J. Biochem. 121, 849-853 (1997)

Role of Histidine 46 in the Hydrolysis and the Reverse Transphosphorylation Reaction of RNase Rh from *Rhizopus niveus*[1]

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Received for publication, November 15, 1996

In order to study the reaction mechanism of RNase Rh from *Rhizopus niveus*, the rates of cleavage of four 2′,3′-cyclic nucleotides by mutant enzymes of RNase Rh, H46F, H109F, E105Q, and K108L were measured. H46F is virtually inactive towards cyclic nucleotides, but H109F hydrolyzed these substrates at 0.7-4.5% of the rates of the native RNase Rh. The other mutants hydrolyzed 2′,3′-cyclic nucleotides at 15-20% of the rates of the native enzyme. Relative enzymatic activities towards four cyclic nucleotides of H109F in the hydrolysis reaction (2nd step) were much higher than in the transphosphorylation reaction (the 1st step). In the presence of a 13-fold excess of uridine, H109F catalyzed the transphosphorylation reaction of 2′,3′-cyclic AMP (A>p) to ApU. However, this reaction was not catalyzed by H46F mutant or native RNase Rh. These results showed that His46 is crucial to the hydrolysis reaction, and to the reversed reaction of the transphosphorylation reaction. We suggest that His46 in RNase Rh plays a major role in these reactions by acting as a base catalyst to activate water and the 5′-hydroxyl group of nucleosides, respectively.

Key words: base non-specific ribonuclease, histidine residue, mechanism of enzymatic action, *Rhizopus niveus*, second step reaction.

Base non-specific acid RNases which cleave 3′-5′ nucleotidyl linkages of RNA, forming 2′,3′-cyclic phosphates at the 3′-terminal of the RNA, are widely distributed in living creatures, such as viruses (*1*), bacteria (*2, 3*), fungi (*4-8*), plants (*9-15*), protozoan (*16*), and animals (*17-20*). These RNases (RNase T_2 family enzymes) have protein moieties with a molecular mass of about 24 kDa. The mechanism of action of the RNases in this group has been studied mostly for RNase Rh from *Rhizopus niveus* (*21-29*), RNase M from *Aspergillus saitoi* (*30, 31*), and RNase T_2 from *A. oryzae* (*32*) and *Nicotiana alata* (*33*).

Kinetic and protein engineering studies on RNase Rh have revealed that its active site consists of His46, His109, His104 (*22*), Glu105 (*23*), and Lys108 (*25*). The former two histidine residues function as general acid and base catalysts in the transfer reaction of RNase Rh (the first step reaction, Fig. 1) and His104 as a phosphate binding site, while the latter two facilitate catalysis, probably by stabilizing the intermediate or polarizing the P=O bond (*23-25*). The results of X-ray crystallographic analysis of RNase Rh and its 2′-AMP complex (*28, 29*) are consistent with the results of the protein engineering studies. However, the roles of these functional groups, in the hydrolysis reaction (the 2nd step reaction) have not yet been studied. We investigated the mode of action of histidine mutants, H46F and H109F, a Glu105 mutant and a Lys108 mutant on the rates of hydrolysis and the reverse of the transphosphorylation reaction (the first step reaction), the reaction of 2′,3′-cyclic nucleotides (X>p) with nucleosides.[3]

MATERIALS AND METHODS

Substrates and Other Reagents—Dinucleoside phosphates (XpY) and 2′,3′-cyclic nucleotides (X>p) used as substrates were purchased from Sigma (St. Louis, MO).

Enzyme—RNase RNAP Rh, which is excreted by yeast cells, was purified as described in the previous paper (*21*). RNase RNAP Rh is known to have virtually the same enzymatic properties as RNase Rh (*21*). The mutant RNase RNAP Rh enzymes, H46F and H109F, were prepared as described in a previous paper (*22*). RNase RNAP Rh K108L and E105Q were prepared as described in previous papers (Refs. *25* and *23*, respectively).

Protein Concentration—Protein concentrations were

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.
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Abbreviations and nomenclature: Pu>p, 2′,3′-cyclic purine nucleotide; Py>p, 2′,3′-cyclic pyrimidine nucleotide; RNase Rh, base non-specific and purine nucleotide-preferential RNase from *Rhizopus niveus*; RNase RNAP Rh, RNase Rh with three extra amino acid residues, Ala-Ser-Gly, at its N-terminus; X>p, 2′,3′-cyclic nucleotide (X is A, G, U, or C); XpY, dinucleoside phosphate which has X and Y bases at the 5′- and 3′-side of the phosphate, respectively. Nomenclature for mutant enzymes produced by site-directed mutagenesis of RNase RNAP Rh: Mutant enzyme in which the amino acid at the *i*th residue (X) is replaced by Y as a result of site-directed mutagenesis is abbreviated as X*i*Y. Thus, RNase Rh H46F is a mutant RNase Rh in which the histidine residue at the 46th position has been replaced by phenylalanine.

[3] As already discussed in previous papers (*22, 23, 25*) we selected the Phe mutant enzymes because Phe has a similar size to His, and has no ionizable group. Similarly, to eliminate the effect of charge while retaining a similar size, we used Gln and Leu to replace Glu105 and Lys108 residues, respectively, in this work.

determined spectrophotometrically based on the molar absorbance calculated from the amino acid composition (*4*).

Enzyme Assay—(a) The rates of hydrolysis of dinucleoside phosphates (XpY) were measured according to the methods of Imazawa *et al.* (*34*) and of Witzel and Barnard (*35*) by following the changes in absorbance during the course of the reaction at 22°C. Changes in absorbance were monitored with a Shimadzu UV200 spectrophotometer. (b) Hydrolysis of 2′,3′-cyclic pyrimidine nucleotide (Py>p) was assayed by the methods of Richards (*36*) and Crook *et al.* (*37*) at 22°C and in 0.1 M sodium acetate buffer (pH 5.0). The substrate concentrations used were 80-240 μM. The final enzyme concentration was 0.1-4 μM. (c) The rates of hydrolysis of 2′,3′-cyclic purine nucleotides (Pu>p) upon incubation with enzyme were evaluated by measuring the amount of 3′-nucleotides formed on a Hewlett Packard model 30CE capillary electrophoresis apparatus with a 50 μm×50 cm capillary. The reaction mixture consisted of 20 μl of Pu>p (20-100 μM) in 0.1 M acetate buffer (pH 5.5). To the reaction mixture, 1-2 μl of enzyme (1-29 μM) was added and the mixture was incubated at 22°C for 10 min. Then 20 μl aliquots were withdrawn and subjected to electrophoresis. Electrophoresis was performed at 50°C, 39 kV and 60 μA; Pu>p and 3′-nucleotides formed were detected under UV light at 210 nm.

Synthesis of ApU from A>p and Uridine by RNase Rh and Its Mutant Enzymes—A reaction mixture consisting of A>p (6.25 mM) and uridine (83 mM) in 0.1 M sodium acetate buffer (pH 5.0) was incubated at 22°C. To this solution (1 ml) was added about 5 μl of 24 μM RNase Rh or a His mutant enzyme. The reaction mixture (200 μl) was applied to Toyo Roshi No. 51A filter paper at the appropriate time, and the paper was developed in a descending fashion with the solvent system: 2-propanol, concentrated NH_4OH, and water (7 : 1 : 2, v/v). The UV-absorbing zone corresponding to ApU was cut out and eluted with distilled water (5 ml). The extract was concentrated *in vacuo* to dryness, and the residue was dissolved in acetate buffer (1 ml, pH 5.0). The amount of ApU was determined from the UV change at 265 nm upon addition of a small amount of RNase Rh (24 μM) (*34*).

The Kinetic Constants—The K_m and V_{max} values of RNase RNAP Rh and its mutants were calculated from the Lineweaver-Burk plots (*38*).

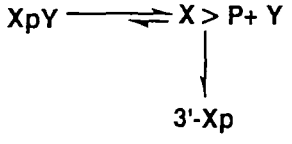

Fig. 1. **The scheme of the two-step ribonuclease reaction.**

RESULTS

pH Profile of the RNase Rh Reaction with U>p as Substrate—The pH profile of the RNase Rh reaction with U>p is shown in Fig. 2. The profile is very similar to that of UpU (substrate for the transphosphorylation reaction), although the absolute v_0 value is about 3 orders smaller. The results in Fig. 2 show that histidine residues and a glutamic acid residue are involved in the hydrolysis of U>p, as in the case of UpU hydrolysis (*24*) (Fig. 2).

Kinetic Constants for Hydrolysis of X>p's by RNase Rh and Its Mutant Enzymes—The rates of hydrolysis of Py>p and Pu>p by RNase Rh and its two histidine mutants (H46F and H109F), and by E105Q and K108L were determined. The results are shown in Table I. The cleavage reaction of Py>p was measured by spectrophotometric methods according to Richards (*36*) and Crook *et al.* (*37*). Spectrophotometric assays are not applicable to Pu>p. Titrimetric assay with a pH stat is also unsuitable for acid RNases, because of the very small increment in the ionizable group produced by hydrolysis of cyclic phosphates in the acidic pH range, and we therefore selected capillary electrophoresis to quantitate the increase in newly formed 3′-nucleotide or the decrease in Pu>p. The results are shown in Table I. The Michaelis constants of RNase Rh for X>p's are slightly larger than for XpYs (*26*). The V_{max} values of these substrates are about 0.01-0.7% of those of the dinucleoside phosphates, depending, on the base in the nucleosides.

The rates of cleavage of four X>p's by the two histidine

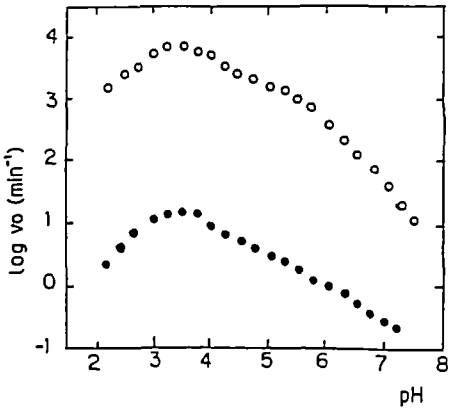

Fig. 2. **pH-profile of the RNase Rh reaction with UpU and U>p as substrates.** ○, UpU; ●, U>p. Reaction conditions: substrate concentration, 24 μM; temperature, 22°C. Enzymatic activity was measured spectrophotometrically as described in "MATERIALS AND METHODS." Initial velocity (v_0) is expressed in min^{-1}.

TABLE I. **Kinetic constants for RNase Rh and several of its mutant towards four 2′,3′-cyclic nucleotides (X>p).**

	A>p			G>p			U>p			C>p		
	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	0.94	31	33	1.2	8.2	7.1	6.3	105	17	2.4	43	19
H46F		n.d.			n.d.			n.d.			n.d.	
H109F	2.2	0.76	0.34	2.1	0.29	0.14	8.7	1.1	0.13	4.0	3.3	0.84
E105Q							2.0	6.7	3.4	2.8	8.0	2.9
K108L							2.8	9.7	3.5	1.6	8.0	4.9

n.d.: Not detected with a tenfold larger amount of H46F (4 μM) as compared with that of H109F (0.4 μM). K_m, V_{max}, and V_{max}/K_m values are expressed as $\times 10^4$ M, min^{-1}, and $min^{-1}\cdot M^{-1}\times 10^{-4}$, respectively.

mutants, H46F and H109F, were measured, and these results are also shown in Table I. H109F hydrolyzed X>p's at rates of 0.7-4.5% of the native RNase Rh, as judged from the V_{max}/K_m values, but H46F hardly hydrolyzed the substrates under the experimental conditions used. When yeast RNA was used as the substrate, the ratio of specific activity of H109F/RNase Rh measured by acid-soluble nucleotides formed was 1 : 10,000 or less (*22*). However, with X>p as the substrate, the ratio was 1/22-140. These values indicated that the enzymatic activity of the hydrolysis reaction catalyzed by the H109F mutant remained greater than that of the transphosphorylation reaction. However, H46F was virtually inactive towards X>p. These results seem to suggest that His46 is very important in the hydrolysis reaction, but that His109 is not.

The mutant enzymes at putatively important functional groups other than the two histidine in the transphosphorylation, Glu105 and Lys108 mutants, displayed approximately 10-20% of the activity of the native RNase Rh. Thus, these functional groups participated in the transphosphorylation reaction, but were not crucial.

Synthesis of XpY by RNase Rh and Its His Mutants—RNases such as bovine pancreatic RNase A can catalyze the synthesis of XpY from X>p in the presence of excess nucleosides (Y) (*39, 40*) by the reverse reaction of the transphosphorylation reaction (Fig. 1). However, in the case of RNase Rh, several trials to force the reverse reaction, *i.e.*, formation of XpY from X>p+Y, failed. Since, as mentioned above, the H109F mutant has very little activity in the transphosphorylation reaction and retains some activity for the hydrolysis of X>p, we investigated the reverse reaction with the H109F and H46F mutants. We incubated 6.25 mM A>p with excess uridine (about 13-fold) in the presence of H109F or H46F. Aliquots were subjected to paper chromatography at appropriate intervals, and a typical chromatogram pattern of the reaction mixture and the standard samples is shown in Fig. 3a. The amount of ApU formed was quantitated as described in "MATERIALS AND METHODS," and the results are shown in Fig. 3, a and b. Only the H109F mutant could catalyze the synthesis of ApU, yielding 0.05% under these experimental conditions. The reaction was maximum at around 6-24 h at 25°C (Fig. 3). These findings suggested that His46 and not His109 is crucial in the reverse transphosphorylation reaction.

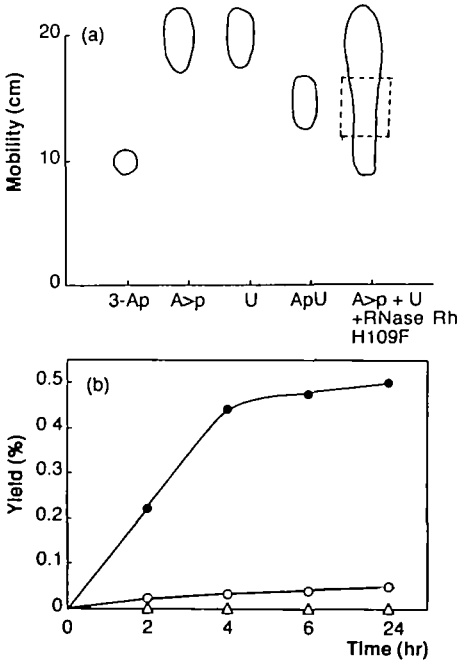

Fig. 3. **Reverse-reaction of the 1st step reaction of RNase Rh and its histidine mutants.** (a) Paper chromatogram of the reaction of A>p and uridine catalyzed by RNase Rh and H109F at pH 5.0 and 22°C. The dotted lines indicate the area used to quantitate ApU. Reaction conditions are described in "MATERIALS AND METHODS." (b) The time course of the reaction analyzed in terms of the formation of ApU. Enzymes used were: △, RNase Rh; ○, H46F; ●, H109F.

DISCUSSION

In the previous paper (*22, 24*), we reported that RNase Rh catalyzes the hydrolysis of RNA by general acid-base catalysis and we suggested that in the transphosphorylation, His109 plays the role of base catalyst and His46 that of acid catalyst, based on the following evidence. The pK_a values of His46 and His109 estimated by NMR were 6.7 and 6.3, respectively. Thus, at pH 5.0 and even at the optimal pH, His109 exists as a base to a greater degree than His46. Chemical modification of RNase M, an analog of RNase Rh, with iodoacetate at pH 5.0 occurs on both histidine residues, His46 and His109 (RNase Rh numbering), in a ratio of 45:55, so His109 exists to a greater extent as a base (*30*). X-Ray crystallographic analysis of RNase Rh-2′-AMP complex (*29*) showed that the 2′-oxygen of the ribose moiety is closer to His109. That means that in transphosphorylation, His109 activates the 2′-OH group as a base catalyst.

The pH profile of U>p suggested that the His residues, His46 and His109, and the Glu105 of RNase Rh are probably involved in the hydrolysis of X>p as well as the cleavage of UpU. However, kinetic studies on several mutant enzymes have shown that the H46F mutant loses X>p hydrolyzing activity almost completely, while H109F was 0.7-4.5% as active as the native RNase Rh. These results as well as the finding that the synthesis of ApU is catalyzed only by the H109F mutant indicate the importance of His46 for the reaction with the water molecule and the 5′-hydroxyl group of nucleosides. This is the first evidence for the crucial role of a histidine residue (His46).

As regards the role of His46 in hydrolysis reaction of 2′,3′-cyclic nucleotides by RNase Rh and its H109F mutant enzyme, there seem to be two possibilities: (i) His46 works as an acid catalyst as in the case of transfer reaction, and polarizes the P=O bond, then a water molecule or $HOCH_2$- attacks the P^+-O^-. However, H109F has no base catalyst to activate the water and $HOCH_2$- group, and it is unlikely that neutral water or hydroxyl group of ribose would attack P^+-O^-. In RNase Rh-d(ApC) complex, the $HOCH_2$- group of cytidine is very close to His46 (*29*), so protonation of water and $HOCH_2$- by His46 may occur. However, attack on the polarized P atom by protonated water or the hydroxyl group of ribose would be unlikely because of their positive charge. (ii) His46 works as a base catalyst: There is a small fraction of unprotonated His46, and this attacks or activates water and $HOCH_2$- of ribose, then the activat-

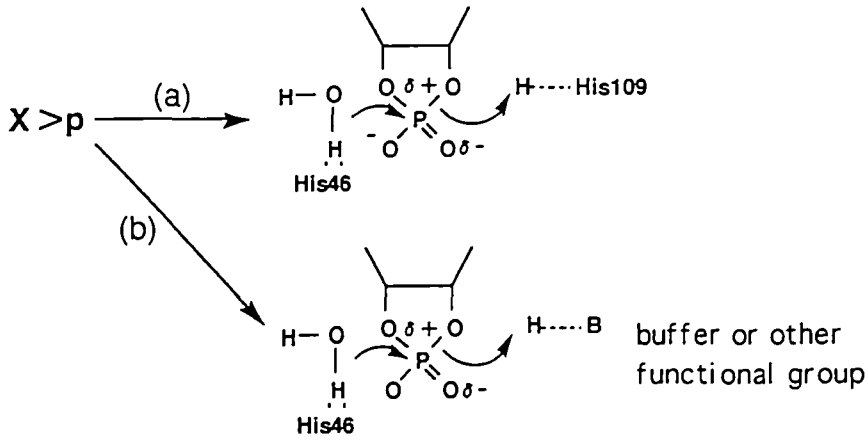

Fig. 4. **Possible routes of the 2nd step RNase Rh reaction.** (a) Major route for RNase Rh; (b) Route for the H109F mutant and minor route for RNase Rh. H··B represents a proton donated by the buffer or some other functional group, such as Lys108.

ed hydroxyl group attacks the P=O group (polarized by Glu105 or Lys108). The proton may be donated from H109 (protonated fraction of H109) in RNase Rh or from the buffer or some other functional group in the case of H109F as shown in Fig. 4. This mechanism is supported by the results of X-ray crystallographic data of RNase Rh-d(ApC) complex, and could explain, at least in part, the very slow hydrolysis of 2′,3′-cyclic nucleotide as compared to transphosphorylation of dinucleoside phosphate, because only the small fraction of base from (His46) is available for the activation.

This reaction catalyzed by H109F seems to be very unusual, because this mutant lacks the proton donor for general acid-base catalysis (Fig. 4).

A similar example was reported by Bernfield (*40*) in the case of RNase S protein. RNase S protein lacks the 21 N-terminal residues of RNase A, which include the His12 residue important for the catalysis. RNase S-protein has only His119 at its active site. The S-protein catalyzed the synthesis of homooligonucleotides, such as UpU>p and UpUpU>p, more efficiently than the hydrolysis of U>p, when compared to native RNase A. This phenomenon seemed to suggest that the reversed phosphorylation reaction step is catalyzed mainly by His119 (*40*).

Jackson *et al.* (*41*), on the other hand, synthesized RNase A analogs in which His12 and/or His119 was replaced by a 4-fluorohistidine residue and then analyzed the pH-profile of the cleavage of UpU and hydrolysis of U>p by these analogs. They concluded that both histidine residues are essential for the cleavage of UpU (transphosphorylation reaction), but for the hydrolysis reaction, only His119 is crucial. In the latter reaction, Lys41 or buffer may donate a proton to the 2′-hydroxyl group of ribose.

These results are very similar to those obtained for the cleavage reaction or synthesis of ApU by RNase Rh RNAP H109F reported here. However, since the rate of hydrolysis of X>p by RNase Rh is several fold higher than that by H109F, the hydrolysis should occur mainly *via* route A shown in Fig. 4, though route B, in which the buffer or a protonated side chain such as that of Lys108 donates a proton, may make a minor contribution.

The authors wish to thank Prof. K.T. Nakamura of Showa University, and Prof. Y. Mitsui, Dr. T. Nonaka, and Mr. M. Hamashima of Nagaoka University of Technology for information on the three-dimensional structures of RNase RNAP Rh and its-2′-AMP complex.

REFERENCES

1. Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E., and Thiel, H.-T. (1993) Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science* **261**, 1169-1171
2. Meador, J., III and Kennel, D. (1990) Cloning and sequencing of the gene encoding *Escherichia coli* ribonuclease I, exact physical mapping using a genome library. *Gene* **95**, 1-7
3. Favre, D., Nagai, P.K., and Timmis, K. (1993) Relatedness of a periplasmic broad specificity RNase from *Aeromonas hydrophila* to RNase I of *Escherichia coli* and to a family of eucaryotic RNases. *J. Bacteriol.* **175**, 3710-3722
4. Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K., and Irie, M. (1988) Primary structure of a base non-specific ribonuclease from *Rhizopus niveus*. *J. Biochem.* **103**, 408-418
5. Kawata, Y., Sakiyama, F., and Tamaoki, T. (1988) Amino acid sequence of ribonuclease T_2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **176**, 683-697
6. Watanabe, H., Naitoh, A., Suyama, Y., Inokuchi, N., Shimada, H., Koyama, T., Ohgi, K., and Irie, M. (1990) Primary structure of a base non-specific and adenylic acid preferential ribonuclease from *Aspergillus saitoi*. *J. Biochem.* **108**, 303-310
7. Kobayashi (Shimada), H., Inokuchi, N., Koyama, T., Watanabe, H., Iwama, M., Ohgi, K., and Irie, M. (1992) Primary structure of a base non-specific and adenylic acid preferential ribonuclease from the fruit bodies of *Lentinus edodes*. *Biosci. Biotech. Biochem.* **56**, 2003-2010
8. Inada, Y., Watanabe, H., Ohgi, K., and Irie, M. (1991) Isolation, characterization, and primary structure of a base non-specific and adenylic acid preferential ribonuclease with higher specific activity. *J. Biochem.* **110**, 896-904
9. Jost, W., Bak, H., Glund, K., Terpstra, P., and Beintema, J.J. (1991) Amino acid sequence of an extracellular, phosphate starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*). *Eur. J. Biochem.* **198**, 1-6
10. Löffler, A., Glund, K., and Irie, M. (1993) Amino acid sequence of an intracellular, phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells. *Eur. J. Biochem.* **214**, 627-633
11. Ide, H., Kimura, M., Arai, M., and Funatsu, G. (1991) The complete amino acid sequence of ribonuclease from the seeds of bitter gourd (*Momordica charantia*). *FEBS Lett.* **284**, 161-164
12. Ai, Y., Singh, A., Coleman, C.E., Ioerger, T.P., Kheyer-Pour, A., and Kao, T.-H. (1990) Self-incompatibility in *Petunia inflata*: isolation and characterization of cDNAs encoding three S-allele associated proteins. *Sex Plant Reprod.* **3**, 130-138
13. McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F., and Clarke, A.E. (1989) Style self-in-

compatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* **342**, 955-957
14. Tayler, C.B. and Green, P.J. (1991) Genes with homology to fungal and S-gene RNases are expressed in *Arabidopsis thaliana*. *Plant Physiol.* **96**, 980-989
15. Kaufmann, A., Salamini, F., and Thompson, R.D. (1991) Sequence variability and gene structure at the self-incompatibility locus of *Solanum tuberoscum*. *Mol. Gen. Genet.* **226**, 457-466
16. Inokuchi, N., Koyama, T., Sawada, F., and Irie, M. (1993) Purification, some properties, and primary structure of base non-specific ribonuclease from *Physarum polycephalum*. *J. Biochem.* **113**, 425-430
17. Watanabe, H., Narumi, H., Inaba, T., Ohgi, K., and Irie, M. (1993) Purification, some properties, and primary structure of a base non-specific ribonuclease from oyster (*Crassostrea gigas*). *J. Biochem.* **114**, 800-807
18. Yagi, H., Kobayashi, H., Inokuchi, N., Koyama, T., and Irie, M. (1995) Purification and properties of a base non-specific ribonuclease from bullfrog (*Rana catesbeiana*). *Biol. Pharm. Bull.* **18**, 219-222
19. Hime, G., Prior, L., and Saint, R. (1995) The *Drosophila melanogaster* genome contains a member of the Rh/T2/S-glycoprotein family of ribonuclease-encoding genes. *Gene* **158**, 203-207
20. Uchida, T., Hayano, K., Iwama, M., Watanabe, H., Sanda, A., Ohgi, K., and Irie, M. (1996) Base specificity and primary structure of poly U preferential ribonuclease from chicken liver. *Biosci. Biotech. Biochem.* **60**, 1982-1988
21. Ohgi, K., Horiuchi, H., Watanabe, H., Takagi, M., Yano, K., and Irie, M. (1991) Expression of RNase Rh from *Rhizopus niveus* in yeast and characterization of the secreted proteins. *J. Biochem.* **109**, 776-785
22. Ohgi, K., Horiuchi, H., Iwama, M., Watanabe, H., Takagi, M., and Irie, M. (1992) Evidence that three histidine residues of a base non-specific and adenylic acid preferential ribonuclease from *Rhizopus niveus* are involved in the catalytic function. *J. Biochem.* **112**, 132-138
23. Ohgi, K., Horiuchi, H., Watanabe, H., Iwama, M., Takagi, M., and Irie, M. (1993) Role of Asp51 and Glu105 in the enzymatic activity of a ribonuclease from *Rhizopus niveus*. *J. Biochem.* **113**, 219-224
24. Irie, M., Ohgi, K., Watanabe, H., Iwama, M., Nakamura, K.T., Kurihara, H., Nonaka, T., Mitsui, Y., Horiuchi, H., and Takagi, M. (1994) pH-profile of kinetic constants of RNase Rh from *Rhizopus niveus* and its mutant enzymes towards UpU, and possible mechanisms of RNase Rh. *J. Biochem.* **115**, 1083-1087
25. Ohgi, K., Iwama, M., Tada, K., Takizawa, R., and Irie, M. (1995) Role of Lys108 in the activity of RNase Rh from *Rhizopus niveus*. *J. Biochem.* **117**, 27-33
26. Ohgi, K., Takeuchi, M., Iwama, M., and Irie, M. (1996) Enzymatic properties of mutant enzymes of RNase Rh from *Rhizopus niveus* at Trp49 and Tyr57. *J. Biochem.* **119**, 9-15
27. Ohgi, K., Takeuchi, M., Iwama, M., and Irie, M. (1996) Enzymatic properties of mutant forms of RNase Rh from *Rhizopus niveus* as to Asp51. *J. Biochem.* **119**, 548-552
28. Kurihara, H., Mitsui, Y., Ohgi, K., Irie, M., Mizuno, H., and Nakamura, K.T. (1992) Crystal and molecular structure of RNase Rh, a new class of microbial ribonuclease from *Rhizopus niveus*. *FEBS Lett.* **306**, 189-192
29. Nakamura, T.K., Ishikawa, N., Hamashima, M., Kurihara, H., Nonaka, T., Mitsui, Y., Ohgi, K., and Irie, M. (1993) Protein nucleic acid recognition in ribonuclease Rh. The 3rd International Meeting on Ribonuclease, Chemistry, Biology, Biotechnology (Capri, Italy, May 9-11), p. 56
30. Irie, M., Watanabe, H., Ohgi, K., and Harada, M. (1986) Site of alkylation of the major ribonuclease from *Aspergillus saitoi* with iodoacetate. *J. Biochem.* **99**, 627-632
31. Ohgi, K. and Irie, M. (1977) Circular dichroism studies on the *N*-bromosuccinimide oxidation of ribonuclease from *Aspergillus saitoi*. *J. Biochem.* **81**, 1031-1039
32. Kawata, Y., Sakiyama, F., Hayashi, K., and Kyogoku, Y. (1990) Essential histidine residues of ribonuclease T_2 from *Aspergillus saitoi*. *Eur. J. Biochem.* **187**, 255-262
33. Ishimizu, T., Miyagi, M., Norioka, S., Liu, Y.-H., Clarke, A.E., and Sakiyama, F. (1995) Identification of histidine 31 and cysteine 95 in the active site of self-incompatibility associated S6 RNase in *Nicotiana alata*. *J. Biochem.* **118**, 1007-1013
34. Imazawa, M., Irie, M., and Ukita, T. (1968) Substrate specificity of ribonuclease from *Aspergillus saitoi*. *J. Biochem.* **64**, 595-602
35. Witzel, H. and Barnard, E.A. (1962) Mechanism and binding sites in the ribonuclease reaction. II. Kinetic studies on the first step of the reaction. *Biochem. Biophys. Res. Commun.* **7**, 295-299
36. Richards, F.M. (1955) An assay for ribonuclease activity using a cyclic phosphate substrate. *Compt. Rend Trav. Lab. Carlsberg ser chim.* **29**, 315-321
37. Crook, E.M., Mathias, A.P., and Rabin, B.R. (1960) Spectrophotometric assay of bovine pancreatic ribonuclease by the use of cytidine 2′:3′-phosphate. *Biochem. J.* **74**, 234-238
38. Lineweaver, M. and Burk, B. (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658-662
39. Richards, F.M. and Wyckoff, H.W. (1971) Bovine pancreatic ribonuclease in *The Enzymes* IV (Boyer, P.D., ed.) 3rd ed., pp. 647-806, Academic Press, New York
40. Bernfield, M.R. (1965) Ribonuclease and oligonucleotide synthesis. I. Synthetic activity of bovine pancreatic ribonuclease derivatives. *J. Biol. Chem.* **240**, 4753-4762
41. Jackson, D.Y., Bumier, J., Quan, C., Stanley, M., Tom, J., and Wells, J.A. (1994) A designed peptide ligase for total synthesis of ribonuclease A with unnatural catalytic residues. *Science* **266**, 243-247