Site-specific cleavage reaction catalyzed by leadzyme is enhanced by combined effect of lead and rare earth ions

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Abstract Lead-dependent ribozyme (leadzyme) is a ribozyme working with Pb^{2+}. In this paper, we have investigated the combined effect of metal ions, especially rare earth ions, on the cleavage reaction by the leadzyme. As a result, it was observed that although only a rare earth ion or another divalent ion except Pb^{2+} did not play a role as the catalyst, the addition of a rare earth ion in the presence of Pb^{2+} increased significantly the yield of the cleavage reaction. The result suggests that the complex between the leadzyme and the substrate should have two classes of metal ion binding sites.

Key words: Leadzyme; Ribozyme; Rare earth ion; Reaction mechanism

1. Introduction

Ribozyme is an RNA enzyme which can catalyze biochemical reactions [1,2]. Because a ribozyme requires metal ions for catalytic function, ribozyme is considered as one of the metalloenzymes [3]. Many studies on the relation between ribozyme and metal ions have been carried out so that it has become clear that a metal ion plays several roles in the reaction catalyzed by the ribozyme [4–9]. However, little is known about the combined effect of the metal ions on the ribozyme reaction, despite the metal ions having several important functions in RNA catalysis. Therefore, we have investigated the combined effect of metal ions, especially rare earth ions, on the cleavage reaction by a lead-dependent ribozyme (leadzyme) as shown in Fig. 1. The leadzyme consisting of 10 nucleotides, CUGGGAGUCC, was chosen in this study on the basis of its secondary structure being smaller than a small hammerhead ribozyme or a hairpin ribozyme [10,11]. Thus, this system is the simplest system in the ribozymes. This paper deals with our new finding that the addition of rare earth ion in the presence of Pb^{2+} significantly increases the yield of the cleavage reaction, although only a rare earth ion or another divalent ion except Pb^{2+} did not cleave the substrate RNA. The result suggests that the complex between the leadzyme and the substrate RNA should have two classes of metal ion binding sites and that the ribozyme reaction may be controlled by the combination of metal ions.

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Abbreviations: HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid

2. Materials and methods

2.1. Preparation of RNA oligonucleotides

An substrate RNA, GGACCGAGCCAG, and a leadzyme, CUGGGAGUCC, were synthesized on a solid support employing the phosphoramidite method on an DNA/RNA synthesizer. The synthesized oligomers were removed from the solid support and base blocking groups were removed by treatment with concentrated ammonia in ethanol (3:1, v/v) at 55°C for 3 h. After drying in vacuum, the 2′-silyl protection groups were removed by resuspending the pellet in 50 equivalents of tetrabutylammonium fluoride (TBAF) per equivalent of silyl, and the reaction mixture was incubated at room temperature overnight in the dark. Following deprotection, the samples were passed through a C18 Sep-Pak cartridge (Waters) for desalting and purified by HPLC on a C18 column (TOSOH) with a gradient of 0–50% methanol/H_2O containing 0.1 M triethylammonium acetate (TEAA) (pH 7.0). After purification on HPLC, the oligomers were desalted again with a C18 Sep-Pak cartridge. Final purities of the oligomers were rechecked by HPLC and were greater than at least 98%. Concentrations of purified RNA oligonucleotides were determined by absorption spectrophotometry. The RNA substrate with 5′-OH was 5′ end-labeled in 25 μl reaction mixture containing 25 pmol substrate RNA, [γ-^{32}P]ATP (6000 Ci/mol, New England Nuclear), 10 U T4 polynucleotide kinase (Pharmacia Biotech), 70 mM Tris-HCl buffer, pH 7.6, 10 mM $MgCl_2$. The reaction mixture was incubated for 30 min at 37°C. The labeled RNA substrate was precipitated with 2.5 vols. of ethanol and washed with 70% ethanol.

2.2. Cleavage reaction

Cleavage experiments with the ribozyme in excess over the substrate RNA were carried out in 15 mM MOPS buffer (pH 7.5) at 25°C. The ribozyme and 5′-end labeled substrate RNA were heated together to 90°C for 2 min, cooled slowly, and incubated at 25°C for 30 min in 7 μl of 15 mM MOPS buffer (pH7.5). Cleavage reactions were initiated by the addition of 7 μl of 15 mM MOPS buffer containing metal ions and rare earth ions. Reactions were terminated by the addition of an equal volume of 200 mM Na_2EDTA, 7 M urea, 0.02% bromophenol blue, and 0.02% xylene cyanol to the reaction mixture. The substrate and the products were separated by 20% non-denaturing gel electrophoresis. The radio activities of the substrate and the product were analyzed by Bio-Image Analyzer (BAS 2000; Fuji Film, Tokyo).

3. Results

The complex between the leadzyme and the substrate RNA used in our experiments is shown in Fig. 1. The expected cleavage site is between CpG in the asymmetrical internal loop as indicated by an arrow in the figure. This reaction gives a terminal 5′-hydroxyl and a 3′-phosphate product [10,11]. Fig. 2 shows the autoradiogram of the cleavage reaction of the substrate by the leadzyme in the presence of various concentrations of Pb^{2+}. The cleavage reaction was performed in 15 mM MOPS buffer (pH 7.5) and various concentrations of Pb^{2+} at 25°C. Reaction time was 20 min. For the various concentrations of Pb^{2+}, only one product band was observed. Thus, the reaction is a site-specific cleavage. Considering the position of the maker (dT_4 and dT_{20}), the cleavage site would be in agreement with the result of the previous experiment

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which was carried out in the presence of excess Mg^{2+} over Pb^{2+} [11]. This result in this study would indicate that CUGGGAGUCC acts as a ribozyme in the presence of Pb^{2+} only. Fig. 2 shows that the cleavage yield decreased abruptly at high Pb^{2+} concentrations. Thus, the cleavage yield of the substrate is not simply proportional to the concentration of Pb^{2+}. The decrease in reaction yield at Pb^{2+} concentrations higher than 200 μM would be due to the formation of Pb^{2+} polyhydroxides or polyhydrates as in the case of the Pb^{2+} cleavage motifs [11]. Because our purpose in this study was to increase the cleavage yield at the lowest concentration of combined metal ions, the subsequent cleavage experiments were carried out in the presence of 25 μM of various ions. The concentration of 25 μM is the lowest concentration at which the cleavage reaction of the substrate by the leadzyme was observed for 20 min in the presence of Pb^{2+} only as shown in Fig. 2.

It is known that divalent ions such as Mn^{2+} can substitute for Mg^{2+} in the cleavage reaction catalyzed by the hammerhead ribozyme or *Tetrahymena* ribozyme [4,8,9]. On the other hand, the rare earth ions promote non-enzymatic hydrolysis of RNA at natural pH [12–14]. Therefore, we have investigated whether Pb^{2+} can be replaced by other divalent ions (Mg^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}) except Pb^{2+}, trivalent ions (Al^{3+}, Cr^{3+}, and Fe^{3+}) or rare earth ions (Eu^{3+}, Ce^{3+}, Gd^{3+}, Dy^{3+}, Tm^{3+}, Tb^{3+}, Lu^{3+}, Pr^{3+}, La^{3+}, Sm^{3+}, and Nd^{3+}). Although other reaction conditions were the same as described above, none of the divalent, trivalent, or rare earth ions showed any cleavage of the substrate under the same conditions as used at 25 μM Pb^{2+} (data not shown).

The reaction catalyzed by the ribozyme may be accelerated by two or more metal ions, if each metal ion plays the most suitable role, respectively. So, the combined effects of the metal ions on the Pb^{2+}-dependent reaction were also studied in the presence of 25 μM Pb^{2+} and 25 μM of various metal ions. Fig. 3A shows the effect of the addition of 25 μM Mg^{2+} in the presence of 25 μM Pb^{2+}. The cleavage site was not changed by the addition of Mg^{2+}. The cleavage yield in the presence of both 25 μM Pb^{2+} and 25 μM Mg^{2+} was 14.0%. Although the value is 2-fold greater than 7.0% at 25 μM Pb^{2+} only, this is almost equal to 13.5% at 50 μM Pb^{2+}. Thus, Mg^{2+} addition shows a similar effect in the case of Pb^{2+} addition. The effect of 25 μM Nd^{3+} in the presence of 25 μM Pb^{2+} is shown in Fig. 3B. In analogy with Mg^{2+}, the

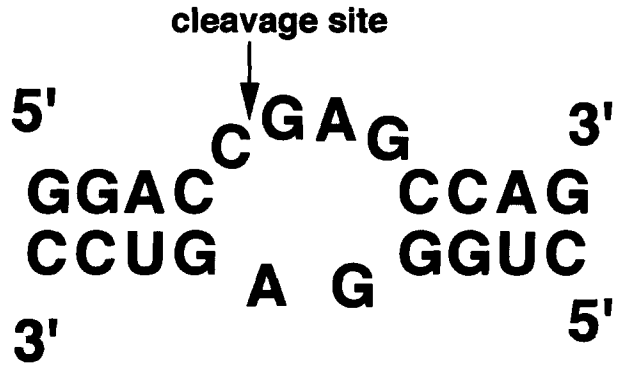

Fig. 1. Secondary structure of the complex between the leadzyme and the substrate RNA used in this experiment. The arrow indicates the cleavage site.

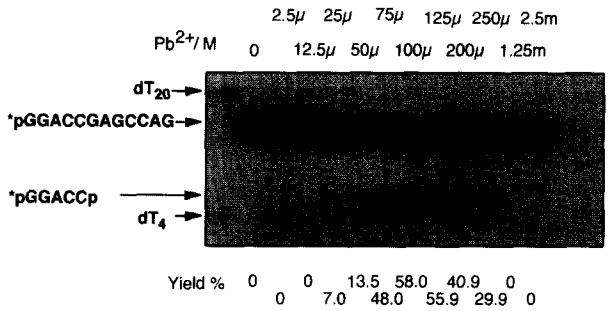

Fig. 2. Autoradiogram of denaturing 20% polyacrylamide gel showing the result of the cleavage reaction for 5 μM leadzyme, 250 nM substrate RNA, and various concentrations of Pb^{2+} in 15 mM MOPS buffer (pH 7.5) at 25°C and for 20 min reaction time.

cleavage site did not change. Surprisingly, the addition of Nd^{3+} in the presence of Pb^{2+} led to a significant increase in the yield as shown in Fig. 3B. This cleavage yield was 40.0%. This value is about 6-fold larger than that of 25 μM Pb^{2+}. We have also investigated the effects of other metal ions on the Pb^{2+}-dependent reaction. The cleavage site was not changed by the addition of various metal ions like Mg^{2+} or Nd^{3+}. The relation between the cleavage yield and metal ions is displayed in Fig. 4. When Ba^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, or Ca^{2+} was added, the reaction yield amounted to 13.0, 10.0, 9.1, 7.0, or 3.1%, respectively. No other divalent ions (Fe^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}) or trivalent ions (Al^{3+}, Cr^{3+}, and Fe^{3+}) showed any cleavage of the substrate even in the presence of Pb^{2+}. Thus, divalent and trivalent ions did not increase the reaction yield. On the other hand, the addition of the rare earth ions in the presence of Pb^{2+} increased the reaction yield as in the case of Nd^{3+}. For example, when Gd^{3+}, Tm^{3+}, or La^{3+} was added, the yield was 25.0, 32.0, or 35.0%, respectively, as shown in Fig. 4. No cleavage reaction by only the divalent ion or the rare earth ion was observed as described above. The combinations of Nd^{3+} and divalent ions except for Pb^{2+} or trivalent ions also showed no cleavage of the substrate (data not shown). Therefore, the increase in cleavage yield should be due to the combined effect of Pb^{2+} and the rare earth ions. The ionic strength of 25 μM Pb^{2+} and 25 μM Mg^{2+} or 50 μM Pb^{2+} is 2.0×10^{-4} mol kg^{-1}. That of 25 μM Pb^{2+} and 25 μM Nd^{3+} is 3.25×10^{-4} mol kg^{-1}. The ionic strength of 25 μM Pb^{2+} and 25 μM Nd^{3+} is about 1.6-fold greater than that of 25 μM Pb^{2+} and 25 μM Mg^{2+} or 50 μM Pb^{2+}. The increase in the reaction yield is much larger than that of the ionic strength. Therefore, the increase in the reaction yield should be the result of not only the increase in charge but also the direct contribution of the ions to the reaction mechanism.

4. Discussion

Ribozymes act to cleave phosphodiester linkages in nucleic acid substrates via transesterification or hydrolytic mechanisms [3]. In these mechanisms, metal ions activate the attacking water or sugar hydroxyl group and stabilize the oxygen leaving group in the active site [3]. The metal ions also possess the function of neutralizing the negative charge of the backbone of the nucleic acids to promote ribozyme folding and substrate binding [15,16]. Thus, the metal ions play several roles in chemistry and in the folding of the complex between the ribozyme and the substrate.

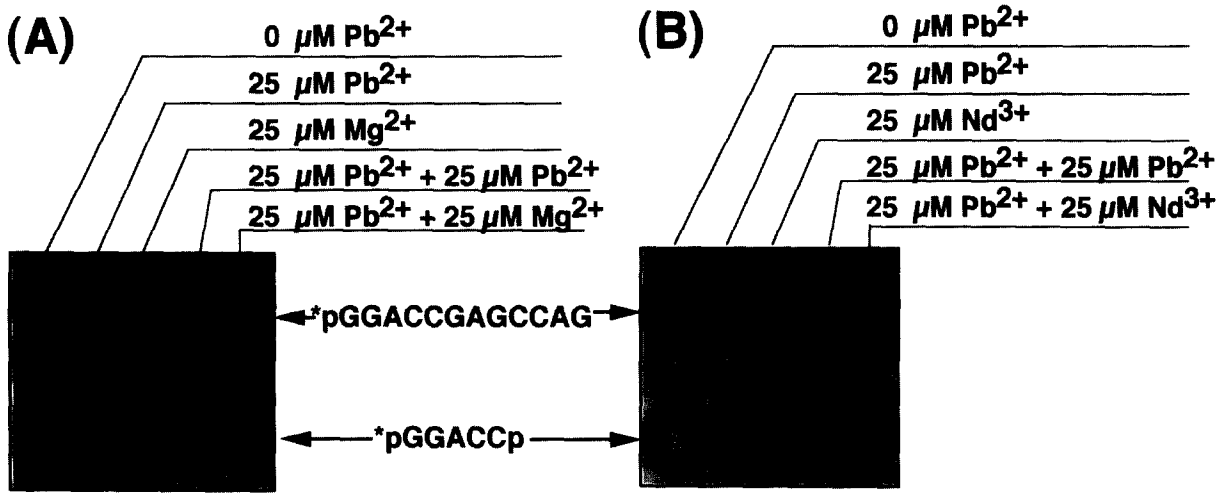

Fig. 3. Autoradiograms of denaturing 20% polyacrylamide gel showing the cleavages of the substrate RNA by the leadzyme in the presence of (A) Pb^{2+} and Mg^{2+}, and (B) Pb^{2+} and Nd^{3+}. Reaction buffer (pH 7.5) contained 15 mM MOPS, 5 μM leadzyme, and 250 nM substrate RNA. Reaction time and temperature were 20 min and at 25°C, respectively.

If the dependence of the reaction yield on the metal ions is the result of the direct contributions of the metal ions to the reaction mechanism, our results should provide effective information about the binding and function of metal ions. Increases in the reaction yield were observed only in the presence of Pb^{2+} and the rare earth ions. When the concentration ratio of Nd^{3+} with Pb^{2+} was 1:1, the cleavage yield reached a maximum value (data not shown). These results would suggest that the complex of the leadzyme and the substrate has the two classes of metal ion binding sites, i.e. the strong binding site for Pb^{2+} and that for the rare earth ion, and each ion at each site plays the most suitable role in the reaction mechanism. Previously, the binding of metal ions to RNA was studied for tRNA and it was reported that tRNA had many different binding sites for metal ions [17]. For example, in the presence of both Pb^{2+} and Mg^{2+}, Pb^{2+} coordinated directly to O^4 of U and N^3 of C in the T loop at the corner of the L-structure of tRNA, while Mg^{2+} located near the site of Pb^{2+} [18]. The two classes of metal ion binding sites were also observed in *Tetrahymena* self-splicing intron [6,7,19]. Considering the above examples, it may be general that the complex of the leadzyme and the substrate containing the internal loop, in which the rare earth ion and Pb^{2+} could bind to the different purine residues, has two binding sites of the metal ions. Although the roles of the rare earth ions remain unclear based on the present results only, either Pb^{2+} or the rare earth ion may activate the attacking water or sugar hydroxyl group, while the other ion stabilizes the oxygen leaving group such as in the mechanism of the phosphoryl transfer reaction catalyzed by the 3′,5′-exonuclease of DNA polymerase I [20]. In fact, NMR has demonstrated the possibility that 5 purine residues in the internal loop near the active site do not form stable non-Watson-Crick base pairs under low salt conditions like 0.1 M NaCl and hence heterocyclic nitrogens of the bases to which metal ions can bind directly may be free [21,22]. Moreover, other NMR results suggests that Pb^{2+} may bind near G residue close to the cleavage site in the leadzyme [23].

In conclusion, although only a rare earth ion or another divalent ion except Pb^{2+} did not play a role as the catalyst, a rare earth ion like Nd^{3+} in combination with Pb^{2+} enhances the ribozyme reaction. To our knowledge, this is the first result showing that the combined action of metal ions increases the yield of the cleavage reaction. The result suggests

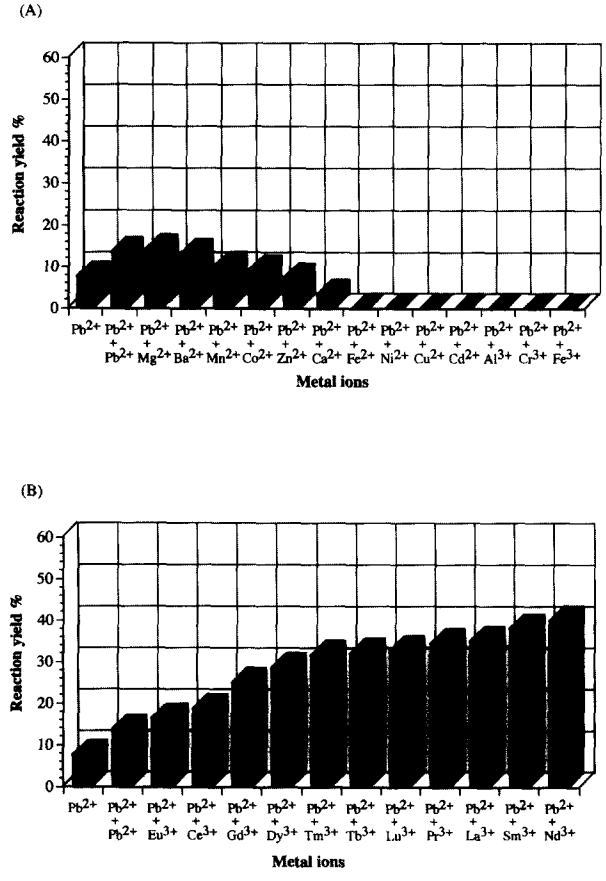

Fig. 4. Dependence of cleavage yield on (A) divalent and trivalent ions, and (B) rare earth ions. Reaction buffer (pH 7.5) contained 15 mM MOPS, 5 μM leadzyme, 250 nM substrate RNA, 25 μM Pb^{2+}, and 25 μM of the indicated metal ions. Reaction time and temperature were 20 min and at 25°C, respectively.

that the complex between the leadzyme and the substrate has two binding sites of metal ions as well as tRNA or *Tetrahymena* self-splicing intron, and each ion plays the most suitable role in the cleavage reaction.

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