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Enzymatic Properties of Mutant Enzymes at Trp49 and Tyr57 of RNase Rh from *Rhizopus niveus*[1]

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In order to establish the role of Tyr57 and Trp49 in the enzymatic reaction of RNase Rh, several mutant enzymes at Tyr57 and Trp49 were prepared by protein engineering and their enzymatic properties were investigated. Among the four mutant enzymes at Trp49 (W49F, W49Y, W49A, and W49I), W49F showed 16% of the activity of the native enzyme, but the others (W49Y, W49A, and W49I) showed greatly decreased activity. The data showed that Trp49 is very important for the enzyme activity. Among 8 mutant enzymes at the 57th position, Y57F and Y57W showed similar enzymatic activity toward RNA to that of the wild-type enzyme, but the others (Y57G, Y57A, Y57V, Y57M, and Y57K) are more active toward RNA and less active toward XpGs. The reason for the apparent increase for RNA activity is discussed from the view point of substrate inhibition. It is noteworthy that W49F and Y57W became more pyrimidine base- and purine base-preferential, respectively.

Key words: base non-specific ribonuclease, mechanism of enzymatic action, *Rhizopus niveus*, ribonuclease, tyrosine residue, tryptophan residue.

Base non-specific RNases which cleave 3′-5′ nucleotidyl linkages of RNA, forming 2′,3′-cyclic phosphate at the 3′-terminal of RNA, are widely distributed in living things, such as virus (*1*), bacteria (*2, 3*), fungi (*4-8*), plants (*9-15*), amoeba (*16*), and animals (*17, 18*). These RNases (RNase T_2 family enzymes) have a protein molecular mass of about 24,000 Da. The mechanism of action of RNases of this group has mostly been studied with RNase Rh from *Rhizopus niveus* (*19-23*), RNase M from *Aspergillus saitoi* (*24, 25*) and RNase T_2 from *A. oryzae* (*26*).

Kinetic and protein engineering studies on RNase Rh revealed that the active site of this enzyme consists of His46, His109, His104 (*20*), Glu105 (*21*), and Lys108 (*23*) and the former two residues work as general acid and base catalysts, and His104 as the phosphate binding site, while the latter two probably help the catalysis by stabilizing the intermediate or polarizing the P=O bond (*22, 23*). The three-dimensional structures of RNase Rh and its 2′-AMP complex were determined by X-ray crystallography by Kurihara *et al.* (*27*) and Nakamura *et al.* (*28*). The X-ray crystallographic data showed that the base moiety stacks between Try57 and Trp49 at the B_1 site (the 5′-side base of the scissile nucleotidic bond), and interacts with Asp51 through hydrogen bonds (Fig. 1). In the sequences of RNases belonging to the RNase T_2 family, the Trp49 residue is conserved without exception (Fig. 2). Although Tyr57 is also conserved in many enzymes of this family, substitutions of Phe, Leu, and Lys for Tyr57 are observed in the enzymes from some fungi, such as *Trichoderma viride* (*8*) and *Lentinus edodes* (*7*), as well as plant (*11, 12*), animals (*17*), and squid (M. Iwama, unpublished data). In this paper, we describe the effect of substitution of these two aromatic amino acid residues by site-directed mutagenesis on the enzymatic activity and the base specificity of the RNase Rh.

MATERIALS AND METHODS

Substrates and Other Reagents—Yeast RNA was a product of Kojin (Tokyo). Dinucleoside phosphates used as substrates were purchased from Sigma (St. Louis, MO).

Enzyme—Nucleic acid modifying enzymes were obtained from Takara Shuzo (Kyoto) and used as recommended by the supplier. *Staphylococcus aureus* V8 protease was a product of ICN Immuno Biologicals (Lisle, IL, USA).

Site-Directed Mutagenesis—The mutant enzymes were prepared by site-directed mutagenesis according to the method of Kunkel *et al.* (*29*) with a Muta-Gene™ *in vitro* mutagenesis kit (Bio-Rad Japan, Tokyo). The synthetic oligonucleotides used were listed in Table I.

The structures of mutant enzymes were confirmed by DNA sequence determination by the dideoxy method of Sanger and Coulson (*30*).

Expression of the Mutant Genes and Purification of Mutant RNase Rh—The site-specific mutants of pYE RNAP Rh, a plasmid for expression of RNase Rh, were transformed into *Saccharomyces cerevisiae* R27-7C-1C and the transformants were cultivated as previously reported

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Abbreviations and nomenclature: RNase Rh, a base non-specific and purine nucleotides-preferential RNase from *Rhizopus niveus*; RNase RNAP Rh, RNase Rh with an extra 3 amino acid residues, Ala-Ser-Gly, at the N-terminus.

Nomenclature of mutant enzymes produced by site-directed mutagenesis of RNase RNAP Rh: A mutant enzyme whose amino acid at the *i*th residue (X) is substituted by Y as a result of site-directed mutagenesis is abbreviated as X*i*Y. Thus, Y57L is a mutant enzyme in which the tyrosine residue at the 57th position has been replaced by leucine.

(*19*). Two liters of the culture supernatant was applied to a DEAE-cellulose column (3×25 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and eluted with a 1.6-liter linear gradient of NaCl (0-0.5 M) in the starting buffer. The fractions containing mutant RNase RNAP Rh were pooled, and concentrated in a rotary evaporator. The preparation was dialyzed thoroughly against 0.1 M NH_4HCO_3 buffer (pH 7.8). Limited digestion of mutant enzyme with Staphylococcal V8 protease and purification of mutant enzyme from the digest were performed as previously reported (*19-21*). The elution profiles of on column chromatographies of the mutant enzymes were verified by SDS-PAGE stained with Coomassie Brilliant Blue.

Protein Concentration—The protein concentration was determined spectrophotometrically assuming the molar absorbancy calculated from the amino acid composition.

Electrophoresis—SDS-PAGE was performed in 12% polyacrylamide gel by Laemmli's method (*31*) with protein molecular weight markers (Promega, Madison, WI, USA).

Enzyme Assay—(a) RNA as substrate: The enzymatic activity was measured by following the increase in acid-soluble nucleotides after digestion of yeast RNA at pH 5.0 and 37°C, as reported previously (*19*). (b) The rate of hydrolysis of RNA was monitored by following the change in the absorbancy at 300 nm (*32*) or 260 nm. (c) Dinucleoside phosphates as substrates: The rates of hydrolysis of dinucleoside phosphates were measured according to the method of Imazawa *et al.* (*33*) and Witzel and Barnard (*34*) by following the changes in absorbancy during the course of reaction at 20°C. Change in absorbancy was monitored with a Shimadzu UV200 spectrophotometer. The kinetic constants, K_m and V_{max}, of RNase RNAP Rh and its mutant enzymes were calculated from Lineweaver-Burk plots (*35*).

Circular Dichroism Spectra—CD spectra were measured with a JASCO J-600 spectropolarimeter at room temperature. The cells used for the wavelength regions of 200-250 and of 250-300 nm has light paths of 0.05 and 1 cm, respectively.

Release of Four Nucleotides from RNA—A small volume of the enzyme was added to the reaction mixture (2 ml) containing 5 mg of yeast RNA in 20 mM sodium acetate buffer (pH 5.0). The final concentration of the enzymes was about 45-344 nM for RNase RNAP Rh and the mutant enzymes. The reaction was carried out at 37°C. Samples (100 μl) were withdrawn at appropriate intervals. Ten

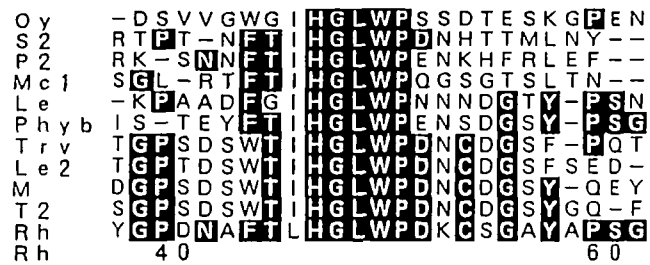

Fig. 2. **Comparison of the sequences of one of two common segments containing active site amino acids in RNase T_2 family RNases.** RNase Oy, oyster RNase (*17*); S2, self-incompatibility factor with RNase activity from *Nicotiana alata* (*13*); P2, self-incompatibility factor with RNase activity from *Petunia inflata* (*12*); Mc1, an RNase from *Momoridica charantia* (*11*); Le, an RNase from *Lycopersicon esculentum* (tomato) (*9*); Phyb, an RNase from *Physarum polycephalum* (*16*); Trv, an RNase from *Trichoderma viride* (*8*); Le2, an RNase from *Lentinus edodes* (shiitake) (*7*); M, an RNase from *Aspergillus saitoi* (*6*); T2, an RNase from *Aspergillus oryzae* (*6*); Rh, *Rhizopus niveus* RNase (*4*).

TABLE I. **Oligonucleotides for site-directed mutagenesis.**

Position in RNase Rh	Created mutation	Mutagenic oligonucleotide
Trp 49	Phe	5′-CATGGTCTTTGGCCCGATAAAT-3′
	Tyr	5′-CATGGTCTTTATCCCGATAAAT-3′
	Ala	5′-TCATGGTCTTGCTCCCGATAAAT-3′
	Ile	5′-TCATGGTCTTATTCCCGATAAAT-3′
Tyr 57	Phe	5′-CTGGTGCTTTTGCTCCTAG-3′
	Trp	5′-TCTGGTGCTTGGGCTCCTAGC-3′
	Gly	5′-TGTTCTGGTGCTGGTGCTCCTAGC-3′
	Ala	5′-TGTTCTGGTGCTGCTGCTCCTAGCG-3′
	Val	5′-TTCTGGTGCTGTCGCTCCTAGC-3′
	Ile	5′-TCTGGTGCTATTGCTCCTAG-3′
	Met	5′-TTCTGGTGCTATGGCTCCTAGC-3′
	Lys	5′-TTCTGGTGCTAAGGCTCCTAGC-3′

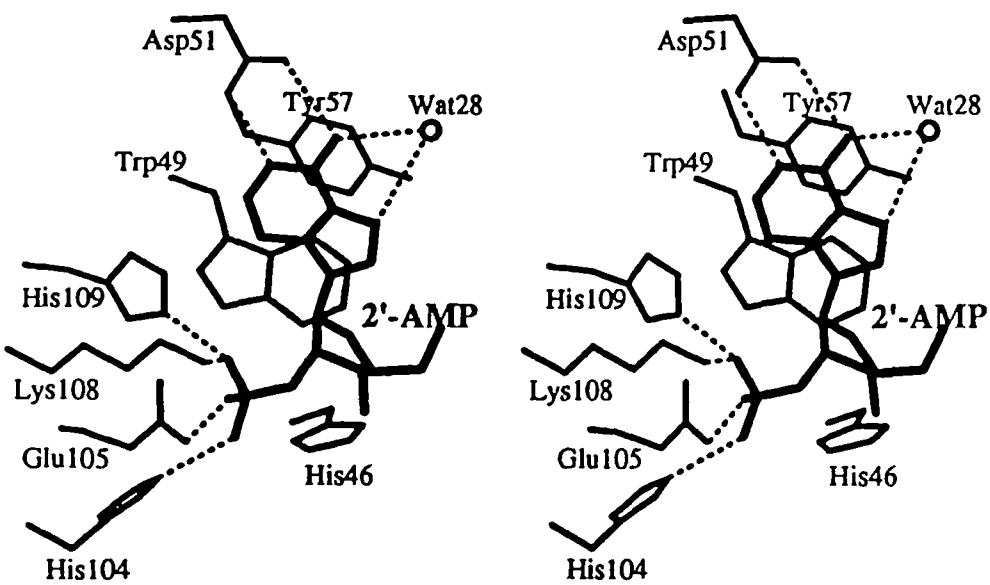

Fig. 1. **Stereo view of the active site of RNase Rh-2′-AMP complex.** The figure is taken from Ref. *28*.

volumes of ice-cold alcohol were added to the samples, which were then kept in a freezer overnight. The precipitate formed was removed by centrifugation, and the supernatant was dried *in vacuo*. The dried samples were dissolved in the buffer for HPLC, 20 mM sodium phosphate buffer (pH 6.45). The nucleotides were separated by HPLC on a column of TSKgel 80^{TM} (4.4×250 mm). The column was eluted with a linear gradient of acetonitrile (0.8%/15 min) in the same buffer. The flow rate was 1 ml/min. The eluate was monitored by measuring the absorption at 254 nm.

RESULTS

Preparation of Mutant Enzymes at Trp49 and Tyr57—In order to investigate the roles of Tyr57 and Trp49 in RNase Rh action by means of site-directed mutagenesis, we prepared various mutants in which Tyr57 was replaced by Phe, Trp, Gly, Ala, Val, Ile, Met, and Lys, and Trp49 was replaced by Phe, Tyr, Ala, and Ile. We investigated their specific activity and base specificity.

All mutant enzymes described in this paper were prepared by the procedures described in "MATERIALS AND METHODS" using oligonucleotides listed in Table I. All enzymes were purified to homogeneity on SDS-PAGE and their secondary structures were confirmed by the CD spectra between 200 and 250 nm to be the same as those of the native enzyme within the experimental error (data are not shown).

Enzymatic Properties of Trp49 Mutants—The enzymatic activities of Trp49 mutant enzymes with RNA as the substrate at pH 5.0 are shown in Table II. The pH optima of the Trp49 mutants were pH 5.0-5.5. These values are similar to that of the wild-type enzyme (pH 5.0). Therefore, the decrease in enzymatic activity was not due to the change in optimal pH. The RNA hydrolysis activities of all mutant enzymes are markedly lower than that of the native RNase Rh. The enzymatic activity of W49F was 16% of that of the native enzyme. However, substitution of the other amino acids, Tyr, Ala, and Ile, for Trp49 resulted in markedly decreased enzymatic activity. These data suggest that the Trp residue is very important for the efficient expression of activity.

The relative activities of the four mutant enzymes, W49Y, W49F, W49A, and W49I towards dinucleoside phosphates with the G base at the 3′-side (XpG, where X is one of A, G, U, and C) were smaller than those of RNase Rh (Table III). These results are essentially coincident with the case of RNA as a substrate. Kinetic constants indicated that only W49F has comparable activity to the native RNase Rh, and the K_m values of the mutant enzymes are very similar to each other. Among these mutant enzymes, the base specificity of W49F is unique; that is, the V_{max}/K_m values for CpG and UpG are comparable with those of native RNase Rh, but those for ApG and GpG are decreased markedly. Therefore, it became more pyrimidine base specific.

The rates of hydrolysis of four homopolynucleotides, poly A, poly I, poly U, and poly C by W49F and RNase RNAP Rh are shown in Table II. The rates of hydrolysis of poly A and poly I by W49F were decreased markedly, but those of poly U and poly C remained unchanged. Therefore, W49F was more pyrimidine base-specific, as anticipated from the experiments in Table III.

It is also very interesting that in contrast to the case of substitution of Phe for Trp, that for Tyr is unfavorable for enzymatic activity. We have no explanation for this phenomenon, though possibly the hydroxy group of tyrosine residue interacts with some neighboring amino acid side chain, such as that of Asp51 and Glu105, interfering with the substrate binding or catalysis.

Enzymatic Properties of Tyr57 Mutant Enzymes—The substitution of aromatic amino acid residues for Tyr57 did not have any marked influence on the enzymatic activity of RNase Rh toward RNA (Table II).

TABLE II. **Relative rates of hydrolysis of RNA and homopolynucleotides by RNase RNAP Rh and its mutant enzymes at Trp49 and Tyr57.**[a]

Enzyme	Relative rate of hydrolysis				
	RNA	Poly A	Poly I	Poly U	Poly C
RNase RNAP Rh	1.00	1.00	1.00	1.00	1.00
W49F	0.16	0.24	0.04	1.33	1.08
W49Y	0.02	0.11	0.01	0.13	0.18
W49A	0.06	0.08	0.03	0.08	0.07
W49I	0.07	0.07	0.03	0.11	0.08
Y57F	0.84	0.91	1.56	1.78	0.93
Y57W	0.93	1.70	3.47	1.45	0.83
Y57G	1.29	0.96	3.29	1.09	0.29
Y57A	1.86	2.18	6.71	1.17	0.38
Y57V	1.31	2.83	3.98	0.98	0.81
Y57I	3.97	1.57	0.97	2.47	1.01
Y57M	1.78	1.22	0.99	0.90	1.11
Y57K	0.94	1.28	0.51	0.71	0.91

[a]Enzymatic activity is expressed relative to that of native RNase Rh as 1.00.

TABLE III. **Kinetic constants of mutant enzymes at Trp49 and Tyr57 derived from RNase Rh.**

	ApG			GpG			CpG			UpG		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Rh	0.52	13,400	25,700	0.45	40,900	90,900	1.08	55,400	51,300	0.63	39,900	65,300
W49F	0.10	1,650	16,500	0.40	2,750	6,900	0.86	59,000	58,100	0.40	14,600	36,700
W49Y	0.26	41	157	0.60	64	105	0.40	137	342	0.23	115	500
W49A	0.23	161	709	0.26	207	796	0.48	75	156	0.71	99	140
W49I	0.26	258	1,010	0.27	196	725	0.53	222	418	0.51	229	449
Y57F	0.37	22,100	59,800	0.37	11,640	31,400	0.50	13,400	26,800	1.17	14,000	12,000
Y57W	0.38	36,200	95,300	0.30	39,000	130,000	0.57	25,000	44,000	0.40	22,800	57,000
Y57I	0.93	9,400	10,100	0.48	5,270	11,000	1.17	6,260	5,350	0.69	1,170	7,700
Y57M	3.3	36,500	11,000	0.70	11,400	16,300	0.71	6,880	9,700	0.59	2,620	4,440
Y57K	1.28	5,880	4,590	0.67	5,290	14,300	0.95	3,360	3,560	0.56	775	758

K_m, V_{max}, and V_{max}/K_m values are expressed as follows: K_m, $M\times10^4$; V_{max}, min^{-1}; V_{max}/K_m, $M^{-1}\cdot min^{-1}\times10^{-4}$.

The enzymatic activities towards RNA of mutant RNase Rh's having aliphatic amino acid residues at the Y57 position varied in the range of 94-397% of that of the wild type, depending on the side chain. The kinetic constants toward XpG of several typical mutant enzymes are listed in Table III. The enzymatic activities of most of the mutant enzymes towards XpGs are lower than those of RNase Rh. The substitution of Phe and Trp for Tyr57 retained higher enzymatic activity. The K_m values of the mutant RNases with an aliphatic amino acid at this position toward ApG and GpG are inclined to be increased from the value of the native enzyme. The results indicated that a Tyr or Trp residue at the 57th position (B_1 site) seems to be favorable for the cleavage of ApG and GpG, possibly owing to stacking and/or other factors. The mutant enzymes in which the Tyr57 was replaced by amino acid with an aliphatic side chain are active, though the change is unfavorable for the binding of purine base and for enzymatic activity towards XpGs. This is probably due to the weak interaction of the side chains with the base moiety of the substrate or some conformational change induced by the side chain.

All mutant enzymes with an aromatic side chain amino acid at the 57th position seem to be purine-preferential. The base specificity of Y57W mutant is unique. The K_m values for four substrates were decreased slightly and the V_{max}/K_m values for ApG and GpG were greater than the other two, indicating that Y57W had become more purine base-specific.

In the hydrolysis of four polynucleotides by RNase Y57W (Table II), the rates of hydrolysis of poly A and poly I by RNase Y57W were increased markedly, but those of poly U and poly C were not much changed. The reason for the higher enzymatic activities towards RNA of several mutant enzymes such as Y57I, Y57A, Y57V, is unclear. However one possible explanation is suggested by the following experiment. Enzymatic activities of several mutant enzymes, Y57I, Y57A, and Y57G, at different concentrations of RNA are shown in Fig. 3. At higher concentration of the substrate, RNase Rh was inhibited, even at less than 0.01 mg/ml of RNA. Since the three

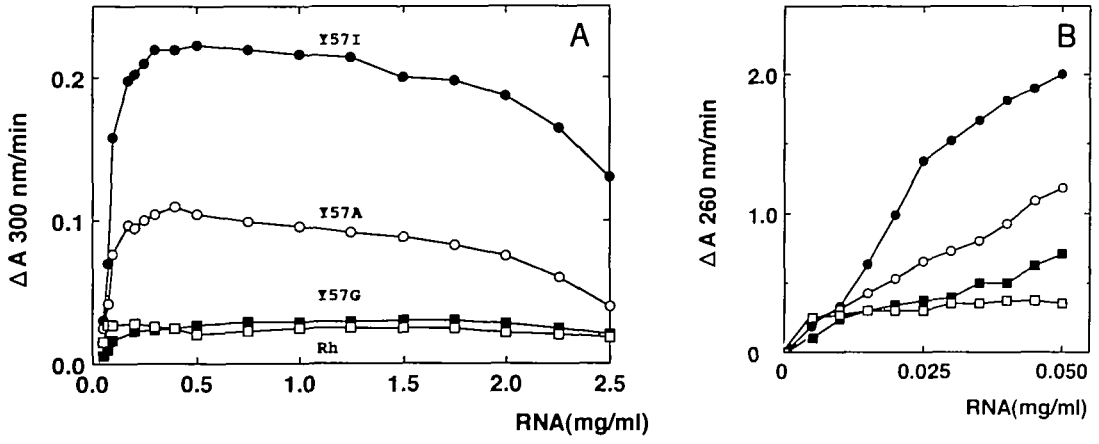

Fig. 3. **Enzymatic activity of RNase Rh and its Tyr57 mutant enzymes at various RNA concentrations.** The enzymatic activity was measured by following the decrease in absorbancy at 300 nm and increase in absorbancy at 260 nm upon hydrolysis of RNA (*32*), respectively. A: RNA concentration 0-2.5 mg/ml. The reaction was followed in terms of the decrease in absorbancy at 300 nm. B: RNA concentration, 0-0.05 mg/ml. The reaction was followed in terms of increase in absorbancy at 260 nm. ●, Y57I; ○, Y57A; ■, Y57G; □, RNase Rh. Enzyme concentration was *ca.* 40 nM.

TABLE IV. **Kinetic constants of RNase Rh, Y57W, and W49F towards XpYs at pH 5.0 and 20°C.**

	RNase Rh			Y57W			W49F		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
ApG	0.52	13,400	25.7	0.38	36,200	95.3	0.10	1,650	16.4
GpG	0.45	40,900	90.9	0.30	39,000	130	0.40	2,750	6.9
CpG	1.08	55,400	51.3	0.57	25,000	44	0.86	50,000	58.1
UpG	0.63	39,900	65.3	0.40	22,800	57	0.40	14,600	36.7
ApA	0.65	4,240	6.5	0.15	3,050	20	0.11	2,830	2.6
GpA	0.72	1,930	2.7	0.43	4,800	22.7	0.80	226	0.3
CpA	1.32	13,900	10.5	0.61	8,280	13.5	0.56	22,900	40.1
UpA	1.44	27,500	19.1	1.08	11,200	10.3	0.58	15,700	27.1
ApC	1.30	58,900	45.3	0.78	48,600	62.3	0.46	12,500	27.1
GpC	1.14	58,500	51.3	0.63	77,300	122	0.58	9,620	16.5
CpC	0.49	10,600	21.7	1.21	21,000	17.5	0.84	33,200	39.5
UpC	0.68	18,000	26.5	0.48	15,900	33.0	0.77	35,700	46.3
ApU	0.78	12,800	16.4	0.59	14,700	24.9	1.00	2,360	2.4
GpU	0.79	11,600	14.6	0.86	45,400	52.7	0.56	597	1.1
CpU	0.76	25,600	33.9	0.85	25,600	30.9	1.13	35,900	31.7
UpU	1.26	19,200	15.2	2.53	19,600	4.6	1.26	19,800	15.7

K_m, V_{max}, and V_{max}/K_m are expressed as follows: K_m, $M \times 10^4$; V_{max}, min^{-1}; V_{max}/K_m, $min^{-1} \cdot M^{-1} \times 10^{-7}$.

TABLE V. **Release of four nucleotides from RNA by treatment with RNase Rh mutants at Tyr57 and Trp49.**

Enzyme	Released nucleotides
RNase Rh	A>G≫U>C
W49F	A>C>U,G
W49Y	A≫C>U,G
W49A	G>C,U,A
W49I	G≫A>U,C
Y57F	G>U>C,A
Y57W	A,G≫C>U
Y57G	G>U>A>C
Y57A	G>A>U>C
Y57I	G≫U,A>C
Y57M	G>C≫U,A
Y57K	G>C>U,A

mutant enzymes are less sensitive to the substrate inhibition, they have higher activity under the usual assay condition (2.5 mg/ml) than RNase Rh. Since substrate inhibitions is often observed in the case of a higher-affinity substrate, apparent activation by the mutant enzyme is possibly due to the lower affinity to RNA of mutant enzymes with an aliphatic side chain at the 57th position.

Release of Four 2′,3′-Cyclic Nucleotides and 3′-Nucleotides from RNA by Digestion of RNA with Mutant Enzymes at Tyr57 and Trp49—The release of four 2′,3′-cyclic nucleotides and 3′-nucleotides from RNA by digestion with Y57 mutant enzymes except for Y57V was measured, and the results are shown in Table V. The order of nucleotide release by the wild-type enzyme was A>G>U,C. The order for Y57W is A,G>C,U showing preference for A and G. This phenomenon can be well understood in terms of the base specificity shown in Tables III and IV. The mutant enzymes with a longer side chain released guanylic acid faster than the other ones, suggesting some change in base specificity. Although the results in Table V reflect the cleavage at the 3′-side and 5′-side of the nucleotide released, it is difficult to interpret the changes in the order of nucleotide release simply in terms of the specificity of the B_1 site (major binding site). However, it is evident that the modification of this site could influence the base specificity.

On the other hand, mutation at W49 caused a marked decrease in enzymatic activity. However, the mutants with an aromatic amino acid residue, such as Tyr or Phe showed a different order of release of nucleotides, A>C>U,G, as compared to that of RNase Rh (A>G>C,U). Mutants, with substitution for aliphatic amino acid at this site, showed a preference for guanine base. To elucidate this complex perturbation of base specificity, we need more information on the specificity of the B_2 site [the site responsible for the binding of the base (B_2 base) at the 3′-side of the scissile bond] of RNase Rh. As described above, W49F released nucleotides in the order of A>C>U>G. The most rapid release of adenylic acid is not consistent with the observed kinetic constants of dinucleoside phosphates, but increased preference for U and C as compared to G could be expected from the results of Table III.

The kinetic constants of two unique enzymes with relatively higher activity, W49F and Y57W, toward 16 dinucleoside phosphates showed that the base preferences of W49F and Y57W are significantly different from that of RNase Rh. The former is more purine-specific and the latter is more pyrimidine-specific. To confirm this, the kinetic constants for XpA, XpC, and XpU were studied. The results are summarized, together with those of XpG, in Table IV. The results also showed the preference of W49F for pyrimidine bases and of Y57W for purine bases at the B_1 site.

DISCUSSION

X-ray crystallography of RNase Rh-2′-AMP complex showed that Tyr57 and Trp49 are stacked with the B_1 base of the substrates. We first of all prepared Y57I as an example of non-aromatic substitution and found a marked increase in RNA depolymerization activity. The substitution of a non-aromatic amino acid for Tyr57 causes some decrease in the enzymatic activity towards dinucleoside phosphates, in contrast to an apparent increase in the activity for RNA.

In the mutant enzymes with an aliphatic side chain at the 57th position, the K_m values for XpGs are slightly increased in the cases of ApG and GpG. Enzymatic activity of these mutant enzymes towards XpGs was decreased markedly. That is, enzymatic activities were influenced more sensitively by substitution at this position than K_m values. This phenomenon could be explained in several possible ways: (i) V_{max} of the native enzyme is so large that the K_m values of RNase Rh are larger than true K_s (dissociation constant of enzyme-substrate complex) values, so the apparent K_m values are not much affected by decrease in binding ability; (ii) the binding constants of the mutant enzymes with an aliphatic side chain and an aromatic amino acid residue at the 57th position are not so different, but the conformation induced by binding of substrate markedly influences on the activity, directly or indirectly. The stacking of the aromatic amino acid residues of the base recognition site seems to be related to the catalytic activity of the enzyme. It is evident that the introduction of the aliphatic side chain amino acid at this position profoundly affects the V_{max} value. The change in base specificity caused by introducing Trp in place of Tyr57 indicated some interaction of the base and the aromatic amino acid side chain, in spite of the very small change in K_m values. The X-ray crystallographic data of the Y57W mutant with 2′-AMP, showed that Trp57 is properly stacked with purine base too (Nakamura *et al.*, unpublished data). These data suggest some role of stacking in the enzymatic activity.

From the data described above, we concluded that an aromatic residue at the 57th position, Tyr, Phe, or Trp, was necessary to keep higher activity against dinucleoside phosphates, that is, the stacking with B_1 base is favorable for the activity. The X-ray crystallography data also suggested the possibility of stacking of these amino acid side chains (*28*). As mentioned above, proper stacking by Trp residue with B_1 base possibly increased the purine specificity. However, in mutant enzymes with an aliphatic side chain at the 57th position, CH/π interaction (*36*) could be important, although such amino acid resides are less effective than Tyr57 as a component of base recognition site. These results indicate that Tyr57 is an effective lid for the active site cleft, but not an essential one. The discrepancies between the effect of substitution at the 57th position on relative activities for RNA and dinucleoside phosphate suggested the presence of different factors which affect the interaction of polynucleotides with RNase Rh,

such as polyanion-enzyme interaction.

On the other hand, the W49 residue is conserved in all RNases in RNase T_2 family enzyme. The substitution of this residue for other amino acids markedly decreased the enzymatic activity toward both RNA and dinucleoside phosphates. Therefore, the Trp residue seems to be very important for the active conformation of the enzyme, because it is located near the central part of the active site (*27*) and local conformational change induced by mutation may affect the mutual relation of the active site amino acid residues. Among the mutants tested, W49F is most active and becomes more pyrimidine base-specific, indicating a direct or an indirect interaction between Trp49 and pyrimidine bases, though this was not apparent from the K_m values. This property could be very useful for further studies to modify the base specificity of RNase Rh by site-directed mutagenesis. All these experiments suggested that we can modify the base specificity of RNase Rh by protein engineering.

RNase Rh released four nucleotides from RNA in the order of A>G>C,U (*37*). However, we could not explain the adenylic acid preference of RNase Rh from the kinetic data shown in Tables II and III in terms of a simple rule, such as specificity at B_1 (B_2), base dependence of K_m value, or V_{max}. It seems to be necessary to consider the combination of B_1 and B_2 bases in relation to the cleavage of XpYs. A similar indication was noted in the cases of plant RNases (*38*).

In terms of the V_{max}/K_m values in Table IV, the order of base preference of B_1 and B_2 of RNase Rh is G>U>C>A and G>C>U>A, and that of the B_1 and B_2 sites of Y57W is G>A>U,C and G>C>U>A, respectively. The changes in base specificities in Y57W and W49F should be helpful for understanding the factors determining the base specificity of RNase Rh.

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