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Studies on the Specificity of Ribonuclease from *Rhizopus* sp.

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In order to investigate the base specificity of the RNase from *Rhizopus* sp. (RNase Rh), its kinetic parameters were measured with 16 dinucleoside phosphates (XpY, where X or Y represents one of adenosine, guanosine, uridine and cytidine) as substrates at pH 5.5 and 25°. The average K_m values of ApY, GpY, CpY and UpY increased in the order A, G, C and U. The average K_m values of XpA, XpG, XpC and XpU increased in the order A, G, U and C. The average V_{max} values of ApY, GpY, CpY and UpY were larger for dinucleoside phosphates having A and G at X. However, the average V_{max} values of XpA, XpG, XpC and XpU were relatively constant. It was concluded that the X base in XpY contributes mainly to the specificity of the enzyme and the Y base may modify this somewhat.

The K_i values of various nucleotides towards RNase Rh were measured at pH 5.5. These compounds inhibited RNase Rh competitively. Although the inhibitory effect depends on the base, sugar and location of the phosphate moiety, for a given location of phosphate on the sugar, the K_i values of ribonucleotides decreased in the order U, C, G and A and those of deoxyribonucleotides decreased in the order T, C, G and A. These data also show that purine bases, especially adenine, have high affinities for RNase Rh.

Keywords——Ribonuclease; *Rhizopus*; base-specificity of ribonuclease; hydrolysis of dinucleoside phosphates; binding of nucleotides to ribonuclease

Introduction

It has been reported by Tomoyeda *et al.*[2)] that a species of *Rhizopus* produces a base non-specific ribonuclease which cleaves the phosphodiester linkages between the nucleoside 3′-phosphate and 5′-hydroxyl group of the neighboring nucleoside of polynucleotides. They also reported that the RNase (RNase Rh) liberated four 3′-nucleotides from RNA in the order 3′-AMP>3′-GMP>3′-CMP≧3′-UMP.

In this work, the kinetic constants of the enzymatic cleavage of 16 dinucleoside phosphates by RNase Rh were measured at pH 5.5 in order to compare the base specificity of RNase Rh with those of the other base non-specific RNases, such as RNase M and RNase Ms from *Aspergillus saitoi*,[3)] and RNase T_2 from *Asp. oryzae*[4)].

The inhibitory effects of various nucleotides (substrate analog) were also studied to obtain information on the required substrate structure (base, sugar and phosphate moiety) of nucleotides.

Experimental

Enzyme——RNase Rh was obtained from a commercial digestive "Gluczyme," from *Rhizopus sp.*, by the method of Komiyama and Irie.[5)] The enzyme preparation was homogeneous as determined by disc electrophoresis on polyacrylamide gel. The enzyme concentration was determined from the absorbancy at 280 nm assuming that the absorbancy of a 0.1% protein solution was 1.91.

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2) M. Tomoyeda, Y. Eto, and T. Yoshino, *Arch. Biochem. Biophys.*, **131**, 191 (1969).
3) K. Ohgi and M. Irie, *J. Biochem.*, **77**, 1085 (1975).
4) G.W. Rushizky and H.A. Sober, *J. Biol. Chem.*, **238**, 371 (1963).
5) T. Komiyama and M. Irie, *J. Biochem.*, **70**, 765 (1971).

Substrate——Dinucleoside phosphates (XpY) were purchased from Sigma Chem. Co., St. Louis, Mo., U.S.A.

Competitive Inhibitors——2′-AMP, 3′-AMP, 2′-GMP, 3′-GMP, 5′- and 3′-deoxyribonucleotides were products of Sigma Chem. Co. All 5′-ribonucleotides and 2′,(3′)-UMP were purchased from Kojin Co. Isomers of CMP, 2′-CMP and 3′-CMP, were separated from commercial 2′,(3′)-CMP by chromatography on a column of Dowex 1 (formate, ×10) according to the procedure of Cohn.[6]

Measurement of Enzymatic Activity——The enzymatic activity was measured principally according to the method reported by Imazawa *et al.*[7] The reaction mixture, consisting of 1.0 ml of acetate buffer (pH 5.5, 0.1 M) containing dinucleoside phosphate (20—68 μM) and 0.13—0.52 nmol of enzyme, was incubated at 25° and the reaction was followed in terms of the absorbancy, using a Shimadzu UV 200 spectrophotometer. The wavelength used to measure the enzymatic activity, and the final absorbancy increments after complete cleavage were as described previously.[7] K_m and V_{max} values were obtained from Lineweaver-Burk double reciprocal plots.[8]

Determination of K_i Values of Competitive Inhibitors——The reaction mixture, consisting of 0.1 M acetate buffer (pH 5.0), GpU (21—52 μM) and nucleotide inhibitor, was incubated at 25°. The reaction was initiated by the addition of 10 μl of RNase Rh (0.13—0.26 nmol). The assay was performed as described above. The nucleotide concentrations used were 20 μM for 2′-AMP and 5′-dAMP, 30 μM for 3′-AMP, 5′-AMP, 2′-GMP, 3′-GMP, 5′-GMP, 3′-dGMP and 5′-dGMP, 40 μM for 3′-dCMP and 5′-dCMP, 55 μM for 3′-dCMP, 5′-CMP and 5′-UMP and 80 μM for 3′-dTMP and 2′,(3′)-UMP.

Results

Cleavage of Dinucleoside Phosphates with RNase Rh

Since the optimum pH for the reaction catalyzed by RNase Rh was about 4.0—5.0, and most of the kinetic data for various RNases activity on XpY's have been measured at pH 5.0—6.0, the rates of cleavage of 16 dinucleoside phosphates (XpY, where X and Y represent one of A, G, U and C) were measured spectrophotometrically at pH 5.5 and 25°.

Table Ia. K_m Values of Various Dinucleoside Phosphates

X	Y				Average
	A	G	U	C	
A	0.66	0.93	1.15	1.42	1.04
G	1.09	1.20	1.72	3.90	1.98
U	1.23	1.34	2.40	4.80	2.44
C	0.71	1.12	2.16	4.40	2.09
Average	0.92	1.15	1.86	3.63	

K_m is expressed as $M \times 10^4$.
The experimental conditions are described in the text.

Table Ib. V_{max} Values of Various Dinucleoside Phosphates

X	Y				Average
	A	G	U	C	
A	660	760	2100	960	1120
G	150	1250	175	1950	881
U	82	170	165	330	187
C	67	175	204	220	167
Average	240	589	661	865	

V_{max} is expressed as mol substrate/mol enzyme, min.
The experimental conditions are described in the text.

6) W.E. Cohn, "The Nucleic Acids," Vol. 1. ed. by J.N. Davidson and E.A. Chargaff, Academic Press, New York, 1955, p. 230.
7) M. Imazawa, M. Irie, and T. Ukita, *J. Biochem.*, **64**, 595 (1968).
8) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

The kinetic constants, K_m and V_{max}, of each reaction were obtained from a double reciprocal plot.[8] The results are summarized in Table Ia and Ib.

As shown in Table Ia, when X was the same, K_m values increased in the order XpA< XpG<XpU<XpC. Similarly, when Y was the same, the K_m value of ApY was the smallest and that of UpY was the largest. It appears that the contribution of adenine base to the affinity of XpY is greatest among the 4 bases, irrespective of X or Y location.

The results in Table Ib show that the V_{max} value of XpY having the same X bases was smallest when Y was adenosine. In contrast, the V_{max} value of XpY having same Y base was larger when X was a purine base than when it was a pyrimidine base. However, the average V_{max} of the 4 dinucleoside phosphates having adenosine at X of XpY was larger than those of G, C and U. On the other hand, the average V_{max} of XpC, XpU, XpA and XpG was in the order C, U G and A, but the differences were relatively small.

In view of the rates of release of 3′-nucleotides from RNA, that is, A>G>C≧U, the base specificity seems to be determined by higher affinities of adenosine and guanosine at the X and Y sites and a higher rate of cleavage of XpY when X is A or G.

Inhibition of RNase Rh by Various Nucleotides

In order to estimate the affinities of various nucleotides and to estimate the base specificity, the inhibitory effects of various ribonucleotides and deoxyribonucleotides on RNase Rh were studied using GpU as a substrate at pH 5.0. All the nucleotides tested inhibited RNase Rh competitively. The K_i values of the nucleotides are listed in Table II. The inhibitory effect depends on the base, sugar and location of the phosphate group on the sugar moiety. For a given location of the phosphate group on the sugar, K_i values of ribonucleotides were in the order U>C>G>A and those of deoxyribonucleotides were in the order T>C>G>A. Thus among the bases, A showed the highest affinity for RNase Rh for both ribo- and deoxyribonucleotides, followed by G in both cases. These results are very similar to those for the K_m values for XpY. That is, for a particular X or Y, K_m values decreased with change of Y or X in the order U or C, G and A. On the other hand, the K_i values of nucleotides having the same base and sugar were in the order 5′->3′->2′- for ribonucleotides and 5′->3′- for deoxyribonucleotides. These relations are the same as those already reported for RNase A,[9] RNase T_1,[10] and RNase M.[11]

Table II. K_i Values of Various Nucleotides

Ribonucleotide	K_i ($\times 10^4$, M)	Deoxyribonucleotide	K_i ($\times 10^4$, M)
2′-AMP	0.24		
3′-AMP	0.48	3′-dAMP	0.13
5′-AMP	0.62	5′-dAMP	0.27
2′-GMP	0.59		
3′-GMP	0.89	3′-dGMP	0.19
5′-GMP	0.77	5′-dGMP	0.63
2′-CMP	0.79		
3′-CMP	1.15	3′-dCMP	0.44
5′-CMP	2.44	5′-dCMP	2.00
2′,(3′)-UMP	2.30	3′-dTMP	0.52
5′-UMP	4.45	5′-dTMP	2.40

The experimental conditions are described in the text.

9) T. Ukita, K. Waku, M. Irie, and O. Hoshino, *J. Biochem.*, **50**, 405 (1961).
10) M. Irie, *J. Biochem.*, **56**, 495 (1964).
11) M. Irie, *J. Biochem.*, **65**, 133 (1969).

It is interesting that, when the base and location of the phosphate group on the sugar were the same, the K_i value of deoxyribonucleotides was always smaller than those of ribonucleotides. Similar results were observed for a base nonspecific RNase of *Aspergillus saitoi* (RNase Ms).[12]

Discussion

When RNA was hydrolyzed by RNase Rh, nucleotides were liberated in the order 3′-AMP>3′-GMP>3′-CMP≧3′-UMP.[2] The order of nucleotide liberation was similar to that of $1/K_i$ values of ribonucleotides and that of $1/K_m$ values of dinucleoside phosphates having a given X base, except for minor differences in the order of UpY and CpY. These results suggest that the rate of release of nucleotides is related to the affinity of the X base moiety. However, small differences in the average K_m values of GpY and dinucleoside phosphates having pyrimidine bases at X seem to be insufficient to explain the differences in the rates of release of 3′-GMP and pyrimidine nucleotides. The order of rate of release of 3′-nucleotides is very similar to that of average V_{max}/K_m values of ApY, GpY, UpY and CpY, which might be related to the rate constants at substrate concentrations lower than K_m. The order of V_{max}/K_m depends largely on the low K_m values of ApY and high V_{max} values of ApY and GpY (Table III).

Table III. V_{max}/K_m Values of Various Dinucleoside Phosphates

X	Y				Average
	A	G	U	C	
A	1000	817	1826	676	1079
G	138	1041	102	500	445
U	67	127	69	69	83
C	94	156	94	50	99
Average	325	535	523	324	

The figures were calculated from the data in Table Ia and Ib.

The results indicate that the specificity for dinucleoside phosphates is related to the X moiety of XpY, and thus that the site for X in RNase Rh is probably the major site of binding. This is the case for base-specific RNases, such as RNase A[13] and RNase T_1.[14] Therefore, in view of the results already reported for RNases having no strict base specificity, such as RNase M[7,10] and RNase Ms from *Aspergillus saitoi*,[3] strong binding of X base to RNases seems to be a general feature of RNases, irrespective of the base specificity.

Though the Y base has some effect on the K_m and V_{max} values of XpY, the role of the Y base should be considered as that of a modifier of the specificity.

RNases which are base non-specific, but preferential for adenine, such as RNase M and RNase Rh, and different in molecular weight, show some difference in the mode of interaction with XpY. Since RNase M liberates 3′-nucleotides in the order A>C>G>U,[10] it is preferential for adenine but not for purine, as RNase Rh is. For a given X, the Michaelis constants of RNase M for the 4 dinucleoside phosphates were in the order U>G>C>A.[7] A similar tendency was observed for given Y. A very similar tendency is seen in the release of

12) M. Irie and K. Ohgi, *J. Biochem.*, **80**, 39 (1976).
13) F.M. Richards and H.W. Wyckoff, "The Enzymes," Vol. 4, ed. D.B. Boyer, Academic Press, New York, 1971, p. 647.
14) K. Takahashi, T. Uchida, and F. Egami, "Advances in Biophysics," Vol. 1, ed., M. Kotani, Tokyo University Press, 1970, p. 53.

3′-nucleotides from RNA by RNase M. The most marked difference between RNase Rh and RNase M is that for RNase M the average V_{max} value of the four dinucleoside phosphates having the same X base is smallest when X is A. Thus, the base specificity is mainly due to the binding of bases.[7] These data indicate that even in base non-specific RNases, there are marked differences in the precise mechanism of hydrolysis.

It is also very interesting that RNase Rh(molecular weight, *ca.* 25,000) and RNase M (molecular weight, *ca.* 35,000) are very similar base non-specific and adenine-preferential enzymes; their modes of modification by iodoacetate and iodoacetamide are also very similar. That is, RNase M is inactivated by both reagents and an alkyl group is incorporated into the N_3-position of histidine.[15] RNase Rh is also alkylated by iodoacetamide and iodoacetate, forming N_3-substituted histidine.[16,17] These results are markedly different from the alkylation of pyrimidine-specific RNase A,[18] which forms N_1-carboxymethylhistidine with iodoacetate, and from that of guanine-specific RNase T_1, which forms carboxamidomethylhistidine with iodoacetamide[19] and a carboxymethyl ester of glutamic acid with iodoacetate.[20] Therefore, RNase M and RNase Rh, both of which are base non-specific and adenylate-preferential RNases, may have very similar active site structures.

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16) K. Komiyama and M. Irie, *J. Biochem.*, **75**, 419 (1973).
17) A. Sanda and M. Irie, unpublished work.
18) A.M. Crestfield, W.H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2421 (1963).
19) K. Takahashi, *J. Biochem.*, **80**, 1267 (1976).
20) K. Takahashi, W.H. Stein, and S. Moore, *J. Biol. Chem.*, **242**, 4682 (1967).