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Evidence for the Presence of a Histidine Residue having pK_a 7 in the Active Site of a Ribonuclease from a *Rhizopus* sp.

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The kinetic parameters, K_m and log V_{max}, of the cleavage of dinucleoside phosphates, GpU, GpC and ApU, by RNase Rh from a *Rhizopus* sp. were measured at various pH's. Analysis of the pH profiles of K_m and V_{max} of these dinucleoside phosphates according to Dixon's theory suggested that two functional groups having pK_a values of 7.0—7.3 and 3.25—3.5 are present in the active site of RNase Rh. The former value may correspond to a histidine residue. The pH dependence of the rates of inactivation of RNase Rh by photooxidation and carbethoxylation also indicated that a functional group having pK_a about 7.0 was involved in the active site. Amino acid analysis of photooxidized RNase Rh showed that only a histidine residue had been destroyed. In the carbethoxylation of RNase Rh, the formation of carbethoxyhistidine caused a corresponding loss of activity of RNase Rh without modification of tyrosyl residues. It was concluded that a histidine residue having pK_a about 7.0 is involved in the active site of RNase Rh.

Keywords——ribonuclease; *Rhizopus*; photooxidation of ribonuclease; carbethoxylation of ribonuclease; histidine residue in the active site of ribonuclease; kinetic study of ribonuclease; active site of ribonuclease; hydrolysis of dinucleoside phosphate by ribonuclease

A base non-specific ribonuclease (RNase Rh) has been purified from a *Rhizopus* sp. by Tomoyeda *et al.*[2] The RNase liberated 3′-nucleotides from RNA in the order 3′-AMP, 3′-GMP, 3′-UMP and 3′-CMP. Since the enzyme can be purified very easily and the molecular weight of the enzyme is relatively low (molecular weight, 24000),[2] it appeared to be suitable for studies on the nature of the active site of base non-specific ribonuclease.

In the previous paper, we reported the presence of a histidine residue having pK_a 6.2 in the active site of RNase Rh on the basis of photooxidation studies at various pH's.[3] However, further studies on the photooxidative inactivation of RNase Rh revealed that the pK_a value of histidine residue had been underestimated because of underestimation of the rates of inactivation at alkaline pH's. In the present paper the presence of a histidine residue having pK_a 7.0 in the active site was demonstrated by means of pH dependence studies of the rates of cleavage of dinucleoside phosphates by the enzyme as well as those of chemical modifications of RNase Rh.

Experimental

Enzyme——A base non-specific RNase from a Rhizopus sp. (RNase Rh) was purified from a commercial digestive "Gluczyme" by the method reported previously.[4]

Chemicals——RNA was obtained from Kojin Co. 2′-AMP, guanylyl (3′—5′)cytidine (GpC), guanylyl (3′—5′)uridine (GpU) and adenylyl(3′—5′)uridine (ApU) were obtained from Sigma Biochem. Co., and diethylpyrocarbonate from Tokyo Kasei.

Enzyme Assay——(a) Measurement of Acid-soluble Nucleotides: The enzymatic activity was measured by following the increase of acid-soluble nucleotide after digestion of RNA at pH 5.0 and 37°, as reported previously.[4]

1) Location: *2-4-41, Ebara, Shinagawa-ku, Tokyo 142, Japan.*
2) M. Tomoyeda, Y. Eto, and T. Yoshino, *Arch. Biochem. Biophys.*, **131**, 191 (1969).
3) T. Komiyama and M. Irie, *J. Biochem.*, **75**, 419 (1973).
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(b) Cleavage of Dinucleoside Phosphate: The cleavage of dinucleoside phosphate was measured spectrophotometrically according to the method of Imazawa *et al.*[5] which is based on the hyperchromicity on the formation of nucleoside and 2′,3′-cyclic nucleotide from the substrate. The reaction mixture (1 ml) consisted of 16—54 μM substrate in 0.1 M buffer solution and 0.13—0.52 μM enzyme. The reaction was followed at 23° in a Shimadzu UV 200 spectrophotometer equipped with a 10-fold magnification scale. The wavelengths used to follow the reaction were 280, 275 and 265 nm for GpC, GpU and ApU, respectively. The difference in molar extinction coefficient between dinucleoside phosphate and the sum of nucleoside and 2′,3′-cyclic nucleotide was obtained from the literature.[5] The changes in molar extinction coefficient difference at various pH's were measured separately and corrected. The buffers used were 0.1 M formate for pH 2.5, 0.1 M acetate for pH 3—6.5 and 0.1 M Tris-HCl buffers for pH 7.0—8.0.

Kinetic Parameters——The kinetic parameters, K_m and V_{max} were obtained from Lineweaver–Burk plots.[6]

Determination of the Inhibitor Constant (K_i) of 2′-AMP——The K_i value of 2′-AMP was measured at 23° using GpC (10—40 μM) as a substrate, as described above. The concentration of 2′-AMP was 20 μM. The K_i values were also calculated from Lineweaver–Burk plots.

Protein Concentration——Protein concentration was determined from the absorbance at 280 nm, taking $A_{1\,cm}^{1\%}$ to be 1.91.[4]

Photooxidation——(a) pH dependence of the rate of inactivation of RNase Rh by photooxidation: Buffer solution (1 ml, 0.1 M) containing 156 μM RNase Rh and 0.005% methylene blue was kept at 28° in a circulating water bath with constant stirring. The reaction mixture was illuminated vertically from a distance of 25 cm with a tungsten lamp (200 W). The enzymatic activities of aliquots (5 μl) were determined by assay method (a). The buffers used were 0.1 M acetate buffer for pH 5—6.5 and 0.1 M Tris-HCl buffer for pH 7.0—9.0.

(b) Photooxidation to determine the amino acid composition of photooxidized RNase Rh: Buffer solution (4 ml) containing 0.8 mM enzyme and 0.005% methylene blue was treated as described above. At appropriate intervals, aliquots (1 ml each) were withdrawn and applied to columns of DEAE Sephadex A-50 (1×1 cm) equilibrated with 50 mM Tris-buffer (pH 7.5). The columns were washed with the same buffer to eliminate methylene blue. The enzyme was then eluted with the same buffer containing 0.6 M NaCl. The eluates containing protein were collected and dialyzed against distilled water overnight, then used for amino acid analysis.

Chemical Modification of RNase Rh by Diethylpyrocarbonate——Diethylpyrocarbonate (50 μl) diluted 50 fold with ice-cold water was added to a 0.15 mM RNase Rh solution containing 0.1 M buffer (1 ml) kept at 26° and the increase in absorbancy at 250 nm was measured. Aliquots (5 μl each) were withdrawn at appropriate intervals and used for measurement of the RNase activity by assay method (a) described above. Carbethoxyhistidine formed was calculated using a molar extinction coefficient, $\varepsilon_{250\,nm}$, of 3600.[7] The buffers used were acetate buffer for pH 5—6.5, 2-(N-morpholino)ethanesulfonate buffer for pH 7.0 and N-2-hydroxyethylpiperidine N′-ethanesulfonate buffer for pH 7.5—9.0.

Determination of Amino Acid Composition——Photooxidized samples were hydrolyzed in 6 N HCl in evacuated, sealed tubes for 24 hr at 110°. The amino acid compositions of hydrolysates were determined by the method of Spackman *et al.*[8] using a JEOL 6AH amino acid analyzer. To estimate the methionine content in photooxidized enzyme, protein was hydrolyzed in 6 N $Ba(OH)_2$ (1 ml) in a sealed tube at 110°. The hydrolyzed sample was neutralized with dil. sulfuric acid and the barium sulfate formed was eliminated by centrifugation. The precipitate was washed with 5 ml of water and the precipitate was eliminated by centrifugation. The two supernatants were combined and analyzed for amino acids as described above.

Estimation of Tryptophan Content——The tryptophan contents of the protein samples were estimated by the method of Dalby and Tasi.[9]

Results

pH Dependence of the Kinetic Parameters of the Cleavage of Dinucleoside Phosphate by RNase Rh

The kinetic parameters (K_m and V_{max}) of the RNase Rh cleavage of 16 dinucleoside phosphates (XpY), where X and Y represent any common base in RNA, U, C, A or G, have been reported in the previous paper.[10] It was found that the K_m values were small when

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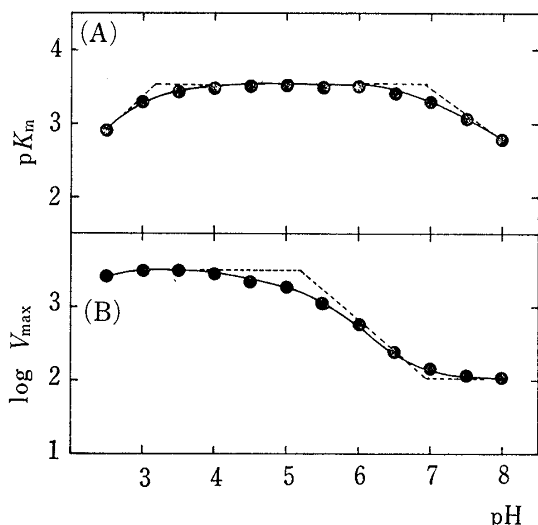

Fig. 1. pH-Profiles of K_m and log V_{max} measured with ApU as a Substrate

The experimental conditions were as described in the text.

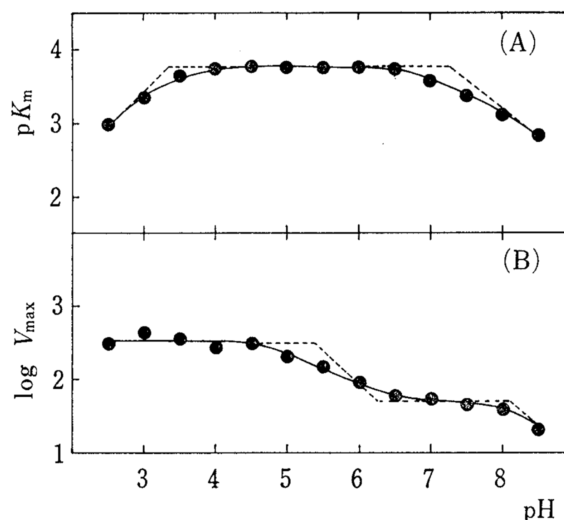

Fig. 2. pH-Profiles of pK_m and log V_{max} measured with GpU as a Substrate

The experimental conditions were as described in the text.

X was A. Therefore, the pH dependence of the kinetic parameters, K_m and V_{max}, of RNase Rh was measured using ApU as a substrate. The results are shown in Fig. 1. V_{max} was maximum at pH 3.5 and pK_m was maximum between 4.5 and 6.0. The maximum of pK_m in this pH range is common to other RNases, including base-specific and non-specific RNases such as bovine pancreatic RNase A (pyrimidine specific),[11] RNase T_1 from *Asp. oryzae* (guanine specific)[12] and RNase M from *Asp. saitoi* (base non-specific).[13] Based on the pH dependence of the kinetic parameters, the functional groups in the active site of RNase Rh were estimated according to Dixon's theory.[14] With the aid of guide lines of integral slope on the pK_m–pH profiles, two concave-upwards bends can be seen at pH 7.0 and 3.25. These values probably correspond to the pK_a value of free enzyme or substrate. Similarly, in the log V_{max}–pH plot, a concave-upwards bend was found at pH 5.2. Since ApU has a pK_a value 3.4 in the adenine moiety, it is difficult to determine whether the pK_a value of 3.25 is due to the nitrogen of the adenine moiety or not. However, the bend at pH 7.0 in the pK_m–pH profile may be that of the active site of the free enzyme. The bend at pH 5.2 is a pK_a of the enzyme-substrate complex.

To eliminate the effect of ionization of the adenine moiety of the substrate, the pH dependence of the kinetic parameters of GpU was measured, since the pK_a of the amino group of GpU is around 2.0, far below the pH region used in this experiment. pH-profiles of pK_m and log V_{max} for GpU are shown in Fig. 2. The curves are very similar to those of ApU and show two concave-upwards bends at pH 3.3 and 7.25. Although the log V_{max}–pH plot gave a more complex curve than that for ApU, a similar pK_a value (pK_a 5.3) in addition to that around 8.2 was observed. Since pK_a 3.3 is much higher than that of the free amino group in GpU, a functional group having pK_a 3.3, which is common for GpU and ApU as substrates, might belong to the free enzyme.

The pH dependence of the kinetic parameters of GpC which has a dissociable functional group on the 3′-side nucleoside was then investigated. The results are shown in Fig. 3. In this case, the pK_m–pH profile of GpC was very similar to those of ApU and GpU. However,

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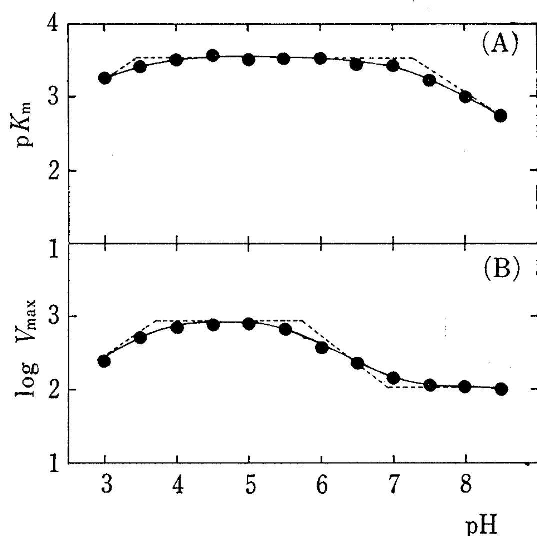

Fig. 3. pH-Profiles of pK_m and log V_{max} measured with GpC as a Substrate

The experimental conditions were as described in the text.

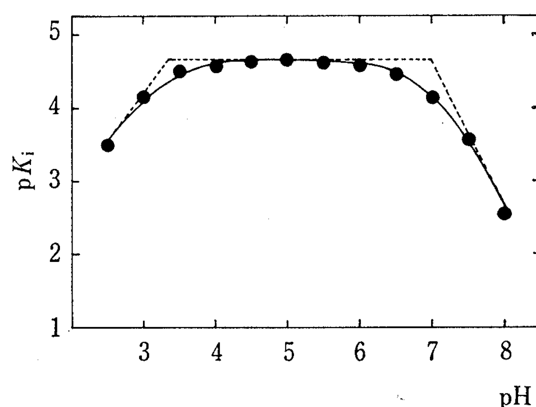

Fig. 4. The pK_i-pH Profile of 2′-AMP

The experimental conditions were as described in the text.

the log V_{max}-pH profile was concave-upwards at pH 3.75 as well as at pH 5.80. This is not seen in Fig. 1 and 2 and seems to correspond to the pK_a of the cytidine moiety. These data indicate that protonation of the cytidine moiety markedly influences the catalytic function of the enzyme.

pH Dependence of the K_i Value of 2′-AMP towards RNase Rh

To confirm the results deduced from the pK_a-pH profiles described above, the pH dependence of 2′-AMP binding with RNase Rh was studied using GpC as a substrate. The results are shown in Fig. 4. Two concave-upwards bends were observed at pH 7.0 and 3.3. These results are very similar to those obtained with dinucleoside phosphates as substrates. From this experimental evidence, that functional groups involved in the active site of RNase Rh have pK_a values of *ca.* 3.3 and *ca.* 7.0, it is possible to deduce that the functional group having pK_a 7.0 is an imidazole group. The other functional group having pK_a 3.3 might

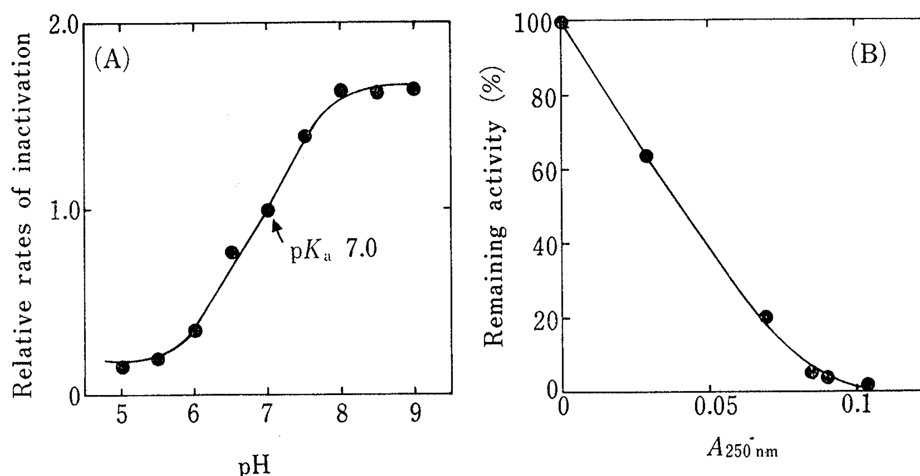

Fig. 5. Effect of pH on the Rate of Inactivation of RNase Rh by Diethylpyrocarbonate (A), and the Relation between Enzymatic Activity and Carbethoxyhistidine Formation during the Reaction of RNase Rh with Diethylpyrocarbonate (B)

The experimental conditions were as described in the text.

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be a carboxylic acid. The substrate-dependent pK_a of 5—5.7 observed in the log V_{max}-pH curve might be a pK_a of the ES complex.

Carbethoxylation of RNase Rh

The pH dependence of the rate of inactivation of RNase Rh by diethylpyrocarbonate, which is known to be a good reagent for histidine, showed an inflection point around pH 7.0 (Fig. 5). The value is very similar to that observed in the pK_m-pH profiles shown in Figs. 1—3. The relation between the rate of inactivation of RNase Rh by the reagent and carbethoxyhistidine formed is shown in Fig. 5b. The rate of inactivation and the carbethoxyhistidine

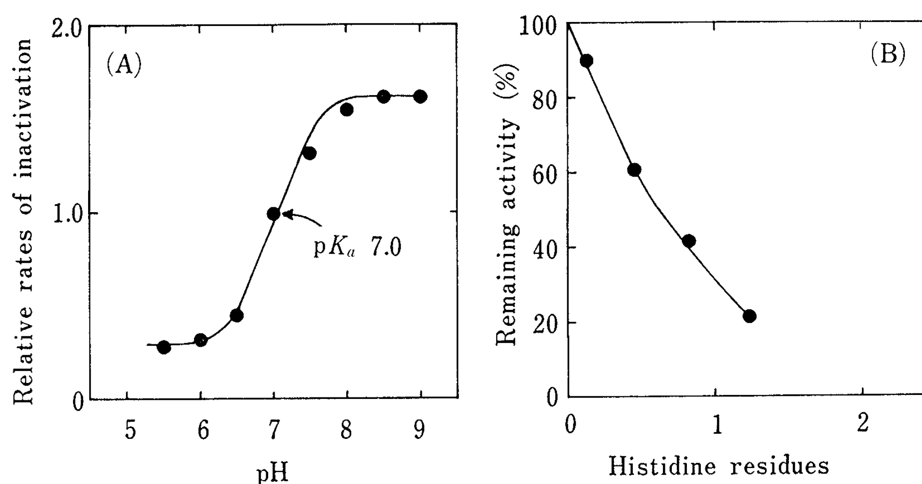

Fig. 6. Effect of pH on the Photoinactivation of RNase Rh (A), and the Relation between Enzymatic Activity and Loss of Histidine (B)

The experimental conditions were as described in the text.

Table I. Amino Acid Compositions of Photoinactivated and Native RNase Rh

Amino acid	Calculated No. of amino acid residues[a]		Theoretical No. of amino acid residues of RNase Rh[b]
	Photoinactivated RNase Rh	Native RNase Rh	
Tryptophan[c]	4.27	4.14	4
Lysine	9.26	8.89	9
Histidine	2.77	4.12	4
Arginine	4.00	4.00	4
Aspartic acid	30.2	29.5	30
Threonine	14.6	14.7	15
Serine	27.1	26.8	27
Glutamic acid	14.7	15.3	15
Proline	10.2	9.8	10
Glycine	20.1	19.9	20
Alanine	15.3	15.7	16
1/2Cystine	4.33	3.89	4
Valine	11.6	11.9	12
Methionine[d]	3.78	4.16	4
Isoleucine	8.97	8.86	9
Leucine	10.5	10.9	11
Tyrosine	14.9	15.3	15
Phenylalanine	5.77	6.12	6

a) Numbers of residues were calculated from amino acid analyses of 24-hour hydrolysates, assuming the amount of arginine to be 4 mol.
b) Taken from reference 4.
c) Obtained colorimetrically as described in the text.
d) Obtained for an alkaline hydrolysate.

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formation proceed in parallel, and loss of about 0.9 histidine residue corresponded to complete loss of the enzymatic activity. Since the absorbance of the enzyme solution at 280 nm did not change at all during the course of this reaction, it can be concluded that tyrosine residues in RNase Rh were not modified by this reagent. When carbethoxylated RNase Rh having 5% or 50% activity was treated with 0.1 M NH_2OH at pH 7.0 for 18 hr, the enzymatic activity of the modified RNase Rh recovered up to 73.5 or 88.7% of the native RNase Rh, respectively. Thus it seems probable that at least one mole of histidine is involved in the active site of the enzyme, and that its pK_a is 7.0.

Photooxidation of RNase Rh

In the previous paper, we deduced from the pH dependence of the photooxidative inactivation of RNase Rh that a functional group having pK_a 6.2 was involved in the active site. This is not consistent with the data described above. Therefore, the photooxidation of RNase Rh was reinvestigated and it became evident that the rates of photooxidative inactivation at alkaline pH's had been underestimated in the previous paper. The results of the reinvestigation are shown in Fig. 6, indicating the contribution of a functional group having pK_a 7.0 in the ac.ive site. This value coincides well with those obtained from the pH-profiles of pK_m and pK_i. Although the pK_a is consistent with that of a histidine residue, this was confirmed by amino acid analysis of photooxidized RNase Rh. The results showed that the inactivation paralleled the decrease in histidine content (Fig. 6); no oxidation of methionine and tryptophan residues was observed (Table I)

Discussion

The pK_a of a histidine residue involved in the active site of RNase Rh was estimated to be *ca.* 7.0 on the basis of the experiments described above. This value is similar to that in base non-specific, but guanine preferential, ribonuclease from *Aspergillus saitoi* (RNase Ms)[15,16] and higher than that in RNase M from the same fungus[17] which is very similar in base specificity to RNase Rh (pK_a about 6.0).

The finding of a functional group having pK_a 5 in the log V_{max}-pH plot indicated the possible contribution of another histidine residue in the active site. However, we have no direct chemical data indicating the presence of another histidine residue in the active site at present.

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