The Modification of Tyrosyl Residues of a Minor Ribonuclease from *Aspergillus saitoi*

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The role of tyrosyl residues in the enzymatic activity and the state of tyrosyl residues of guanine preferential ribonuclease (RNase Ms) from *Aspergillus saitoi* were studied.

1) The solvent perturbation difference spectra of RNase Ms measured with ethylene glycol and polyethylene glycol as perturbants indicated that *ca.* 5 tyrosyl residues and one tryptophan residue were exposed to solvents.

2) The spectrophotometric titration of RNase Ms indicated that *ca.* 5 tyrosyl residues were titrated first, giving an apparent pK_a value of 10.5, and the rest of the tyrosine residues gave higher pK_a values.

3) About 8 tyrosine residues in RNase Ms were acetylated by excess *N*-acetylimidazole and about 6 of them were reactive towards a relatively low concentration of the reagent. Extrapolation of the curve showing the relation between the tyrosyl residues modified and the remaining activity indicated that the enzyme was inactivated when 4—5 tyrosyl residues were modified.

4) The chemical modification of RNase Ms with diazonium 1(*H*)-tetrazole was also studied. The plot of the relationship between the residual activity and the tyrosyl residues modified showed that 1—1.5 tyrosyl residue(s) was responsible for the loss in enzymatic activity.

5) The enzymatic activities of RNase Ms modified by the two kinds of tyrosine-modifying reagents were measured with ribonucleic acid (RNA) and guanyl(3′→5′)cytidine (GpC) as substrates at pH 5.0. The loss in enzymatic activity was more marked when RNA was used as a substrate than with GpC as a substrate.

6) Based on the reported (Heinemann and Saenger, *Nature*, **299**, 27 (1982)) three-dimensional structure of RNase T_1, which is about 60% sequence-homologous with RNase Ms, the possible site of the diazotization is discussed.

Keywords——ribonuclease; *Aspergillus saitoi*; chemical modification; tyrosine; *N*-acetylimidazole; diazonium 1(*H*)-tetrazole; active site; spectrophotometric titration; solvent perturbation

Aspergillus saitoi produces two base non-specific ribonucleases (RNase), an adenine preferential RNase (RNase M) and a guanine preferential RNase (RNase Ms).[1,2] The primary sequence of the latter enzyme has been elucidated in our laboratory and its molecular weight was determined to be 11714.[3] The active site or RNase Ms consisted of two histidine residues,[4,5] a carboxyl group of glutamic acid[5,6] and an arginine residue.[7] The locations of these functional groups on the primary structure of RNase Ms were also determined.[5] The RNase Ms contained 12 tyrosine residues, 1 tryptophan residue and 4 phenylalanine residues as aromatic amino acids. The single tryptophan residue was exposed on the surface of the enzyme, was easily photooxidized in the presence of methylene blue and was not directly related to the active site of RNase Ms.[4]

In this paper, we investigated the state of tyrosyl residues and the role of these residues in the enzymatic activity by chemical modification with *N*-acetylimidazole and diazonium 1(*H*)-

tetrazole.

Materials and Methods

Enzyme——RNase Ms was purified from the commercial digestive "Molsin" (*Asp. saitoi*, Seishin Pharm. Co., Ltd.) as described in the previous paper.[2]

Reagents——Ribonucleic acid (RNA) was purchased from Kojin Co., Ltd. (Tokyo). Homopolymers, poly A, poly I, poly U and poly C were obtained from Yamasa Co., Ltd. (Choshi, Chiba). GpC was a product of Sigma (St. Louis, Mo.). N-Acetylimidazole was obtained from Seikagaku Kogyo (Tokyo). Diazonium $1(H)$-tetrazole (DHT) was prepared as described by Horinishi *et al.*[8] from aminotetrazole (Tokyo Kasei, Tokyo). N-Acetyltyrosinamide and N-acetyltryptophan ethyl ester were obtained from Sigma. Ethylene glycol and polyethylene glycol used as perturbants were the products of Wako Pure Chem. (Tokyo).

Enzyme Assay——(1) RNase activity was measured with RNA as a substrate in terms of the increase in absorbancy at 260 nm of acid-soluble nucleotides formed at pH 5.0 as described previously.[1] (2) RNase activity measured with GpC as a substrate. The assay procedure was essentially the same as that reported by Imazawa *et al.*[9] Reaction mixture (1.1 ml) containing 0.1 M acetate buffer (pH 5.0) and 32—158 μM GpC was incubated at 25 °C. The reaction was followed by measuring the increase in absorbancy at 280 nm after addition of 10—20 μl of enzyme solution.

Kinetic Parameters——K_m and V_{max} for the native RNase Ms and chemically modified RNase Ms were determined by means on Lineweaver and Burk's double reciprocal plot[10] with RNA and GpC as substrates. The substrate concentrations used were 0.5—2.5 mg/ml for RNA and 35—158 μM for GpC. The final enzyme concentrtions used were 0.2—0.5 μM for RNA and 0.17—0.85 μM for GpC.

Modification of RNase Ms with N-Acetylimidazole——RNase Ms (38.7 μM) was dissolved in 20 mM borate buffer (pH 7.5) and mixed with various amounts of the reagents in a small volume (final reagent concentration was 0—174 mM, and the final volume of the reaction mixture was 1.75 ml). The reaction mixture was kept at 37 °C for 1 h, then dialyzed against 0.1 M acetate buffer (pH 5.0) for 5 h to remove the excess reagent. The ultraviolet (UV) spectra of the dialyzates were measured. Tyrosyl residues modified were estimated from the decrease in absorbancy at 287 nm using a molar extinction coefficient of 1160 $M^{-1} \cdot cm^{-1}$ for the modification of one mole of tyrosyl residue.[11] The enzymatic activities were measured by using assay method (1) or (2) as described in the enzyme assay.

Hydroxylamine Treatment of Acetylated RNase Ms——A mixture of the acetylated RNase Ms and an equal volume of 0.2 M NH$_2$OH in 20 mM borate buffer (pH 7.5) was incubated at 37 °C overnight. The increase in absorbancy at 278 nm and the enzymatic activity toward RNA as a substrate were measured.

Modification of RNase Ms with DHT——The modification procedure was essentially the same as that reported by Kasai for the diazotization of RNase T$_1$.[12] RNase Ms in 0.5 M NaHCO$_3$ buffer (pH 8.8) (the final RNase Ms concentration was in the range of 10—100 μM) was mixed with 10 volumes of a solution of DHT prepared according to Horinishi *et al.*[8] and the reaction was terminated by the addition of 0.2 M NaN$_3$. The pH of the reaction mixture was adjusted to 5.0 by the addition of 6 N HCl. To remove excess NaN$_3$, the enzyme solution was dialyzed against 10 mM acetate buffer (pH 5.0) overnight. The contents of mono- and didiazotized tyrosyl residues in RNase Ms were measured spectrophotometrically according to Takenaka *et al.*[13]

Protein Concentration——RNase Ms concentration was determined from the absorbancy at 280 nm taking $A_{cm}^{280 nm}$ (1%) to be 17.[2] The protein concentration of the chemically modified protein was determined from the amino acid analysis, especially the Phe and Ala contents in protein after 6 N HCl hydrolysis taking those of native RNase Ms as standards.

Amino Acid Analyses——Samples of *ca.* 0.1—0.2 mg of protein were hydrolyzed in evacuated tubes with 6 N HCl at 110 °C for 24 h. Amino acid analyses were performed by the method of Spackman *et al.*[14] with a Nihondenshi JEOL 6AH amino acid analyzer.

Edman Degradation——The amino-terminal amino acid was determined by manual Edman degradation as described by Edman.[15]

Solvent Perturbation Difference Spectrum——Solvent perturbation difference spectra were measured according to the procedure of Herskovits and Laskowski[16] with ethylene glycol and polyethylene glycol as perturbants. The model compounds used were N-acetyl-L-tyrosinamide and N-acetyltryptophan ethylester. All spectra were measured with a Shimadzu UV 200 spectrophotometer. The protein concentration used was 50 μM and all the spectra were measured at pH 5.0.

Spectrophotometric Titration——The increase in absorbancy at 295 nm of RNase Ms solution (40 μM) was measured at various pH's. The pH of the solution was adjusted by adding very small amounts of NaOH (2 N). The amount of tyrosyl residues dissociated was calculated from the $\Delta \varepsilon_{295 nm} = 2320$ according to Beaven and Holiday.[17]

Circular Dichroism (CD) Spectra——CD spectra were measured with a JASCO J-40 spectropolarimeter at room temperature in cells of 0.2- and 0.5-cm light path for the wavelength regions of 200—240 nm and 240—320 nm, respectively. The enzyme concentrations used were 5 and 50 μM for short-wavelength and long-wavelength regions, respectively.

Results

Solvent Perturbation Difference Spectrum of RNase Ms

RNase Ms contains 12 tyrosyl residues. In order to estimate the distribution of these tyrosyl residues on the surface of the enzyme molecule, the perturbation difference spectroscopy developed by Herskovits and Laskowski[16] was applied to RNase Ms using ethylene glycol and polyethylene glycol as perturbants at pH 5.0. From the difference spectrum, the numbers of tyrosyl and tryptophyl residues distributed on the surface of the molecule were estimated (Table I and Fig. 1). The single tryptophyl residue in RNase Ms and 5 tyrosyl residues are thought to be exposed on the surface of the molecule (Table I).

Spectrophotometric Titration

The tyrosine residues in RNase Ms were titrated spectrophotometrically by measuring the increase in absorbancy at 295 nm. The results are shown in Fig. 2. About 5 tyrosyl residues were titrated up to pH 11 and their apparent pK's are approximately 10.5. Three tyrosine residues were titrated thereafter, and their pK_a values were about 11.3. The rest of the tyrosyl residues were finally titrated time-dependently by further increase of pH. The reverse titration
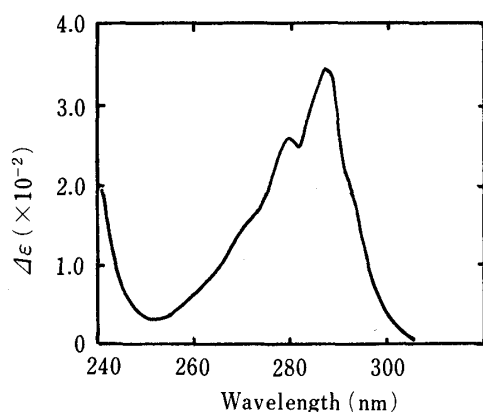

Fig. 1. Solvent Perturbation Difference Spectrum of RNase Ms at pH 5.0

The difference spectrum was measured between RNase Ms in 0.1 M acetate buffer containing 30% ethylene glycol and RNase Ms in the same buffer without ethylene glycol.
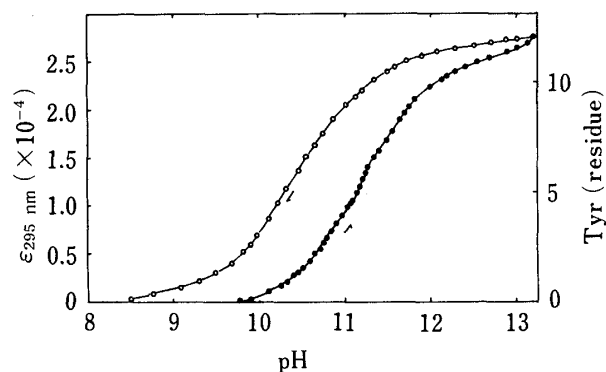
Fig. 2. Spectrophotometric Titration of RNase Ms at 20 °C

The experimental conditions were as described in Materials and Methods. ●, forward titration; ○, backward titration.

TABLE I. $\Delta\varepsilon$ Values per 10% Ethylene Glycol and Polyethylene Glycol for RNase Ms and the Numbers of Tyrosyl and Tryptophyl Residues in RNase Ms Perturbed with Ethylene Glycol and Polyethylene Glycol at pH 5.0

Perturbant	Ethylene glycol		Polyethylene glycol	
Wavelength	285 nm	292 nm	295 nm	292 nm
N-Acetyltryptophan ethylester	86	147	99	166
N-Acetyltyrosinamide	55	7.4	73	9.6
RNase Ms	355	179	530	249
Tyrosyl residues perturbed[a]	4.93		5.65	
Tryptophyl residues perturbed[a]	0.97		1.17	

a) Tyrosyl and tryptophyl residues perturbed in ethylene glycol and polyethylene glycol were calculated from $\Delta\varepsilon/10\%$ solvent and those of the model compounds, N-acetyltryptophan ethyl ester and N-acetyltyrosinamide, at 285 and 292 nm.

revealed that all tyrosyl residues were normalized and their average pK value was about 10.5. These results indicated that several groups of tyrosyl residues exist in RNase Ms, and 5 tyrosyl residues were relatively easily titratable. Since the differences in the pK_a values of the groups of tyrosyl residues were small, it was very difficult to estimate the exact numbers and pK_a values of the groups. However, the results of spectrophotometric titration, which indicated that about 5 tyrosyl residues had an almost normal pK_a value, were qualitatively identical with those of the solvent perturbation study described above.

Modification of RNase Ms with N-Acetylimidazole

In order to investigate the reactivity, distribution and contribution to the enzymatic activity of tyrosyl residues in RNase Ms, a chemical modification study with N-acetylimidazole was carried out. The reagent was developed by Simpson et al. and is known to be reactive with tyrosyl residues under mild conditions.[11] The reaction of tyrosyl residues in RNase Ms increased with increase of the reagent concentration up to 100-fold molar excess of the reagent, and about 6 tyrosyl residues were acetylated under the reaction conditions used (Fig. 3). The residual activity of the modified enzyme was approximately 10% of the original level. By increasing the reagent concentration to 450-fold molar excess, 2 or 3 further tyrosyl residues were modified. All of the tyrosyl residues were easily modified by N-acetylimidazole

TABLE II. The Inactivation of RNase Ms by N-Acetylimidazole
Measured with RNA and GpC as Substrates at pH 5.0

N-Acetylimidazole/enzyme (molar ratio)	Relative activity (%)	
	Substrate	
	RNA[a]	GpC[b]
0	100	100
75	17.5	31.3
100	13	17.5
275	6.0	7.4

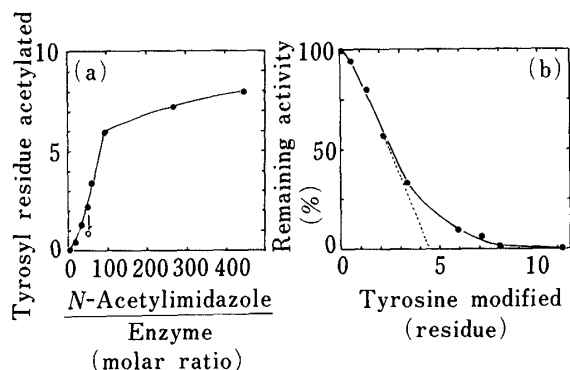Substrate concentration: a) RNA, 2.5 mg/ml; b) GpC, 35.7 μM.

Fig. 3a. Acetylation of RNase Ms with N-Acetylimidazole at pH 7.5

Open circle, RNase Ms modified in the presence of 0.2 M phosphate. The other experimental conditions were as described in Materials and Methods.

Fig. 3b. The Relation between the Residual Activity (%) and the Number of Tyrosyl Residues Acetylated
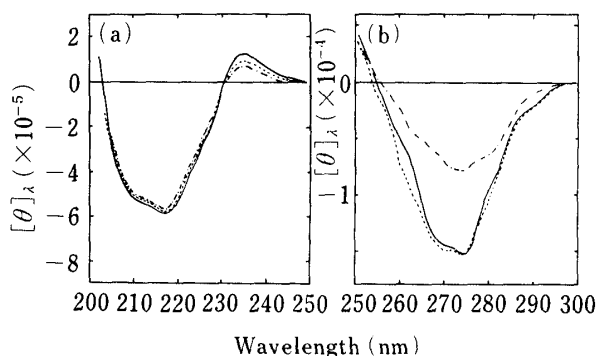

Fig. 4. CD Spectra of RNase Ms, RNase Ms Modified by N-Acetylimidazole (Residual Activity 13%) and Acetylated RNase Ms Treated with Hydroxylamine

The experimental conditions were as described in Materials and Methods. ——, native RNase Ms; – – – –, acetylated RNase Ms (residual activity 13%); ------, acetylated RNase Ms treated with hydroxylamine.

in the presence of 8 M urea (not shown). The initial incorporation of acetyl groups was somewhat depressed in the presence of 0.2 M phosphate buffer. The relation of the residual activity and the extent of tyrosyl residue modification is shown in Fig. 3b. By extrapolation of the curve to zero activity, it was concluded that the modification of about 4—5 tyrosyl residues seemed to induce the inactivation of the RNase Ms. The enzymatic activity of acetylated RNase Ms was measured with GpC as a substrate. The enzymatic activity thus measured was always larger than that measured with RNA as a substrate (Table II). A similar tendency will be described later in the case of the modification of RNase Ms with DHT.

The CD spectrum of the acetylated RNase Ms having 13% residual activity was measured. The CD spectrum of acetylated RNase Ms in the short-wavelength region (205—240 nm), which is considered to attributable to the peptide backbone conformation of the protein, was practically identical with that of the native RNase Ms (Fig. 4). Thus, it could be concluded that, as far as the CD spectrum is concerned, the peptide backbone structure of acetylated RNase Ms remained intact up to this stage of modification. The depth of the CD trough at 275 nm (possibly due to tyrosyl residues) decreased to about 50% of that of the native enzyme. The peak height at 230 nm also decreased. These results indicated a large contribution of acetylatd tyrosyl residues to the CD bands around 250—300 nm. Hydroxylamine treatment of the acetylated RNase Ms completely restored the enzymatic activity and the depth of the trough at 275 nm and partially restored the peak height at 230 nm (ca. 75%) (Fig. 4b).

Modification of RNase Ms with DHT

It is known that DHT can selectively modify several tyrosyl residues of RNase T_1, which is structurally related to RNase Ms.[12] Therefore, we tried to modify RNase Ms with this reagent. The results are shown in Fig. 5. The absorption spectrum of DHT-treated RNase Ms gave an absorption maximum at 495 nm at pH 10.0 (Fig. 5). Although the spectrum strongly indicated that the major product of the modification was monodiazotized tyrosine residues,[8] the amounts of di-DHT and mono-DHT-tyrosyl and mono-DHT-histidyl residues were calculated from the absorption spctrum according to the method of Takenaka et al.[13] The
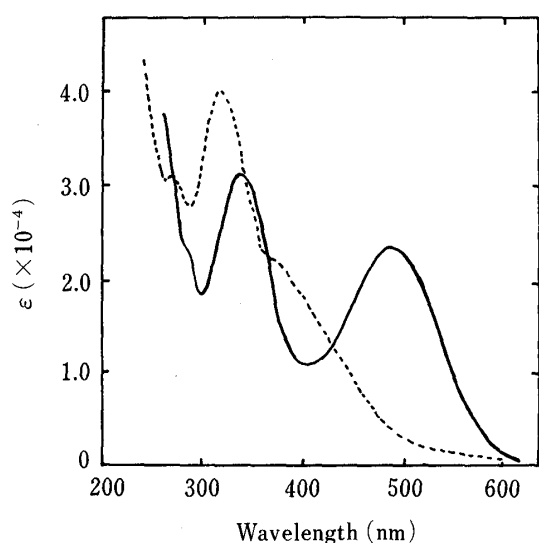
Fig. 5. Absorption Spectra of DHT-Modified RNase Ms (Residual Activity 16%) at pH 3.0 and 10.0
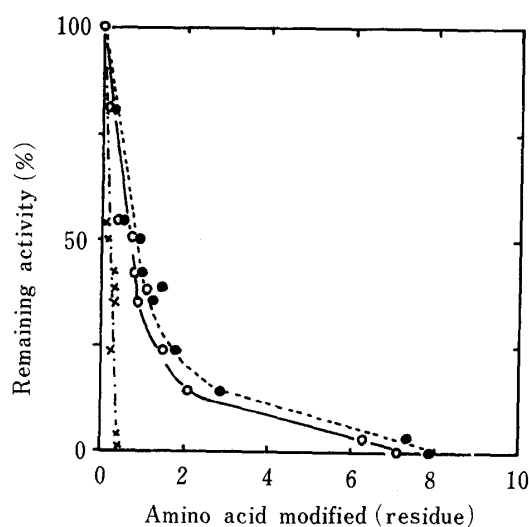
————, pH 3.0; ———, pH 10.0.

Fig. 6. The Formation of DHT-Tyrosine and DHT-Histidine during the Course of Inactivation of RNase Ms with DHT at pH 8.8

---●---, DHT-Tyr; —○—, mono-DHT-Tyr; —×—, DHT-His. The amounts of DHT-modified tyrosine and histidine were calculated from the absorption spectra according to Takenaka et al.[13]
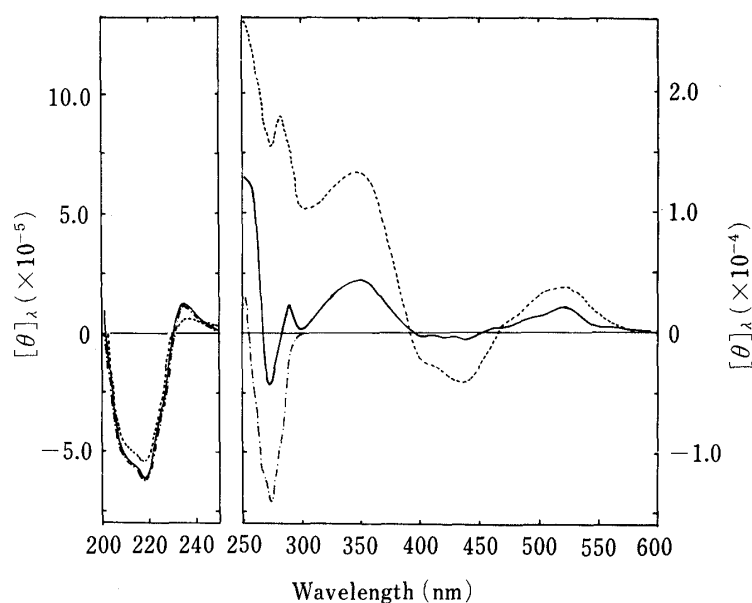
Fig. 7. CD Spectra of DHT-RNase Ms (Residual Activity, 74% and 49%) and RNase Ms

The experimental conditions were as described in Materials and Methods. —·—·—, native RNase Ms; ———, DHT-RNase Ms (residual activity 74%); ————, DHT-RNase Ms (residual activity 49%).

TABLE III. Kinetic Parameters of DHT-RNase Ms

Substrate	RNA[a]		GpC[b]	
	K_m (mg/ml)	V_{max} (OD, $\mu M^{-1} \cdot min^{-1}$)	K_m ($M \times 10^4$)	V_{max} (min^{-1})
Native RNase Ms	0.50	0.36	2.04	3685
DHT-RNase Ms				
32% activity	0.77	0.082	2.27	3685
15% activity	1.11	0.034	2.00	935
3% activity	—	—	2.50	55

a) pH 5.0 (0.05 M, acetate), 37 °C.   b) pH 5.0 (0.05 M, acetate), 22 °C.

results are shown in Fig. 6, and indicate that the diazotization products were mostly mono-DHT-tyrosyl residues, and the formation of DHT-histidine and di-DHT-tyrosine is negligible. The enzymatic activity became almost zero after diazotization of 8 tyrosyl residues. When the curve in Fig. 6 was extrapolated to zero activity, modification of about 1.5 tyrosyl residues seemed to be responsibile for the loss in enzymatic activity. Since DHT is known to react with the NH_2-group of RNase T_1, we examined the N-terminal amino acid residue of DHT-modified RNase Ms having 13% activity by the procedure of Edman.[15] We were unable to detect any phenylthiohydantoin (PTH)-glutamic acid from the DHT-RNase Ms. Therefore, as reported by Kasai[12] for RNase T_1, the N-terminal α-NH_2-group of RNase Ms was modified by DHT. Since the α-NH_2-group of RNase Ms is not important for the enzymatic activity,[18] the inactivation by DHT might be due to the diazotization of tyrosyl residue(s).

CD spectra of diazotized RNase Ms having 74% and 49% activity are shown in Fig. 7. The CD bands between 205—240 nm of these enzymes are very similar to that of the native RNase Ms. The CD bands in the visible light region were very complex, and the Cotton effect

TABLE IV.   Depolymerization of Homopolynucleotides by DHT-RNase Ms[a]

Substrate	Poly G	Poly I	Poly A	Poly C
Native RNase Ms	100	100	100	100
DHT-RNase Ms				
32% activity[b]	30	20	25	36
15% activity	19	7.9	8.5	4.4
3% activity	1.6	—	3.1	0.61

a) Expressed as the relative activity with respect to the native RNase Ms (100%).   b) Enzyme activity measured with RNA as a substrate.

observed at the wavelength region of 300—600 nm might be due to the mono-DHT-tyrosyl residue(s).

Effect of DHT on the Kinetic Parameters of RNase Ms

In the case of RNase T_1, RNA-hydrolyzing activity was decreased more markedly by the progress of DHT modification than GpC-hydrolyzing activity.[12] Similar experiments were performed for RNase Ms. In Table III, both enzymatic activities are shown for DHT-RNase Ms of various degrees of modifications. RNase Ms sample having 32% residual activity towards RNA was almost as active as the native RNase Ms against GpC as a substrate. When RNA was used as a substrate, the K_m value of DHT-modified RNase Ms increased and the V_{max} value decreased. However, K_m's for GpC were little changed by this modification and the V_{max} values decreased.

Hydrolyses of Homopolynucleotides by DHT-Modified RNase Ms

The rates of hydrolyses of 4 homopolynucleotides by RNase Ms modified to various degrees by DHT were measured. The changes in activity caused by chemical modification were similar toward all 4 homopolynucleotides (Table IV). Thus, the decrease in enzymatic activities was not related to particular bases in the substrates. These results show that the different patterns of activity toward RNA and GpC were probably due to the different sizes of the substrates.

Discussion

The data presented in this paper indicate that about 5 tyrosyl residues and one tryptophyl residue are relatively exposed on the surface of the enzyme molecule. The pK_a values of the tyrosyl residues (pK_a ca. 10.5) suggest that these tyrosyl residues exist without any strong interaction with other functional groups, such as carboxyl groups. The chemical modification of RNase Ms with N-acetylimidazole and DHT indicated that modification of some of the tyrosyl residues induced the inactivation of RNase Ms. In particular, the diazotization reaction with DHT indicated that the tyrosyl residue responsible for the loss of enzymatic activity was included in the very reactive 1—2 tyrosine residue(s) among these 5 exposed tyrosine residues.

The established primary structure of RNase Ms[3] shows 60% sequence homology with that of RNase T_1.[19] Nine tyrosyl residues in RNase Ms are located at the same positions as those of RNase T_1 and the other three are located at positions 28, 50 and 64. The three-dimensional structure of RNase T_1 was reported by Heinemann and Saenger.[20] Their data showed that several tyrosyl residues are on the surface of the molecule. Among them, Tyr_{45} was located very close to the active site of RNase T_1 which consists of His_{92}, His_{40}, Glu_{58}, and Arg_{77}. Because of the high homology of RNase Ms and RNase T_1, it may be assumed that the three-dimensional structure of RNase Ms is essentially the same as that of RNase T_1 though

the precise base specificity of RNase Ms is different from that of RNase T_1.[2]

Upon acetylation of RNase Ms with N-acetylimidazole, loss of activity occurred with the modification of about 5 tyrosyl residues. This means that the reagent did not discriminate the reactivity of a special tyrosyl residue(s) involved in the enzymatic activity from those not participating in the activity. However, in the diazotization reaction, RNase Ms activity was decreased almost to zero by the reaction of about 1.5 tyrosyl residue(s). Thus, DHT seems to discriminate probably one tyrosyl residue which is involved in the enzymatic activity from the other 3—4 tyrosine residues located on the surface of the molecule and which do not participate in the activity. The difference between these two reagents is presumably due to the differences in the size of the reagent and the site of the reaction, one being a phenolic hydroxyl group and the other a benzene ring.

The modification of tyrosyl residues strongly influenced the rate of hydrolysis of homopolynucleotides and had a lesser effect in the case of the low molecular weight substrate. Therefore, the tyrosyl residue(s) modified rapidly should be located near the active site of RNase Ms. Assuming similar three-dimensional structures for the two enzymes, as mentioned above, the tyrosyl residues located near the active site might be Tyr_{58} and Tyr_{44} (Tyr_{45} in RNase T_1). However, the Tyr_{58} residue is replaced in RNase T_1 by Trp, and it is known that Trp_{59} in RNase T_1 is in a relatively hydrophobic region and is not reactive to various reagents.[21] Thus, Tyr_{58}, which is located in a similar position to Trp_{59} in RNase T_1, should be solvent-inaccessible. Therefore, the preferred candidate for the tyrosyl residue which is the most reactive and has a marked effect on the enzymatic activity is Tyr_{44} in RNase Ms.

The modification of Tyr_{44} markedly affects the binding with RNA and has less effect on that with GpC. The results indicate that Tyr_{44} is not involved in the active site directly, but is located in a position where the incorporation of a small group (from the reagents) can affect the enzymatic activity.

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