RNase T_1 takes the syn form in the group guanosine, 2′-GMP, and 3′-GMP and the anti form in the group 5′-GMP and guanosine 3′,5′-bis(phosphate). On the other hand, in the present study, we have also analyzed the effects of inhibitor binding on the proton chemical shifts of the methyl groups around the active site of RNase T_1. From such binding analyses, we have found that there are two definite binding sites in RNase T_1, one for the guanine moiety and the other for the phosphate moiety (P1 site) (Richards & Wyckoff, 1971), both of which are common to the two groups of inhibitors. The adaptation of the guanine and phosphate moieties to the specific binding sites will require different overall conformations for the two groups of guanine nucleotide inhibitors. In fact, the relative positions of the guanine and phosphate moieties in the C3′-endo-syn-g^- conformation of 3′-GMP (the first group) is similar to that in the C3′-endo-anti-*gt* conformation of 5′-GMP (the second group).

Structural Feature of Active Site of RNase T_1. The binding of the phosphate moiety of 5′-GMP to the P1 site, as are the cases for 2′-GMP and 3′-GMP, implies that there is no specific binding site (P0 site) for the 5′-phosphate group in RNase T_1. However, the weak binding of 5′-GMP to RNase T_1 is achieved by taking the C3′-endo-anti conformation of the guanosine moiety rather than the C3′-endo-syn conformation as is observed for 2′-GMP and 3′-GMP.

The binding of guanosine 3′,5′-bis(phosphate) to RNase T_1 might be anticipated to be much stronger than that of 3′-GMP, since uridine 3′,5′-bis(phosphate), as an inhibitor, binds to pancreatic ribonuclease A 14 times as strong as uridine 3′-phosphate does (Sawada & Irie, 1969). Actually, however, the binding of guanosine 3′,5′-bis(phosphate) to RNase T_1 is as weak as that of 5′-GMP as found from the gel filtration study (Takahashi, 1972) and the present NMR analyses. We have further found that guanosine 3′,5′-bis(phosphate) as bound to RNase T_1 takes the C3′-endo-anti conformation, as is the case of 5′-GMP. This suggests that guanosine 3′,5′-bis(phosphate) binds to RNase T_1, with the 5′-phosphate group (rather than the 3′-phosphate group) in the P1 site of the enzyme.

The binding to RNase T_1 of a variety of inhibitors in two different types of guanosine conformations (C3′-endo-syn and C3′-endo-anti) further suggests that there is no specific binding site for the ribose moiety of inhibitors. Such a binding site has not been found either in the RNase T_1–2′-GMP complex or in the crystal (Heinemann & Saenger, 1982). In fact, the strong binding of 9-(2-hydroxyethyl)guanine 2′-phosphate (Takahashi, 1972) implies that the ribose ring itself is not essential for the binding to RNase T_1. Thus, the ribose ring of guanine nucleotide inhibitors appears to serve as a spacer between the guanine base and the phosphate group, as is the case for the 2-hydroxyethyl group of 9-(2-hydroxyethyl)-guanine 2′-phosphate.

However, the ribose ring of RNA chains as substrates should be essential for the catalytic activity of RNase T_1. Since the binding mode of 5′-GMP (in the C3′-endo-anti form) should not be of the productive type, the C3′-endo-syn form of the guanosine moiety, as found for 3′-GMP in the present study, is probably required for the productive binding of RNA chains. This is consistent with the previous finding that the 3′-phosphodiester of 8-bromoguanylic acid is a good substrate for RNase T_1 (Takahashi, 1974), where the modified guanosine moiety predominantly takes the syn form because of steric hindrance. It may also be noted that a nonhydrolyzable substrate analogue 2′-deoxy-2′-fluoroguanylyl(3′–5′)uridine has been synthesized (Ikehara & Imura, 1981), and the guanosine moiety of this substrate analogue as bound to RNase T_1 has been found to take the C3′-endo-syn form (Y. Shibata, M. Ikehara, and T. Miyazawa, unpublished results).

Conclusions. In the present study, we have made NMR analyses on the binding of a variety of inhibitors to RNase T_1. The high field shifted proton resonances due to the methyl groups in the active site of the enzyme are useful probes for studying the binding modes of various inhibitors. In particular, the analyses of NOE enhancements for the pair of H8 and H1′ protons, together with analyses of vicinal coupling constants, are important for directly elucidating the conformations of guanine nucleotide inhibitors as bound to RNase T_1. Such analyses will also be useful for some detailed analyses of the binding of appropriate substrate analogues to RNase T_1 and will contribute to the elucidation of the mechanism of rigorous recognition of the guanine base of RNA chains by RNase T_1.

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REFERENCES

Arata, Y., Kimura, S., Matsuo, H., & Narita, K. (1976) *Biochem. Biophys. Res. Commun. 73*, 133–140.

Arata, Y., Kimura, S., Matsuo, H., & Narita, K. (1979) *Biochemistry 18*, 18–24.

Campbell, M. K., & Ts'o, P. O. P. (1971) *Biochim. Biophys. Acta 232*, 427–435.

Chantot, J. F. (1972) *Arch. Biochem. Biophys. 153*, 347–356.
Egami, F., Takahashi, K., & Uchida, T. (1964) *Prog. Nucleic Acid Res. Mol. Biol. 3*, 59–101.
Epinatjeff, C., & Pongs, O. (1972) *Eur. J. Biochem. 26*, 434–441.
Heinemann, U., & Saenger, W. (1982) *Nature (London) 299*, 27–31.
Ikehara, M., & Imura, J. (1981) *Chem. Pharm. Bull. 29*, 2408–2412.
Inagaki, F., Kawano, Y., Shimada, I., Takahashi, K., & Miyazawa, T. (1981) *J. Biochem. (Tokyo) 89*, 1185–1195.
Irie, M. (1967) *J. Biochem. (Tokyo) 61*, 550–554.
Irie, M. (1968) *J. Biochem. (Tokyo) 63*, 649–653.
Irie, M. (1970) *J. Biochem. (Tokyo) 68*, 69–79.
Kanaya, S., & Uchida, T. (1981) *J. Biochem. (Tokyo) 89*, 591–597.
Kimura, S., Matsuo, H., & Narita, K. (1979) *J. Biochem. (Tokyo) 85*, 301–310.
Kyogoku, Y., Watanabe, M., Kainosho, M., & Oshima, T. (1982) *J. Biochem. (Tokyo) 91*, 675–679.
Matsuda, A., & Ueda, T. (1981) *Nippon Kagaku Kaishi 5*, 845–850.
Oshima, T., & Imahori, K. (1971a) *J. Biochem. (Tokyo) 69*, 987–990.
Oshima, T., & Imahori, K. (1971b) *J. Biochem. (Tokyo) 70*, 197–199.
Pongs, O. (1970) *Biochemistry 9*, 2316–2321.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647–806.
Richarz, R., & Wüthrich, K. (1978) *J. Magn. Reson. 30*, 147–150.
Rüterjan, H., & Pongs, O. (1971) *Eur. J. Biochem. 18*, 313–318.
Sato, K., & Egami, F. (1957) *J. Biochem. (Tokyo) 44*, 753–767.
Sawada, F., & Irie, M. (1969) *J. Biochem. (Tokyo) 66*, 415–418.
Son, T. D., Guschlbauer, W., & Guêron, M. (1972) *J. Am. Chem. Soc. 94*, 7903–7911.
Takahashi, K. (1961) *J. Biochem. (Tokyo) 60*, 239–245.
Takahashi, K. (1965) *J. Biol. Chem. 240*, 4117–4119.
Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.
Takahashi, K. (1970a) *J. Biochem. (Tokyo) 67*, 833–839.
Takahashi, K. (1970b) *J. Biochem. (Tokyo) 68*, 659–664.
Takahashi, K. (1971) *J. Biochem. (Tokyo) 69*, 331–338.
Takahashi, K. (1972) *J. Biochem. (Tokyo) 72*, 1469–1481.
Takahashi, K. (1974) *Yuki Gosei Kagaku Kyokaishi 32*, 298–316.
Takahashi, K., Stein, W. H., & Moore, S. (1967) *J. Biol. Chem. 242*, 4682–4690.
Walz, F. G. (1976) *Biochemistry 15*, 4446–4450.
Walz, F. G. (1977) *Biochemistry 16*, 4568–4571.
Zabinski, M., & Walz, F. G. (1976) *Arch. Biochem. Biophys. 175*, 558–564.

Further Investigations on the Inorganic Phosphate Binding Site of Beef Heart Mitochondrial F_1-ATPase†

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ABSTRACT: The possibility that 4-azido-2-nitrophenyl phosphate (ANPP), a photoreactive derivative of inorganic phosphate (P_i) [Lauquin, G., Pougeois, R., & Vignais, P. V. (1980) *Biochemistry 19*, 4620–4626], could mimic ATP was investigated. ANPP was hydrolyzed in the dark by sarcoplasmic reticulum Ca^{2+}-ATPase in the presence of Ca^{2+} but not in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. ANPP was not hydrolyzed by purified mitochondrial F_1-ATPase; however, ADP and ATP protected F_1-ATPase against ANPP photoinactivation. On the other hand, the trinitrophenyl nucleotide analogues (TNP-ADP, TNP-ATP, and TNP-AMP-PNP), which bind specifically at the two catalytic sites of F_1-ATPase [Grubmeyer, C., & Penefsky, H. (1981) *J. Biol. Chem. 256*, 3718–3727], abolished P_i binding on F_1-ATPase; they do not protect F_1-ATPase against ANPP photoinactivation. Furthermore, ANPP-photoinactivated F_1-ATPase binds the TNP analogues in the same way as the native enzyme. The P_i binding site of F_1-ATPase, which is shown to be photolabeled by ANPP, does not appear to be at the γ-phosphate position of the catalytic sites.

Purified beef heart mitochondrial F_1-ATPase is a cold-labile enzyme that contains five distinct subunits [for a review, see Penefsky (1979) and Senior (1979)]. F_1-ATPase[1] exhibits a single binding site for P_i (Penefsky, 1977; Kasahara & Penefsky, 1978). In an attempt to photolabel the P_i binding site of F_1-ATPase, ANPP, an azido derivative of P_i, was prepared; its binding properties were described in a preceding paper (Lauquin et al., 1980). The criteria for the binding of ANPP

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[1] Abbreviations: F_1-ATPase, mitochondrial F_1-ATPase; P_i, inorganic phosphate; ANPP, 4-azido-2-nitrophenyl phosphate; ANP, 4-azido-2-nitrophenol; AMP-PNP, adenyl-5′-yl imidodiphosphate; TNP-ADP, TNP-ATP, and TNP-AMP-PNP, the 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene) derivatives of ADP, ATP, and AMP-PNP (at neutral or basic pH); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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