Comparison of Base Specificity and Other Enzymatic Properties of Two Protozoan Ribonucleases from *Physarum polycephalum* and *Dictyostelium discoides*

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Base specificity and other enzymatic properties of two protozoan RNases, RNase Phyb from a true slime mold (*Physarum polycephalum*) and RNase Ddi from a cellular slime mold (*Dictyostelium discoides*), were compared. These two RNases have high amino acid sequence similarity (83 amino acid residues, 46%). The base specificities of two base recognition sites, the B1 site (base recognition site for the base at 5'-side of scissile phosphodiester bond) and the B2 site (base recognition site for the base at 3'-side of the scissile bond) of the both enzymes were estimated by the rates of hydrolysis of 16 dinucleoside phosphates. The base specificities estimated of B1 and B2 sites of RNase Phyb and RNase Ddi were A, G, U > C and A ≧ G > C > U, and A ≧ G, U > C and G > U > A, C, respectively. The base specificities estimated from the depolymerization of homopolymerolnucleotides and those from the releases of four mononucleotides upon digestion of RNA coincided well with those of the B2 sites of both enzymes. Thus, in these enzymes, the contribution of the B2 site to base specificity seems to be larger than that of the B1 site.

pH-stability, optimum temperature, and temperature stability, of both enzymes are discussed considering that RNase Phyb has one disulfide bridge deleted, compared to the RNase Ddi with four disulfide bridges.

**Key words:** ribonuclease; base specificity; stability; *Dictyostelium discoides*; *Physarum polycephalum*

Base non-specific and acid ribonucleases with molecular masses of about 24 kDa are found in many organisms, from viruses, and bacteria to fungi, plants, and animals. They conserve two unique segments (CAS, conserved active site sequence) containing most of the amino acid residues important for catalysis (Fig. 1). They are called RNase T2 family RNases. Since these enzymes are distributed in many organisms, they are very useful enzymes to trace the evolutionary process by analyzing their primary structures and enzymatic properties. Among the RNases belonging to the RNase T2 family, many fungal RNases are mostly adenylic acid, then guanylic acid preferential enzymes, and plant and animal RNases are guanylic acid or uridylic acid preferential.

In our previous work, we isolated two protozoan RNases from *Physarum polycephalum* (RNase Phyb) and *Dictyostelium discoides* (RNase Ddi) and analyzed their primary structures. It is also demonstrated that the latter enzyme is a lysosomal RNase. The two enzymes are similar in 83 amino acid residues (46%). The two protozoan RNases are very similar to plant/animal type RNases in respect of the location of disulfide bridges. However, the amino acid sequence of RNase Phyb seemed partly similar to those of the fungal RNases, because it lacks an about 10-amino acid residues loop characteristic of plant/animal type of RNases, between the 38 and 39th residues of RNase Rh (a typical fungal RNase). Thus in this respect RNase Phyb has a slightly fungal RNase-like structure.

In this paper we investigated the base specificity and some enzymatic properties of both protozoan enzymes and discussed them in relation to their primary structures.

**Materials and Methods**

**Enzymes.** RNase Phyb and RNase Ddi were prepared as described in our previous papers.

**Substrates.** Yeast RNA was a product of Marin Bio (Tokyo). Four homopolymerolnucleotides, poly A, poly I, poly U and poly C were obtained from Yamasai (Chiba). Sixteen dinucleoside phosphates (XypYs, where X and Y are one of the bases A, G, U, and C) were obtained from Sigma (St. Louis, MO.).

**Enzyme assays.** (a) Enzyme activity towards yeast RNA was measured by following the increase in acid-soluble nucleotides upon digestion of substrate at pH

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**Abbreviations:** RNase Ddi, a base non-specific and acid ribonuclease from *Dictyostelium discoides*; RNase Phyb, a base non-specific and acid ribonuclease from *Physarum polycephalum*
5.0 and 37°C as described in our previous paper. The enzyme unit was defined as the absorbance increase at 260 nm per 5 min under these experimental conditions. (b) Depolymerization of homopolynucleotides and RNA was followed by the change in UV absorption upon depolymerization at 22°C and pH 6.0. The wavelength used were 300, 260, 250, 240, and 270 nm for RNA, poly A, poly U, poly I, and poly C, respectively. The substrate concentration was 30 μg/ml. (c) Enzymatic activity towards XpYs as substrates was measured by the method of Imazawa et al. at pH 5.0 and 22°C. The substrate concentration used was 25 μM.

Circular dichroism. The CD spectrum of RNase DdI was measured between 200–310 nm with a JASCO J600 spectropolarimeter at 20°C. The light path of the cells used for the 200–250 nm and 250–310 nm regions were 1 and 10 mm cell, respectively. Protein concentration was 13 μM.

Results and Discussion
The pH optima of RNase DdI and RNase Phyb have been reported, they were 5.0, 2, 3 Thus the experiments related to base specificity for both RNase DdI and RNase Phyb were done at pH around 5.0 and 22 or 37°C.

The base specificities of RNase DdI and RNase Phyb have been estimated from the rates of release of four mononucleotides upon digestion of RNA to be G > U > A > C and G ≥ A > U, C, 2, 3 respectively. In this study, we estimated their base specificities by two other methods. The rates of depolymerization of four homopolynucleotides and RNA by RNase DdI were followed by the change in UV absorption. The reaction was done at pH 6.0 to exclude the possible association of substrate molecules at acidic pHs. The rates of depolymerization were in the order of poly I > poly U > poly A. Poly C was hardly depolymerized under the experimental conditions used (Table 1). The same experiments done for RNase Phyb are also shown in Table 1. RNase Phyb depolymerized homopolynucleotides in the order of Poly I > poly A > poly C, poly U. These results agreed well with those of the rates of release of mononucleotides from RNA, if we assume that I is equivalent to G.

Sixteen dinucleoside phosphates (XpYs) were hydrolyzed with RNase DdI and RNase Phyb at pH 5.0 at the same substrate concentration (25 μM). The results are shown in Table 2. From the rates of hydrolysis of XpYs having the same Y, we can estimate the base specificity of the B1 site (binding site for the X base, base recogni-
Table 2. Rates of Hydrolysis of Dinucleosidephosphates by RNase DdI and RNase Phyb at pH 5.0 and 22°C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>v0</th>
<th>Substrate</th>
<th>v0</th>
</tr>
</thead>
<tbody>
<tr>
<td>GpG</td>
<td>2,760</td>
<td>RNase DdI</td>
<td>10,500</td>
</tr>
<tr>
<td>GpA</td>
<td>56</td>
<td>12,700</td>
<td>UpG</td>
</tr>
<tr>
<td>GpU</td>
<td>771</td>
<td>1,570</td>
<td>UpA</td>
</tr>
<tr>
<td>GpC</td>
<td>254</td>
<td>9,450</td>
<td>UpU</td>
</tr>
<tr>
<td>ApG</td>
<td>5,340</td>
<td>16,200</td>
<td>UpC</td>
</tr>
<tr>
<td>ApA</td>
<td>92</td>
<td>12,900</td>
<td>CpG</td>
</tr>
<tr>
<td>ApU</td>
<td>494</td>
<td>233</td>
<td>CpA</td>
</tr>
<tr>
<td>ApC</td>
<td>129</td>
<td>5,280</td>
<td>CpU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CpC</td>
</tr>
</tbody>
</table>

The details of the experimental conditions are described in the text. v0 values are expressed as following.

v0 = Increase in absorbancy at given wavelength per min/increase in molar absorbancy upon complete hydrolysis/ enzyme concentration (M).

Initial velocity was measured at the substrate concentration of 25 μM.


<table>
<thead>
<tr>
<th>Enzyme</th>
<th>RNase DdI</th>
<th>RNase Phyb</th>
</tr>
</thead>
<tbody>
<tr>
<td>GpY</td>
<td>3,840</td>
<td>34,200</td>
</tr>
<tr>
<td>ApY</td>
<td>6,060</td>
<td>34,600</td>
</tr>
<tr>
<td>UpY</td>
<td>3,100</td>
<td>35,100</td>
</tr>
<tr>
<td>CpY</td>
<td>365</td>
<td>4,790</td>
</tr>
</tbody>
</table>

GpY is the sum of v0 values of GpA, GpG, GpU and GpC and XpY is the sum of v0 value of ApG, GpG, GpC, and UpG. The other parameters are derived by similar ways as described above.

To analyze the base specificities of two protozoan RNases, we derived parameters, the sum of v0 values for GpYs, ApYs, UpYs, and CpYs of RNase DdI and RNase Phyb (Table 3). The comparison of the sum of v0 values of GpY, ApY, UpY, and CpY for each enzyme may have some correlation to the frequency of cleavage at the 3’-side of the given base in RNA hydrolysis (B1 site specificity), if we assume the random distribution of four bases in RNA. Similarly, the sum of v0 values of XpGs, XpAs, XpUs and XpCs may have correlated to those of the cleavage site at the 5’-side of a given base (B2 site specificity). In RNase DdI, base specificity of B1 and B2 sites were A ≥ G, U ≥ C and G > U > A, C, respectively. On the other hand those of RNase Phyb were A, G, U > C and A > G > U > C, respectively. The base specificity of RNase Phyb estimated by the depolymerization of homopolynucleotides as well as those estimated from the rates of release of mononucleotides from RNA digestion are qualitatively agreed with that of B2 site in respect of A and G base preference. Similarly, the base specificity of B2 site of RNase DdI agreed well with those estimated from the other two methods. Therefore, it could be concluded that the base specificity of the B2 site, but not of the B1 site, plays a major role in total base preference of these RNases, though the B1 site has some effect on the base specificity. The similar results have been reported in RNase MC from bitter gourd. The major role of B2 site specificity in the hydrolysis reaction is not observed in the other families of RNases, RNase A and RNase T1 families.

The results of this study also indicated that RNase Phyb is slightly fungal RNase-like properties in respect of base specificity (A ≥ G preference) and amino acid sequence, in spite of its plant/animal type locations of disulfide bridges.

**CD spectrum of RNase DdI**

The CD spectrum of RNase DdI is shown with that of RNase Phyb in Fig. 2. The spectra of both enzymes at short wavelengths (200-250 nm) showed through at the same wavelength and similar depth, but in RNase DdI a large shoulder was observed at 215 nm, Thus the two RNases have somewhat different gross conformations in-
Fig. 3. Comparison of Some Characterization of RNase Phyb and RNase Ddl.

(a) pH-stability relations of RNase Phyb and RNase Ddl. The enzymes were incubated at given pH and at 37°C for 1 h, and the enzymatic activities were measured at pH 5.0 and 37°C. Ordinate was expressed as percentage of the enzymatic activity at pH 5.0. (b) Heat stability of RNase Phyb and RNase Ddl. RNases were incubated in acetate buffer (pH 5.0) at given temperature for 5 min, and the enzymatic activities were assayed at pH 5.0 and 37°C, as usual. The ordinate was expressed with percentage of enzymatic activity at 20°C as 100%. (c) Optimum temperature of RNase Phyb and RNase Ddl. The enzymatic activities of RNases were measured at given temperature as usual. ⋄, RNase Ddl; ○, RNase Phyb.

stead of higher amino acid similarity of both enzymes, especially near the active site (CAS sequences, Fig. 1). The peak and trough positions of the CD spectrum of RNase Ddl at longer wavelengths (250–310 nm) were very similar to those of RNase Phyb, though their height and depth are quite different, indicating perturbation of aromatic amino acid side chains to different extents.

Comparison of the enzymatic properties of the two protozoan RNases

pH-stability, temperature-stability at pH 5.0, and optimal temperature of both enzymes are shown in Fig. 3(a)–(c).

Both RNases were very labile at acidic pH lower than 3.0. RNase Ddl was very stable at alkaline pH up to 11, but RNase Phyb was only stable up to pH 6.0. The temperature-stability curve of RNase Ddl also showed the higher stability of RNase Ddl over that of RNase Phyb. In addition to these results, the optimal temperature of RNase Ddl was about 10°C higher than that of RNase Phyb. Although we can explain these differences, we have to consider so many factors, one of the big factor which might contribute to these phenomena in general is the number of disulfide bridges in these RNases. The number of disulfide bridges of fungal RNase belonging to the RNase T2 family are 5 and those of plant/animal RNases are mostly four (Fig. 1). Although the two protozoan RNases we are discussing now are very similar to each other, the number of disulfide bridges of RNase Ddl is four and that of RNase Phyb is three. The location of the three disulfide bridges of RNase Phyb are the same as those of RNase Ddl. The location of the one deleted disulfide bridge in RNase Phyb was estimated from the three-dimensional structure of RNase Rh, since RNase Rh is the only enzyme among many RNase T2 family enzymes of which the three-dimensional structure has been described. The one deleted disulfide bridge in RNase Phyb, is near the roots of a loop formed between the 38 and 39th position of RNase Rh (RNase Rh numbering). The sequence corresponds to Tyr20 to Glu31 in RNase Ddl. Since the loop is very closely located to one of the active site amino acid residues, His26 (His 46 in RNase Rh), thus it might be reasonable to assume that enzymatic activity of RNase Phyb lacking one disulfide bridge is more affected by heating.

Among the amino acid residues responsible for catalysis of RNase Rh, most active RNase, all of them, His46, His104, His109, E105, and K108 (RNase Rh numbering), are conserved in RNase Ddl (His26, His74, His79, Glu75, and Lys78 in RNase Phyb) as well as RNase Phyb (His38, His87, His92, Glu88, and Lys91 in RNase Ddl) (Fig. 1). This is probably the reason that RNase Ddl and RNase Phyb have higher specific activity towards RNA. In protozoan RNases two of the B1 site constituents of RNase Rh, Tyr57, and Trp49 are conserved (Y37 and W29, and Y49 and W41, in RNase Phyb and RNase Ddl, respectively) but Asp51 is replaced by Glu (Glu31 and Glu43, in RNase Phyb and RNase Ddl, respectively) in both enzymes. This is one of the possible reason why RNase Ddl and RNase Phyb are more preferential for G base in addition to the B2 site structure. Because, the substitution of Asp51 by Glu in RNase Rh made the mutant enzyme more guanylic acid preferential. The B2 site of RNase Rh consists of Gin32, Pro92, Ser93, Asn94, Gin95, and Phe101. Among these amino acid residues, Gin32, Pro92, Ser93, and Phe101 were conserved in RNase Ddl, and Asn94 and Gin95 are replaced by Leu (Leu77) and Thr (Thr78), respectively. In RNase Phyb, Pro92, Ser93, and Phe101 were conserved, and Gin32, Asn94, and Gin95 were replaced by Glu (Glu 13), Phe (Phe64), and Thr (Thr65), respectively. The base preference of RNase Phyb is very similar to RNase Rh (A ≥ G > U, C), except for a slightly higher guanylic acid preference.

Based on our assumption that the base specificity of B2 site has major role in gross enzyme specificity, the origin of the guanine followed by uracil base specificity of RNases Ddl might be due mostly to Leu77, though we have to await further research on the precise conformation of amino acid side chains in these RNases or protein engineering studies of mutant enzymes at Leu94 (RNase Rh numbering).

References

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