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^{1}H-NMR investigation of the interaction between RNase T_1 and a novel substrate analog, 2′-deoxy-2′-fluoroguanylyl-(3′-5′)uridine

Yasuyuki Shibata*, Ichio Shimada°, Morio Ikehara+, Tatsuo Miyazawa and Fuyuhiko Inagaki°

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, +*Institute of Protein Engineering, Nipponbashi 103, Tokyo* and °*The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan*

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The interaction between RNase T_1 and a non-hydrolysable substrate analog, 2′-deoxy-2′-fluoroguanylyl-(3′-5′)uridine ($G_{fp}U$), was investigated using ^{1}H-NMR spectroscopy. In the complex, the G_{fp} portion takes the *syn* form around the glycosidic bond and the 3′-*endo* form for the ribose moiety, similar to those found in 3′-GMP and 2′-deoxy-2′-fluoroguanosine 3′-monophosphate (G_{fp}). However, in contrast to the cases of these two inhibitors, the complex formation with $G_{fp}U$ at pH 6.0 was found to shift the His-40 C2 proton resonance of RNase T_1 to high field as much as 1 ppm. At pH 6.0, this histidine residue appears to be unprotonated in the complex, but is protonated in the free enzyme (pK_a of His-40 being 7.9). His-40, rather than Glu-58, is probably involved in the catalytic mechanism as a Lewis base, supporting the recent results from site-directed mutagenesis.

NMR; RNase T_1; 2′-Deoxy-2′-fluoroguanylyl-(3′-5′)uridine; Nuclear Overhauser effect

1. INTRODUCTION

RNase T_1 is a small enzyme, produced by *Aspergillus oryzae*, which cleaves RNA chains specifically at guanylic acid residues [1,2]. From extensive investigations, especially by chemical modification studies, His-40, Glu-58, Arg-77 and His-92 have been identified in the active site [2]. Though it is generally believed that Glu-58 and one of these His residues are involved in the catalytic mechanism, there still remain controversies as to the actual roles of these residues [3,4]. In the present study, we analyzed the interaction between RNase T_1 and a novel substrate analog, $G_{fp}U$, using ^{1}H-NMR spectroscopy to gain insight into this problem. $G_{fp}U$ is a non-hydrolysable analog of the substrate, G_pU, in which the 2′-hydroxyl group is replaced by fluorine [5]. As the van der Waals' radius of fluorine is nearly the same as that of the hydroxyl group, $G_{fp}U$ is expected to be a good substrate analog of RNase T_1 in investigating the enzymatic mechanism.

2. EXPERIMENTAL

$G_{fp}U$ and G_{fp} were synthesized as reported previously [5]. RNase T_1 was purified according to Uchida's method [6] with some modifications including the application of an affinity column chromatography (5′-guanylate-aminohexyl-Sepharose 4B [7]) as a final step. ^{1}H-NMR spectra were recorded on a Bruker WH-270 spectrometer. Prior to NMR measurements, labile protons of RNase T_1 were completely exchanged by deuteron at pH 7 and 50°C for 10 min. pH measurements were performed by a radiometer model 26 pH meter equipped with a Nissin Rika CE-103 combination electrode. All pH values were direct pH meter readings at 23°C. DSS was used as an internal standard of chemical shifts.

Correspondence address: F. Inagaki, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan
* *Present address:* Chemistry and Physics Division, National Institute for Environmental Studies, Tsukuba, Ibaraki 305, Japan

Abbreviations: $G_{fp}U$, 2′-deoxy-2′-fluoroguanylyl-(3′-5′)-uridine; NOE, nuclear Overhauser effect; G_{fp}, 2′-deoxy-2′-fluoroguanosine 3′-monophosphate; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate

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3. RESULTS AND DISCUSSION

In our previous paper, we have reported that 3′-GMP binds to RNase T_1 in the *syn* form around the glycosidic bond and the 3′-*endo* form for the ribose moiety [8]. However, the enzyme-bound conformations of inhibitors may not necessarily be the same as those of substrates, so that it is more important to study the binding modes of substrates or substrate analogs in the hope of elucidating the enzymatic mechanism of RNase T_1. This prompted us to study the binding of an non-hydrolysable substrate analog, $G_{fp}U$, to RNase T_1.

At first, we studied the conformation of $G_{fp}U$ as bound to RNase T_1. Fig.1 shows the NOE difference spectra of RNase T_1-$G_{fp}U$ complex (pH 6.0) at the mole ratio of 1.6 ($G_{fp}U$/RNase T_1). Irradiation power and irradiation time were carefully adjusted to minimize the spin-diffusion effect. Upon irradiation of the C8 proton of the guanine base, negative NOE enhancement was observed for the C1′ proton resonance of the G_{fp} moiety of $G_{fp}U$ (fig.1b). Converse irradiation on this C1′ proton resulted in negative NOE enhancement of the C8 proton resonance of the guanine base (fig.1c). Thus, the negative NOEs as observed for the pair of the C1′ and C8 protons indicate that the G_{fp} moiety of this substrate analog takes the *syn* form around the glycosidic bond in the complex with RNase T_1. The puckering of the ribose ring of the G_{fp} moiety in the enzyme-bound state can be studied by the measurement of the spin-spin coupling constants. From the analysis of the NMR spectra obtained on addition of aliquots of $G_{fp}U$ solution to RNase T_1, the spin coupling constant for the pair of the C1′ and C2′ protons was found to be smaller than 3 Hz, while the spin-spin coupling constant for the pair of the C1′ proton and C2′ fluorine in the bound state was as large as 20 Hz. These clearly indicate that the ribose ring in the G_{fp} moiety of $G_{fp}U$ as bound to RNase T_1 takes the 3′-*endo* form. The conformation of G_{fp} in the enzyme-bound state was also found to take the *syn* form around the glycosidic bond and the 3′-*endo* form for the ribose ring. Thus, it is reasonable to conclude that the productive binding of substrates to RNase T_1 also requires the *syn* form around the glycosidic bond and the 3′-*endo* form for the ribose ring of the guanosine moiety as was observed for the cases of inhibitors.

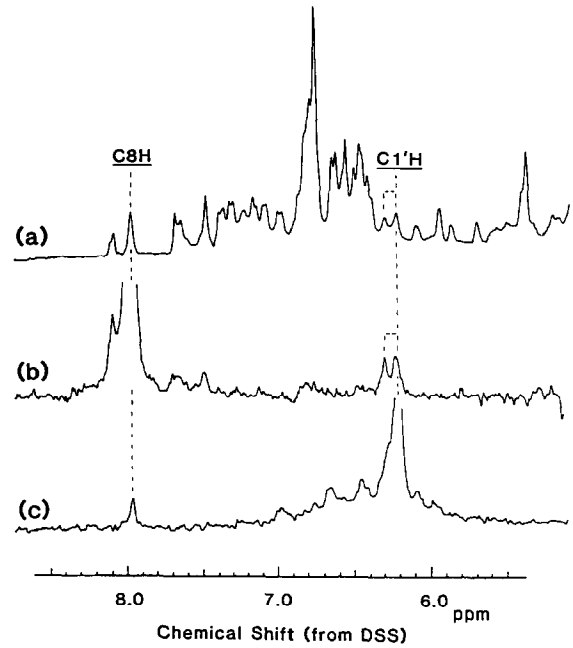

Fig.1. NOE difference spectra of the RNase T_1-$G_{fp}U$ complex. The experimental conditions were as follows: 1.37 mM RNase T_1, 1.73 mM $G_{fp}U$, 0.2 M NaCl, pH 6.0, 30°C in ^2H_2O. Normal spectrum of the aromatic region of the RNase T_1-$G_{fp}U$ complex (a) and the NOE difference spectra on irradiation of the C8 proton of the guanine base (b) and the C1′ proton of the ribose ring (c) of G_{fp} moiety.

Upon complex formation with $G_{fp}U$, the high field shifted methyl proton resonances (A, B, C, F and H) of RNase T_1 were further shifted upfield as shown in fig.2, which is very similar to the case of the inhibitor binding. The upfield shifts of the methyl proton resonances of B, C, F and H are induced by the binding of the guanine base [8]. Since these shifts are sensitive to the relative orientation of the methyl groups and the guanine base, the guanine base in $G_{fp}U$ is found to bind to RNase T_1 in a similar manner as in the case of the inhibitors.

The highest field methyl proton resonance (A, fig.2) of RNase T_1 was recently assigned to Ile-90 located near the phosphate-binding site, which was concluded from NOE data (in preparation) and the crystal structure of the RNase T_1-2′-GMP complex [9,10]. The higher field shift of resonance A on complex formation with $G_{fp}U$ was appreciably smaller than in the cases of the inhibitors. This difference may be ascribed to the effect of the uracil base in $G_{fp}U$. In fact, extensive broadening of the C5 and C6 proton resonances of the uracil base of

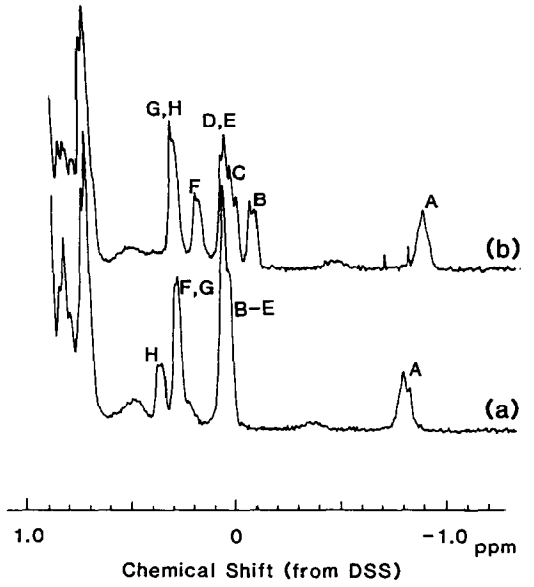

Fig.2. The 270 MHz proton NMR spectra (methyl region) of (a) RNase T_1 and (b) the RNase T_1-$G_{fp}U$ complex. The experimental conditions were the same as in fig.1.

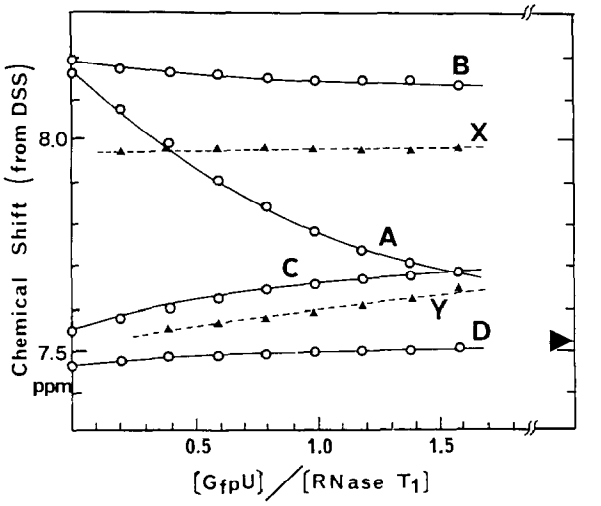

Fig.3. Dependence of the chemical shifts of the His C2 proton resonances (A, B, C), the C8 proton resonance of the guanine base (X) and the C6 proton resonance of uracil base (Y) on the mole ratio of $G_{fp}U$ to RNase T_1 at pH 6.0 and 50°C. A, His-40 C2; B, His-27 C2; C, His-92 C2. The estimated endpoint of the His-40 C2 proton resonance in the complex is indicated by the large arrowhead.

$G_{fp}U$ was observed upon binding to RNase T_1, suggesting that the uracil base also interacts with RNase T_1 (fig.3). This corresponds to the subsite interaction proposed by kinetic studies [11].

Although, the G_{fp} moiety of $G_{fp}U$ binds to RNase T_1 as in the cases of the inhibitors, a significant change was observed in the titration behaviour of the His-40 C2 proton resonance of RNase T_1 upon complex formation with $G_{fp}U$. Fig.3 shows the dependence of proton chemical shifts in the low field region on addition of $G_{fp}U$ at pH 6.0 and 50°C. A large upfield shift of His-40 C2 proton resonance (A) with appreciable line broadening was induced upon binding of $G_{fp}U$, in contrast to the downfield shift of this resonance in the case of the binding of 2'-GMP [8], 3'-GMP [12,13] and G_{fp} (unpublished). From extrapolation of the binding curve, the chemical shift of His-40 C2 proton in the RNase T_1-$G_{fp}U$ complex was found to be nearly the same as that of the unprotonated His-40 in the free enzyme at high pH [12]. This large upfield shift of the His-40 C2 proton resonance of RNase T_1 on the binding of $G_{fp}U$ may not be ascribed to the anisotropic effect of fluorine, since such a shift was not observed on the similar binding of G_{fp}. Rather, this upfield shift is due to the deprotonation of the imidazolium ring of His-40 upon the formation of the RNase T_1-$G_{fp}U$ complex, in which the pK_a of His-40 is much lower than the pK_a (7.9) in the free enzyme [12]. According to the preliminary titration studies, the His-40 C2 proton resonance of the RNase T_1-$G_{fp}U$ complex shifted downfield with a large inflection around pH 5 upon decrease of pH values. The present result shows that His-40 is unprotonated in the complex with $G_{fp}U$ at neutral pH, suggesting that His-40 plays a role as a proton acceptor for the 2'-OH group of guanosine moiety. In fact, a variety of mutant enzymes were prepared to investigate the catalytic mechanism of RNase T_1 using the methods of genetic engineering, and His-40 rather than Glu-58 was found to be indispensable for the catalytic activity [14,15]. Although further studies are required to elucidate the roles of these amino acid residues in detail, non-hydrolysable substrate analogs such as $G_{fp}U$ are quite useful in studying the enzymatic mechanism of RNase T_1 and related enzymes.

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