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Non-cognizable ribonucleotide, 2′AMP, binds to a mutant ribonuclease T_1 (Y45W) at a new base-binding site but not at the guanine-recognition site

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Complex of a mutant ribonuclease T_1 (Y45W) with a non-cognizable ribonucleotide, 2′AMP, has been determined and refined by X-ray diffraction at 1.7 Å resolution. The 2′AMP molecule locates at a new base-binding site which is remote from the guanine-recognition site, where 2′GMP was found to be bound. The nucleotide adopts the *anti* conformation of the glycosidic bond and C3′-*exo* sugar pucker. There exists a single hydrogen bond between the adenine base and the enzyme, and, therefore, the site found is apparently a non-specific binding site. The results indicate that the binding of 2′AMP to the guanine-recognition site is weaker than that to the new binding site.

X-ray; Recognition; Non-specific binding; Mutant RNase T_1

1. INTRODUCTION

Since the specificity of molecular recognition involves complementarity of three-dimensional structures of the molecules, structural studies on complexes between proteins and their cognizable or target nucleic acids have been carried out at many laboratories, using X-ray diffraction and other physical methods. Ribonuclease T_1 (RNase T_1; EC 3.1.27.3) secreted from *Aspergillus oryzae* [1] is one of the most extensively studied proteins. The enzymatic activities are strictly limited to phosphoryltransfer and hydrolysis at 3′-phosphate groups of guanylate residues in single-stranded RNA. The rate of nucleolytic cleavage by RNase T_1 of pGpC is 10^6 to 10^7 times greater than that of pApC in terms of initial velocity. The recognition of nucleotide bases by RNase T_1 seems to be restricted to single base. RNase T_1 binds to 2′GMP molecules, a specific or cognizable inhibitor, with a K_d that ranges from 10^{-5} M to 10^{-6} M, depending on the pH [2,3]. The non-specific binding to the other corresponding ribonucleotides is unexpectedly strong if they are compared with the high specificity in the nucleolytic cleavage described above. The binding is associated with K_d values from 10^{-3} M to 10^{-4} M and the order of affinity binding was estimated as 2′AMP > 2′UMP > 2′CMP. Thus, the three-dimensional structure of a non-specific complex provides an analog of the structural basis of specificity. A full understanding of the true specificity could, therefore, be derived from knowledge of the precision and redundancy of recognition, as clarified by a comparison of the structures formed as a result of non-specific and specific binding, respectively.

Recently, we have succeeded in crystallizing complexes of a mutant RNase T_1 (Y45W) with non-cognizable ribonucleotides, 2′AMP, 2′UMP and 2′CMP by macroscopic seeding of microcrystals of the mutant enzyme complexed with 2′GMP [4,5]. The mutant enzyme produced by protein engineering [6] has a tryptophan residue instead of Tyr^{45} of the wild-type enzyme at the guanine-binding site so as to enhance the binding to ribonucleotides. We report here the first elucidation of three-dimensional structure of one of the non-specific complexes, RNase T_1 (Y45W)/2′AMP complex at an atomic resolution and compare the structure with that of the specific complex, RNase T_1 (Y45W)/2′GMP complex.

Abbreviations: RNase T_1, ribonuclease T_1; K_d, dissociation constant; F_o, observed structure factor; F_c, calculated structure factor; r.m.s. deviation, root-mean-squares deviation.

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2. MATERIALS AND METHODS

2.1. *Crystallization and data collection*

For crystallization, the mutant RNase T_1 (Y45W) was produced in

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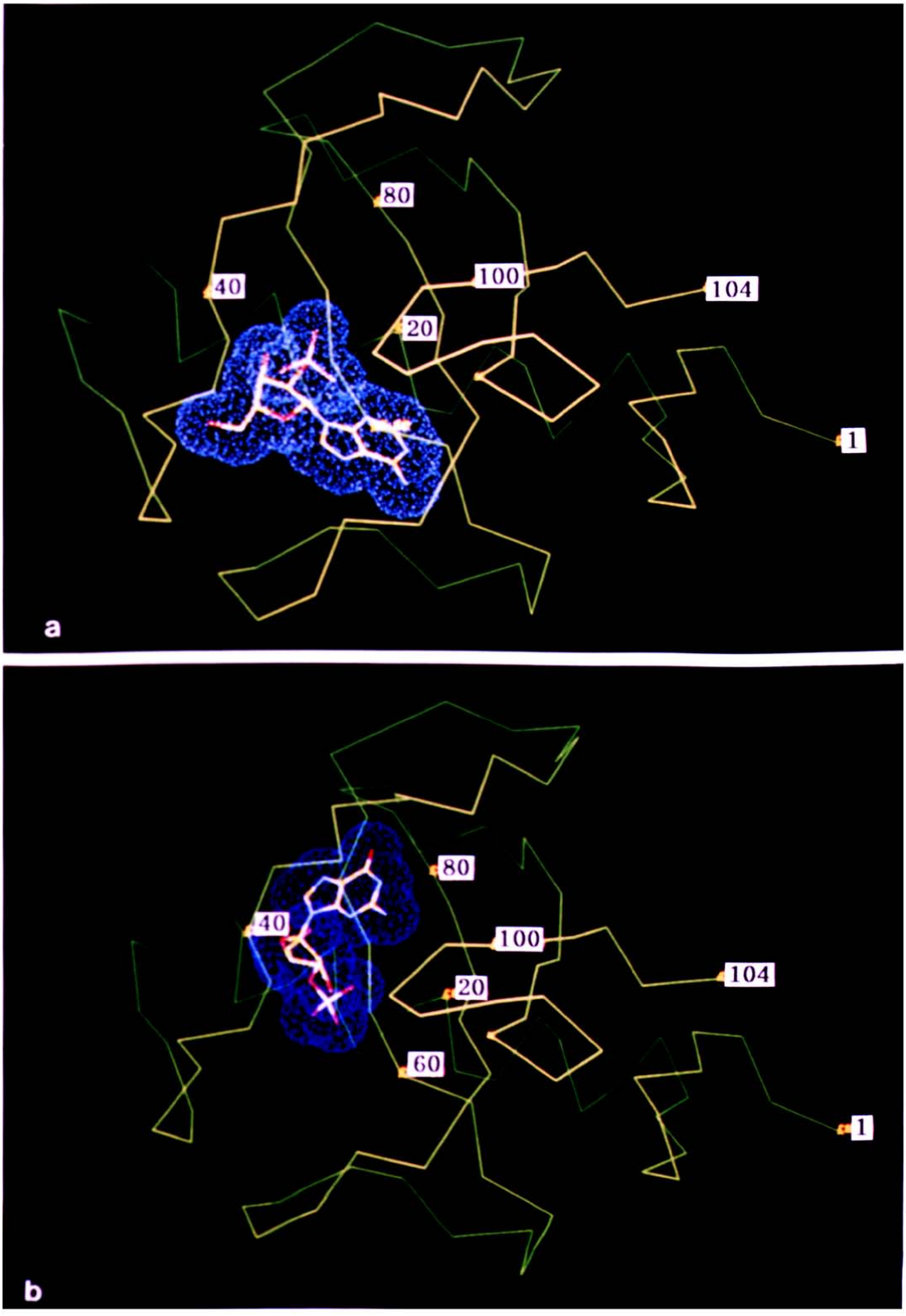

Fig. 1. Nucleotide binding to the mutant RNase T_1 (Y45W) (a) in the complex with 2′AMP crystal and (b) in the complex 2′GMP crystal. The enzyme structures were drawn as tracing C_α-carbon atoms in green and the nucleotides were shown with all non-hydrogen atoms; carbon atoms in yellow, nitrogen atoms in blue and oxygen atoms in red. The van der Waals surfaces of the nucleotides were also plotted with dotted sufaces in blue for 2′GMP and light blue for 2′AMP molecule.

Escherichia coli HB101 that carried plasmid pTP45W2 and purified to homogeneity as described previously [6,7]. The sitting-drop method was employed at 15°C to survey crystallization conditions with 2-methyl-2,4-pentanediol. Details of macroscopic seeding procedure were already reported [4]. The crystals of the RNase T_1 (Y45W) complexed with 2'AMP belong to the space group $P2_12_12_1$ with cell dimensions, $a = 49.38 \pm 0.01$ Å, $b = 46.69 \pm 0.02$ Å, $c = 41.05 \pm 0.02$ Å and $V = 94640 \pm 60$ Å^3. Typical size of the crystals of the complex with 2'AMP obtained is 0.7 mm × 0.3 mm × 0.2 mm. The crystals of the enzyme complexed with 2'GMP also belong to the space group $P2_12_12_1$ with cell dimensions, $a = 48.02 \pm 0.01$ Å, $b = 50.56 \pm 0.01$ Å, $c = 40.28 \pm 0.01$ Å and $V = 97800 \pm 30$ Å^3. Diffraction data were collected on a Rigaku AFC5R-FOS diffractometer equipped to a rotating anode X-ray generator, Rigaku Rota-300, using Cu-Kα radiation at 10°C. Data for the complex with 2'AMP were collected at 1.7 Å resolution using two crystals with all Friedel pairs measured every 100 measurements in addition to the data for merging two crystals. Similarly, data for the complex with 2'GMP were collected at 1.9 Å resolution using one crystal.

2.2. *Structure determination and refinement*

Refined molecular structure of the wild-type RNase T_1 complexed with 2'GMP at 1.9 Å resolution [8] was used as a starting model of the structure determination of the current complexes. Since isomorphism of the current crystals to that of the wild-type enzyme complexed with 2'GMP was poor, rotation and translation search was carried out with the protein coodinates using 3.5 Å resolution data. The initial structure obtained was refined at 3.5 Å resolution using a stereochemically restrained least-squares refinement method with PROLSQ [9]. The resulted structure was inspected and rebuilt on electron density maps with $(2|F_o|-|F_c|)$ as Fourier coefficients using TOM/FRODO [10] on a Silicon Graphics Iris 4D color graphics workstation. The resolution of the data set used in the refinement were gradually raised (3.0 Å, 2.8 Å) to 2.6 Å resolution and refinement of individual temperature factors were begun. The refinement gave a R factor of 0.309. Then, the positional refinement of the structure by simulated annealing with X-PLOR [11] was carried out using reflections between 8 Å and 2.0 Å resolution. Least-squares refinement by PROLSQ was continued including solvent water molecules and 2'AMP. The final structure 783 protein atoms, 23 atoms of 2'AMP molecule, 86 solvent atoms and 1 calcium ion yielded the final R factor of 0.159 against 8932 reflections greater than 1σ (83% completeness) and 0.144 against 7152 reflections greater than 2σ in the resolution range from 8.0 Å to 1.7 Å.

The structural refinement of the complex with 2'GMP was carried out in a similar manner to that of the complex with 2'AMP. The final structure 783 protein atoms, 24 atoms of 2'GMP molecule, 68 solvent atoms and 1 calcium ion yielded the final R factor of 0.173 against 7169 reflections greater than 1σ (98% completeness) and 0.164 against 6285 reflections greater than 2σ in the resolution range from 8.0 Å to 1.9 Å.

3. RESULTS

3.1. *Overall structure*

The r.m.s. deviation of bond distances was 0.018 Å for the complex with 2'AMP and 0.020 Å for the complex with 2'GMP. The mean coordinate error was estimated by analysis of the R values against resolution [12]. The Luzzati plots indicated mean errors in the atomic coordinates of 0.15 Å and 0.175 Å for the complexes with 2'AMP and with 2'GMP, respectively. The whole structures of the enzyme in the two complexes are very similar. The r.m.s. deviation of the C_α-carbon atoms in two complexes is 0.5 Å.

3.2. *Nucleotide-binding sites*

Comparison of the binding of 2'AMP and 2'GMP molecules to the enzyme is shown in Fig. 1. The guanine base was found to the recognition site which consists of a segment from Tyr^{42} to Glu^{46} and Asn^{98} [13] and the phosphate group of the 2'GMP locates at the catalytic site which is a shallow concave surrounding with side chains of Tyr^{38}, His^{40}, Glu^{58}, Arg^{77} and His^{92}. These characteristics are very similar to those in the wild-type RNase T_1 complexed with 2'GMP [8]. The mode of binding of 2'AMP molecule to the enzyme was completely different from that of the 2'GMP molecule. The site is far apart from the recognition site of guanine base by more than 15 Å. As seen in Fig. 2, the guanine-recognition site in the complex with 2'AMP is occupied by

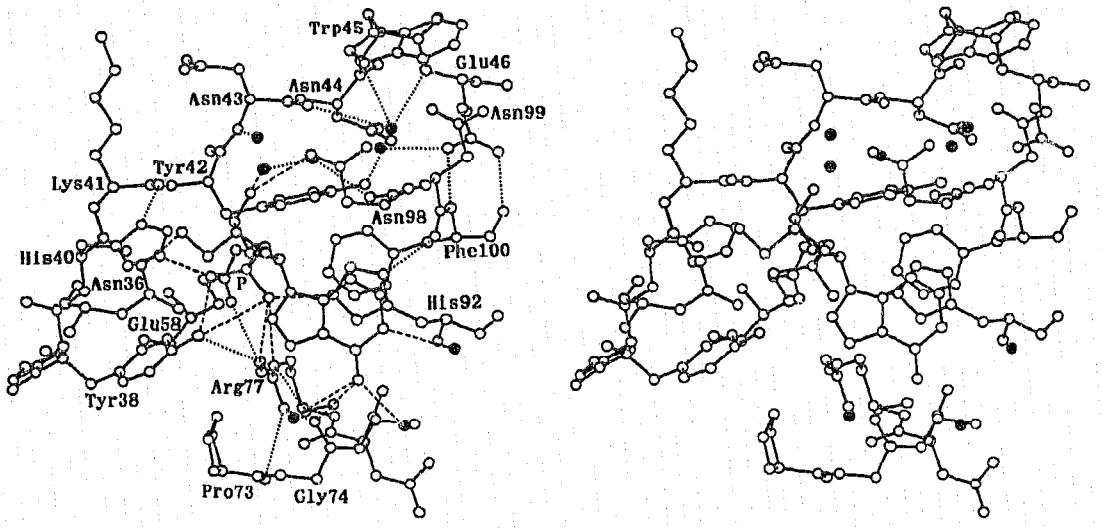

Fig. 2. Stereo view of 2'AMP molecule bound to the mutant RNase T_1 (Y45W). Water molecules are shown by solid circles. Hydrogen bonds between the nucleotide and the enzyme are represented with broken lines and the other hydrogen bonds are with dotted lines.

Table I

Comparison of hydrogen bonds between nucleotide and protein in the RNaseT$_1$ (Y45W)2′AMP and RNase T$_1$ (Y45W)/2′GMP complex crystals

	Atom		Hydrogen bond distance (Å)	
	Nucleotide	Protein	Complex with 2′AMP	Complex with 2′GMP
Base	N1	Glu^{46}Oε1		2.9
	N2	Glu^{46}Oε2	-	3.1
	N2	Asn^{98}O	-	2.8
	N6	Gly^{74}O	3.1	-
	O6	Asn^{43}N	-	3.4
	O6	Asn^{44}N	-	2.8
	O6	Trp^{45}N	-	3.0
	N7	Asn^{43}N		3.2
	N7	Asn^{43}Nδ2		3.5*
Sugar	O3′	Asn^{98}Oδ1	2.8	3.3
	O5′	Asn^{36}Oδ1	2.8	
Phosphate	O1P	Tyr^{38}Oη	2.6	2.4
	O1P	His^{40}Nε2	2.8	3.2
	O2P	Glu^{58}Oε2	2.6	2.5
	O3P	Tyr^{38}Oη	3.3	
	O3P	Arg^{77}Nε	3.3	
	O3P	Arg^{77}Nη1	2.8	3.3
	O3P	His^{92}Nε2	2.6	3.1

*This is longer for a hydrogen bond.

water molecules. It is particularly surprising that the adenine base of the 2′AMP molecule locates at the molecular surface as sitting on the imidazole ring of His^{92}. In contrast to different location of the bases, the phosphate group of the 2′AMP molecule goes inside of the catalytic site in a similar manner as the phosphate group of 2′GMP. The position of phosphorous atom of the 2′AMP is shifted from that of the 2′GMP by only 0.3 Å.

3.3. *Nucleotide conformation and interaction between nucleotide and enzyme*

The hydrogen bonds formed between the nucleotides and enzyme in the complexes are listed and compared in Table I. Only one hydrogen bond was found between the adenine base and the enzyme; between exocyclic amino group (N6) and the main-chain carbonyl group of Gly^{74}. Contrary, the guanine base was locked to the recognition site of the enzyme through seven hydrogen bonds, that resembles to those found in the wild-type RNase T$_1$ complexed 2′GMP (Fig. 3). Nucleotide conformation of 2′AMP is in the *anti* conformation around the glycosidic bond with furanose ring of C3′-*exo* pucker, which is closely related with C2′-*endo* pucker. These conformational features are in contrast to those of 2′GMP molecule which adopts the *syn* conformation and C3′-*endo*-C2′-*exo* pucker. The sugar moiety of the 2′AMP exhibits two hydrogen bonds through 3′- and 5′-hydroxyl groups. One of the hydrogen bonds, i.e. between the 3′-hydroxyl group and the side chain of Asn^{98}, was also formed between the sugar of the 2′GMP and the enzyme. Both of the phosphate groups of the 2′AMP and 2′GMP molecules are tightly linked to the catalytic site through multidentated hydrogen bonds. While two hydrogen bonds formed between the O3P oxygen atom and the side chains of Tyr^{38} and Arg^{77} in the complex with 2′AMP are absent in the complex with 2′GMP, most of these hydrogen bonds are common in the two complexes.

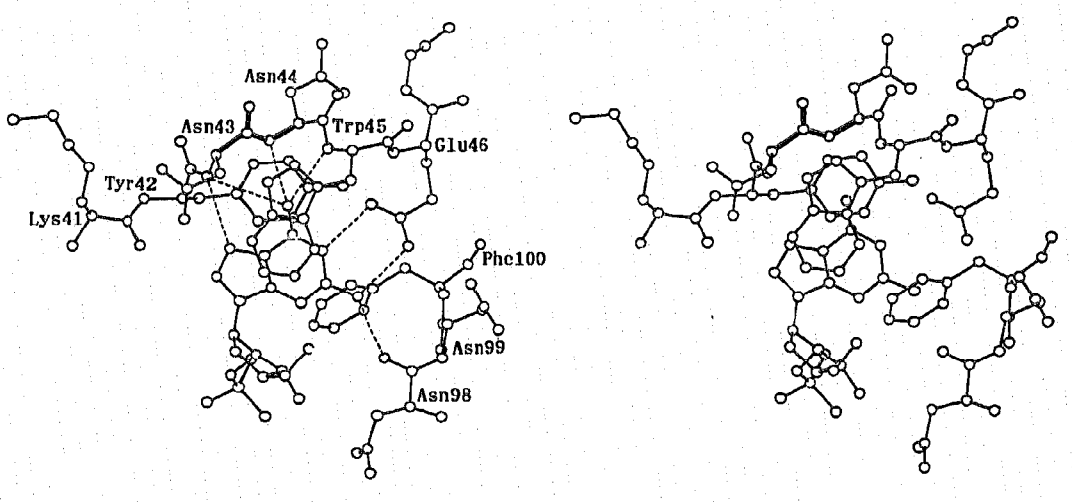

Fig. 3. Stereo view of 2′GMP molecule bound to the guanine-recognition site of the mutant RNase T$_1$ (Y45W). Peptide bond between Asn^{43} and Asn^{44} is indicated by bold lines. Hydrogen bonds between the nucleotide and the enzyme are represented with broken lines.

4. DISCUSSION

The most striking feature of the complex structure with 2'AMP is that the adenine base does not locate at the recognition site. Alternatively, the adenine base locates on a new binding site at the molecular surface. The observation indicates that the unexpectedly strong binding of 2'AMP to the enzyme could be associated with interactions with the new binding site rather than the recognition site. It means that the binding of adenine base to the recognition site must be weaker than that determined so far by gel filtration [2,3].

It is found that the mutation of Tyr^{45} to a tryptophan residue causes little effect on the mode of binding to 2'GMP molecule in addition to that on the structure of the RNase T_1 molecule. The results are in good agreement with those obtained early studies [6] by gel electrophoresis, CD spectra and enzymatic kinetics analyses. The indole ring of Trp^{45} lies over the guanine base with maximum overlapping as shown in Fig. 3. The guanine base sandwiched between two hydrophobic side chains of Tyr^{42} and Trp^{45} seems to be confined in the recognition site while specific recognition of the base through multidentate hydrogen bonds. In contrast to the strong stacking interaction in the complex with 2'GMP, there is no lid like the indole ring over the adenine base which is sitting on the His^{92} on the protein surface. With lack of a multidentated hydrogen bond to the adenine base, the observation suggests that the binding of the adenine base to the new binding site is non-specific rather than specific. Indeed, no structural complementarity between adenine base and the new binding site is recognized.

It is of interest that the phosphate groups of the 2'AMP and 2'GMP molecules found in almost the same position at the catalytic site with many hydrogen bonds conserved in the two complexes. Probably, the site has higher affinity to negatively charged phosphate groups and plays a role of trapping RNA chain followed by specific interaction with guanine bases.

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