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Activation and Repression of the Activity of a Lead Ribozyme by the Combination of Pb^{2+} and Mg^{2+} [1]

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The effect of Pb^{2+} and Mg^{2+} on the activity of a lead ribozyme with modified sequences has been studied. At low Pb^{2+} concentrations, cleavage at a previously reported site (site a) is observed. At higher Pb^{2+} concentrations, cleavage at a new site (site b) adjacent to site a is observed, while the cleavage at site a is repressed. On the addition of a certain amount of Mg^{2+}, the cleavage at site a is enhanced by almost fourfold, while the cleavage at site b is repressed. Further addition of Mg^{2+} represses the cleavage at both sites. CD analysis indicates that the structure and stability of the lead ribozyme change depending on the metal conditions. Activation and repression of the activity by the combination of Pb^{2+} and Mg^{2+} are rationalized by considering that the two metals compete with each other for binding at two metal-binding sites.

Key words: CD, lead ribozyme, metal binding, RNA, RNA cleavage.

A lead ribozyme was isolated by *in vitro* selection using a library of $tRNA^{Phe}$ sequences with randomized mutagenesis (*1, 2*). The lead ribozyme is one of the smallest ribozymes. It consists of an asymmetric internal loop of six residues and short flanking stems. The RNA cleavage by the lead ribozyme terminates with a 3′ phosphomonoester generated from a 2′,3′-cyclic phosphodiester reaction intermediate (*1*). Other ribozymes such as hammerhead, HDV and hairpin ribozymes terminate with a 2′,3′-cyclic phosphodiester (*3-5*). Generally, ribozymes require divalent metal ions for their activities. The metal ions are assumed to promote the proper folding of RNA and/or to directly participate in the catalytic reaction. The lead ribozyme is unique in requiring Pb^{2+} for the RNA cleavage, while the other ribozymes require Mg^{2+} (*3-5*). Mg^{2+} is not essential for the catalytic activity of the lead ribozyme, but it modulates the activity (*6*). Recently, it was reported that rare earth ions such as Nd^{3+} enhance the activity of the lead ribozyme in combination with Pb^{2+} (*7, 8*). The combined effects of Pb^{2+} and Mg^{2+} on the activity have not been clarified so far. We have started a study to elucidate this point.

The lead ribozyme used in our study has minor alterations of sequences in the stems with respect to the original ribozyme reported by Pan and Uhlenbeck (*1*). These alterations were designed originally to give better separated NMR resonances. Unexpectedly, new phenomena of cleavage by the lead ribozyme were observed. At higher Pb^{2+} concentrations, cleavage at a new site was observed, while the cleavage at a canonical site was repressed. The addition of a certain amount of Mg^{2+} enhanced the cleavage at the canonical site, while the cleavage at the new site was repressed. Changes in the structure and stability of the lead ribozyme under varying metal ion conditions were detected on CD analysis. We propose a mechanism for the activation and repression of the cleavage at the two sites by combination of Pb^{2+} and Mg^{2+}.

MATERIALS AND METHODS

Design of the Sequence of the Lead Ribozyme—Figure 1A shows the sequence of the lead ribozyme used in this study. The lower strand (G15-C26) is an enzyme and the upper strand (G1-C14) is a substrate. The central ten residues, composed of an asymmetric internal loop and two flanking base pairs, are the same as those in the original paper (*1*). The sequence of the left stem is identical to that of LZ4 (Fig. 1B) in the original paper except for the insertion of a C2:G25 base pair. The sequence of the right stem is also similar to that in the original paper, but CAG:CUG is replaced by GUC:GAC and a C14:G15 base pair is added at the end. Note that the replacement corresponds to the swapping of the upper and lower sequences in the right stem.

A non-cleavable substrate in which C6 was replaced by 2′-*O*-methylcytidine was used for CD analysis to prevent cleavage during the experiments in the presence of Pb^{2+}.

RNA Synthesis and Purification—All RNA oligomers for the lead ribozyme were synthesized chemically by the solid-phase phosphoramidite method using *o*-nitrobenzyl groups for 2′-OH protection as described previously (*9*). For synthesis of the non-cleavable substrate, N^4-benzoyl-2′-*O*-methylcytidine was prepared and incorporated as

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Abbreviation: MOPS, 3-(*N*-morpholino)propanesulfonic acid.

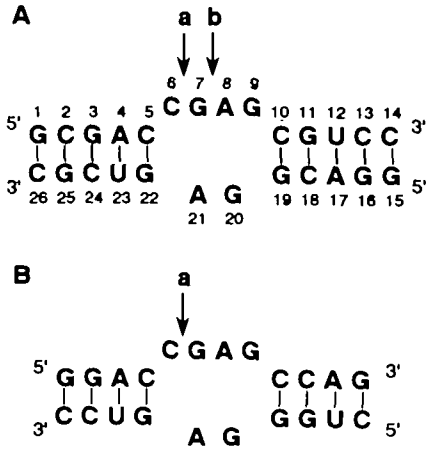

Fig. 1. **Sequence and numbering of the lead ribozyme used in this study (A), and the sequence of LZ4 in Ref. *1* (B).** Arrows indicate the cleavage sites, sites **a** and **b**.

described previously (*9, 10*). As the monomer units of coupling, *N*-acyl-5′-*O*-methoxytritylnucleoside-3′-(2-cyanoethyl)phosphoramidites were prepared according to the published procedure (*9, 11, 12*). We used 5-(*p*-nitrophenyl)tetrazole as a coupling agent. A cytidine resin derivative was prepared using long-chain alkylamine controlled pore glass as a support according to the procedure of Tanaka *et al.* (*11*). The average coupling yield was 98-99%. After treatment of the oligomer-bound resin with concentrated ammonia, the oligomers were isolated by reverse-phase chromatography. After detritylation and photolytic removal of the nitrobenzyl groups, the oligomers were purified by Sephadex G-25 gel filtration and C-18 reverse-phase chromatography, then desalted by treatment on a small DEAE-cellulose column with a volatile elution buffer, triethylammonium acetate. The counter cations, triethylammonium ions, were first replaced with pyridinium ions and then with sodium ions by using Dowex 50 cation-exchange columns as described previously (*9*). The final purity of the oligomers was checked by C-18 reverse-phase HPLC and was greater than 98%.

Cleavage Conditions—The substrate was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The reaction buffer for the cleavage was 15 mM MOPS buffer (pH 7.0). To examine the dependency of the activity of the lead ribozyme on the Pb^{2+} and Mg^{2+} concentrations, cleavage reactions were carried out with 1 μM enzyme and 1 μM substrate for 3 min with varying concentrations of $Pb(OAc)_2$ and $MgCl_2$. The first-order rate constant, k_{obs}, was determined in 0.2 mM $Pb(OAc)_2$ and 10 mM $MgCl_2$ with 10 μM enzyme and 1 μM substrate at 25°C by fitting the time course of the generation of cleavage products with a single exponential curve (*13, 14*).

The enzyme and substrate dissolved in the buffer solution were heated at 90°C for 2 min and cooled gradually to 25°C, then $MgCl_2$ was added to the solution. The cleavage reaction was started by the addition of $Pb(OAc)_2$ to the solution and stopped by the addition of an equal volume of 9 M urea, 50 mM EDTA. The solution was kept at 25°C during the reaction. The substrate and cleavage products were separated by electrophoresis on a denaturing 20% polyacrylamide gel, then their radioactivity was determined with a Bio-Image Analyzer BAS 2000 (Fuji Film, Tokyo).

CD Analysis—CD spectra and thermal CD melting curves were recorded on a JASCO J-720 spectropolarimeter with a 0.1-cm cell. Both the enzyme and the non-cleavable substrate were dissolved in 15 mM MOPS buffer (pH 7.0) and heated at 90°C for 3 min, followed by gradual cooling to 20°C. CD spectra were measured at varying concentrations of Pb^{2+} and Mg^{2+}. Thermal CD melting curves were recorded in the presence of Pb^{2+} and in the presence of both Pb^{2+} and Mg^{2+}. The temperature was raised from 5 to 85°C at a rate of 1°C/min. Changes in CD intensity were monitored at 265 nm. The melting temperatures were determined by the use of the derivatives of the melting curves.

RESULTS AND DISCUSSION

Characterization of the Lead Ribozyme with Modified Sequences—The right panel in Fig. 2A shows the dependency of the cleavage activity on the Pb^{2+} concentration in the presence of 10 mM Mg^{2+}. When the Pb^{2+} concentration was raised, cleavage at site **a** (see Fig. 1) was observed. The site of cleavage was identified by comparing the position of the cleavage product with those of products obtained by partial alkaline hydrolysis of the substrate on gel electrophoresis (data not shown). The positions of two products with a 2′,3′-cyclic phosphodiester and a 3′ phosphomonoester were too close to be resolved on the gel in Fig. 2, but these two products were resolved on the larger gel used for the identification of the products. Site **a** is identical to the cleavage site observed for the original lead ribozyme (*1*). No cleavage was observed in the absence of Pb^{2+} (the first lane of the right panel in Fig. 2A), thus Pb^{2+} is essential for the cleavage. Also no cleavage occurred when the substrate was mixed with Pb^{2+} and Mg^{2+} in the absence of the enzyme (data not shown). The first-order rate constant, k_{obs}, at 0.2 mM Pb^{2+} and 10 mM Mg^{2+} with 10 μM enzyme and 1 μM substrate was determined to be 0.35 min^{-1}, which is comparable to 0.6 min^{-1} (taken from Fig. 1 of Ref. *6*) obtained for the original lead ribozyme under identical metal conditions. These results confirm that our newly designed lead ribozyme possesses the essential characteristics of a lead ribozyme.

*Activation of the Cleavage at Site **b** and Repression of the Cleavage at Site **a** at Higher Pb^{2+} Concentrations*—The left panel in Fig. 2A shows the dependency of the cleavage activity on the Pb^{2+} concentration in the absence of Mg^{2+}. When the Pb^{2+} concentration was raised to 0.025 mM, cleavage at site **a** was observed. When the Pb^{2+} concentration was raised further to 0.1 mM, unexpected cleavage at a new site, site **b** (see Fig. 1), began to occur. The site of cleavage was identified in the same way as described above. The activity of cleavage at site **b** continued to increase with increasing Pb^{2+} concentration. Initiation of the cleavage at site **a** at a lower Pb^{2+} concentration and initiation of the cleavage at site **b** at a higher Pb^{2+} concentration indicates that at low Pb^{2+} concentrations, the first Pb^{2+} binds to the lead ribozyme and causes the cleavage at site **a** (2 of Fig. 3), and at a higher Pb^{2+} concentration, a second Pb^{2+} binds to the lead ribozyme and causes the cleavage at site **b** (3 of Fig. 3). We define the binding site for the first Pb^{2+} ion as site **1** and that for the second Pb^{2+} ion as site **2** (see 1 of Fig. 3). Our NMR study of the lead ribozyme demonstrated that

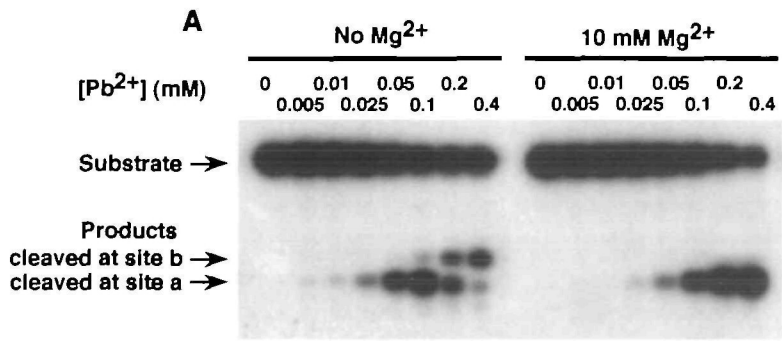

Fig. 2. **Dependency of cleavage yield on Pb^{2+} concentration.** (A) Autoradiograms of denaturing gels showing the cleavage of 1 μM substrate by 1 μM enzyme in 15 mM MOPS buffer (pH 7.0) at 25°C for 3 min with varying concentrations of Pb^{2+} without (left panel) and with (right panel) 10 mM Mg^{2+}. (B) Percentages of products cleaved at site **a** (open circles) and site **b** (open squares) in the absence of Mg^{2+}, and at site **a** in the presence of 10 mM Mg^{2+} (closed circles).

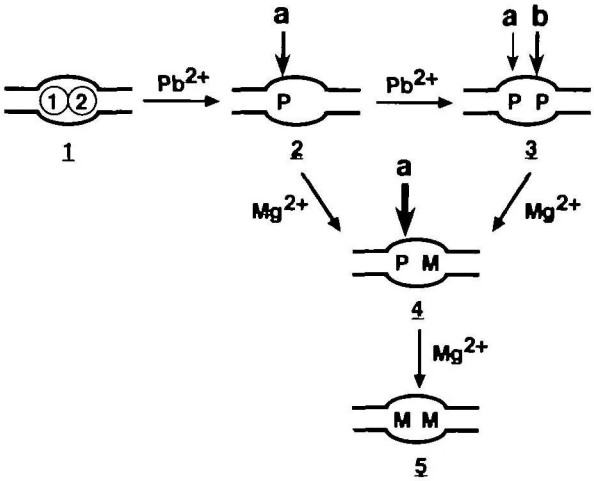

Fig. 3. **Activation and repression of the cleavage at sites a and b by the combination of Pb^{2+} and Mg^{2+}.** Circled **1** and **2** indicate the two metal binding sites in the lead ribozyme. Their relative positions in the lead ribozyme are arbitrary. P and M in the lead ribozyme indicate bound Pb^{2+} and Mg^{2+}, respectively. The thickness of the arrows indicates qualitatively the cleavage activities.

upon the addition of Pb^{2+}, changes in the chemical shift are detected for spatially localized G7, A8, A21, and G22, exclusively (*17, 18*). This indicates that the metal-binding sites are within or close to the asymmetric internal loop. Thus sites **1** and **2** are positioned in the asymmetric internal loop in Fig. 3. The relative position of **1** to that of **2** is arbitrary.

The activity of cleavage at site **a** reached a maximum at 0.1 mM Pb^{2+}, and was repressed by a further increase in the Pb^{2+} concentration (the cleavage yields are plotted in Fig. 2B). It should be noted that the cleavage at site **b** (open squares) begins at nearly the same Pb^{2+} concentration as that where a decrease in the cleavage at site **a** (open circles) begins. This strongly suggests that the binding of the second Pb^{2+} causes the cleavage at site **b** and simultaneously represses the cleavage at site **a**. It is supposed that the binding of the second Pb^{2+} causes a conformational change of the lead ribozyme to a form that is active for the cleavage at site **b** but less active (or even inactive) for that at site **a**. This is indicated by thickness of the arrows in step 3 of Fig. 3. Direct involvement of the second Pb^{2+} ion in the cleavage at site **b** is also likely.

It should be added that gel electrophoresis experiments demonstrated that the cleavages at both site **b** and site **a** terminate with a 3′ phosphomonoester.

The Origin of the Cleavage at the New Site—The cleavage at site **b** was not reported either in the original paper (*1*) or in related papers (*6-8, 16*). The lead ribozyme used in this study has minor alterations in the sequences of the original ribozyme. The alterations are an insertion of a C2:G25 base pair in the left stem and swapping of the upper and lower sequences in the right stem (Fig. 1). The lead ribozyme with the same insertion in the left stem has been examined (*6*), but cleavage at site **b** is not observed. All lead ribozymes examined so far have a C11A12G13:C(U) 16U17G18 segment in the right stem except for one case (*1, 6-8, 16*). Therefore, the swapping of the upper and lower sequences in the right stem seems to be responsible for the cleavage at the new site. In fact, we have confirmed that when the upper and lower sequences in the right stem are swapped back, the cleavage at site **b** is not observed under the conditions in Fig. 2 (data not shown). It is interesting that the alteration of a segment apart from the putative active site affects the cleavage site. We assume that the change in the sequences of the right stem has a long-range effect on the structure of the asymmetric internal loop, providing the space for the binding of the second Pb^{2+} ion, which results in the cleavage at site **b**.

Activation of the Cleavage at Site a and Repression of the Cleavage at Site b by Mg^{2+}—The right panel in Fig. 2A shows the cleavage activity in the presence of 10 mM Mg^{2+}. In the presence of 10 mM Mg^{2+}, a higher concentration of Pb^{2+} (0.05 mM) was required to produce the cleavage at site **a**. The cleavage at site **a** is caused by a Pb^{2+} ion bound at site **1**, and therefore this result suggests that Mg^{2+} competes with Pb^{2+} for binding to site **1**. In the presence of 10 mM Mg^{2+}, the activity continued to increase with increasing Pb^{2+} concentration and it surpassed even the maximum level reached in the absence of Mg^{2+} (closed

circles in Fig. 2B). This indicates that an adequate amount of Mg^{2+} can activate the cleavage at site **a**. In contrast, cleavage at site **b** was not observed in the presence of 10 mM Mg^{2+} at any Pb^{2+} concentration.

To study the activation and repression of the cleavage activities at the two sites by Mg^{2+} in detail, the dependency of the activities on the concentration of Mg^{2+} was examined at 0.05 mM and 0.2 mM Pb^{2+} (Fig. 4). In both cases, the cleavage at site **a** increases with the increase in Mg^{2+} concentration, reaches a maximum level, then decreases toward zero. The enhancement of activity at site **a** by Mg^{2+} reached 3.5–3.8-fold maximally for both Pb^{2+} concentrations. The ability of other metal ions to enhance the cleavage by the lead ribozyme in the presence of Pb^{2+} has been examined intensively, and it was reported that rare earth ions such as Nd^{3+} can enhance the cleavage (*7, 8*). The fact that enhancement by Mg^{2+} was not detected in these studies originates from the fact that the concentration of Mg^{2+} was not varied in these studies and that the Mg^{2+} concentration applied was not high enough to enhance the cleavage. It is demonstrated here that Mg^{2+} can also enhance the activity if it is applied in an adequate concentration.

Concerning site **b**, cleavage was not observed at any Mg^{2+} concentration with 0.05 mM Pb^{2+}, and the cleavage with 0.2 mM Pb^{2+} was repressed by the addition of Mg^{2+}.

Binding of Mg^{2+} at Site 2 Enhances the Cleavage at Site a by Stabilizing the Enzyme-Substrate Complex—The concentration of Mg^{2+} which gave the maximum activity depended on the concentration of Pb^{2+}: 0.5 mM Mg^{2+} for 0.05 mM Pb^{2+}, and 2 mM Mg^{2+} for 0.2 mM Pb^{2+} (Fig. 4B). The higher the concentration of Pb^{2+}, the higher was the concentration of Mg^{2+} required to give the maximum enhancement of the activity. This indicates that a Mg^{2+} ion which enhances the cleavage at site **a** competes with a Pb^{2+} ion for binding to the lead ribozyme. Site **1** is competitively bound by Pb^{2+} and Mg^{2+} as described above, but apparently site **1** is not a binding site for a Mg^{2+} ion which enhances the cleavage at site **a**, because a Pb^{2+} ion at site **1** is responsible for the cleavage at site **a** and its replacement by Mg^{2+} would abolish the cleavage itself. It should be noted that, in the case of 0.2 mM Pb^{2+}, the cleavage at site **a** reaches a maximum level at 2 mM Mg^{2+} (open circles in Fig. 4B), while the cleavage at site **b** reaches nearly zero under the same conditions (open squares in Fig. 4B). In that the cleavage at site **b** is caused by a Pb^{2+} at site **2**, this coincidence strongly suggests that the Mg^{2+} which enhances the cleavage at site **a** binds to site **2**, replacing Pb^{2+} there and abolishing the cleavage at site **b** (4 of Fig. 3). The binding of Mg^{2+} to the sites **1** and **2**, which are defined as the binding sites of Pb^{2+} originally, is supported by the NMR result that on the addition of Mg^{2+}, changes of chemical shifts are observed for the same residues as those on the addition of Pb^{2+} (*17, 18*). The enhancement of the cleavage at site **a** is indicated by a thicker arrow in step 4 in Fig. 3.

Thermal CD melting curves were obtained under different metal conditions with 16 μM of both the enzyme and substrate. In the presence of 0.2 mM Pb^{2+}, the melting point was 47°C. When 10 mM Mg^{2+} was added in the presence of 0.2 mM Pb^{2+}, the melting point increased to 61°C, indicating stabilization of the complex by Mg^{2+}. Thus the enhancement of the cleavage at site **a** by Mg^{2+} is assumed to be brought about by the stabilization of the enzyme-substrate complex. The addition of 100 mM Na^{+} also stabilized the complex, but the addition of Mg^{2+} gave further stabilization. Thus the effect of Mg^{2+} is not only ionic.

In the case of the enhancement by Nd^{3+}, direct involve-

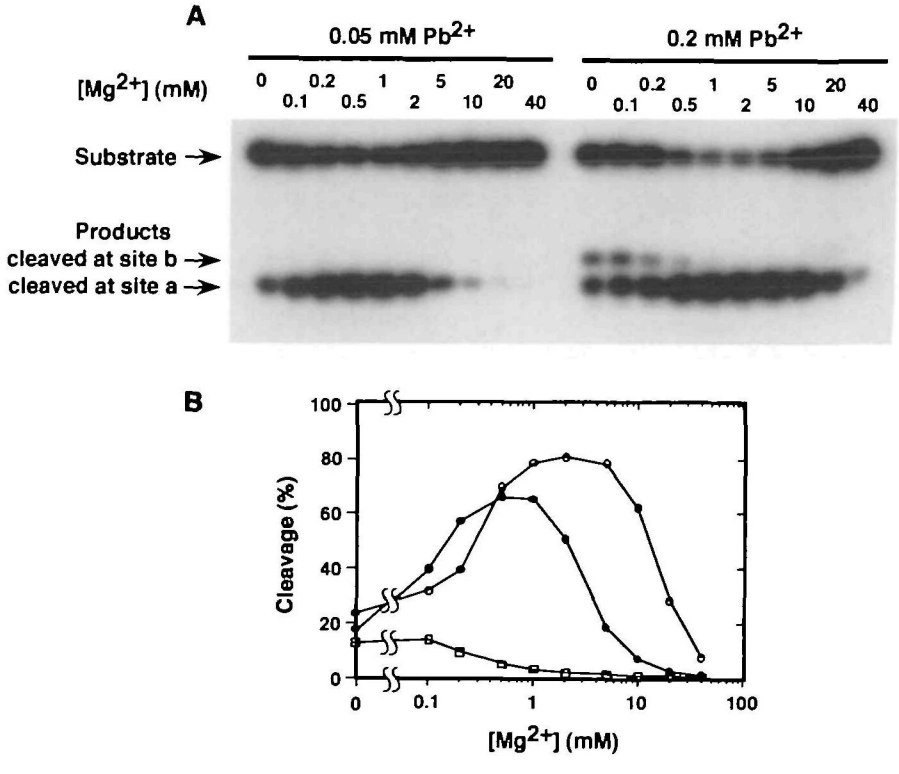

Fig. 4. **Dependency of cleavage yield on Mg^{2+} concentration.** (A) Autoradiograms of denaturing gels showing the cleavage of 1 μM substrate by 1 μM enzyme in 15 mM MOPS buffer (pH 7.0) at 25°C for 3 min with varying concentrations of Mg^{2+} at 0.05 mM Pb^{2+} (left panel) and at 0.2 mM Pb^{2+} (right panel). (B) Percentages of products cleaved at site **a** at 0.05 mM Pb^{2+} (closed circles), and at site **a** (open circles) and site **b** (open squares) at 0.2 mM Pb^{2+}.

ment of Nd^{3+} in the cleavage reaction as an acid catalyst was proposed (*8*). Mg^{2+} could also play the same role in addition to the stabilization of the complex.

Overall Process Involved in the Addition of Mg^{2+}—Further addition of Mg^{2+} repressed the cleavage at site **a**. This may be because Pb^{2+} at site **1**, which is responsible for the cleavage at site **a**, is finally replaced by Mg^{2+} (5 of Fig. 3). More Mg^{2+} is required to completely repress the cleavage at site **a** with 0.2 mM Pb^{2+} (open circles in Fig. 4B) than with 0.05 mM Pb^{2+} (closed circles in Fig. 4B). This is another indication of the competition for binding to site **1** between Pb^{2+} and Mg^{2+}.

The path 2→4→5 (Fig. 3) represents the situation observed on addition of Mg^{2+} at 0.05 mM Pb^{2+} (closed circles of Fig. 4B); the path 3→4→5 represents that observed on addition of Mg^{2+} at 0.2 mM Pb^{2+} (open circles and open squares in Fig. 4B). The latter path needs more Mg^{2+} than the former, because Mg^{2+} has to compete with the higher concentration of Pb^{2+} for binding at both sites 1 and **2** in the latter path, and this is actually what is observed in Fig. 4. Another aspect of the effect of Mg^{2+} addition in the course of 3→4 is the resultant specificity of the cleavage site of the lead ribozyme.

Relative Affinities of Pb^{2+} and Mg^{2+} for Binding to Sites 1 and 2—The relative affinities of the two metals to sites **1** and **2** were estimated as follows. Firstly, in the absence of Mg^{2+} the cleavage at site **a** begins at 0.025 mM Pb^{2+}, while that at site **b** begins at 0.1 mM Pb^{2+} (Fig. 2A). When the slight difference in the amount of the corresponding cleavage product is taken into account, Pb^{2+} binds to site **1** about 5 times more strongly than to site **2**. Secondly, Pb^{2+} binds to site **2** 1-2 times more strongly than Mg^{2+} does, because the Mg^{2+} concentration required to reach the midpoint of the course of cleavage activation at site **a** (the left halves of the bell-shaped curves with closed and open circles in Fig. 4B) is 1-2 times the applied Pb^{2+} concentration, *i.e.*, 0.1 mM Mg^{2+} for 0.05 mM Pb^{2+} and 0.3 mM Mg^{2+} for 0.2 mM Pb^{2+}. A similar ratio is obtained by comparing the Mg^{2+} concentration (0.3 mM) required to reach the midpoint of the course of cleavage repression at site **b** (the curve with open squares in Fig. 4B) with the applied Pb^{2+} concentration (0.2 mM). Thirdly, Pb^{2+} binds to site 1 about 50-100 times more strongly than Mg^{2+} does, because the Mg^{2+} concentration required to reach the midpoint of the course of cleavage repression at site **a** (the right halves of the bell-shaped curves with closed and opened circles in Fig. 4B) is 50-100 times the applied Pb^{2+} concentration, *i.e.*, 3 mM Mg^{2+} for 0.05 mM Pb^{2+} and 15 mM Mg^{2+} for 0.2 mM Pb^{2+}. From these results, the relative affinities are concluded to be as follows: Pb^{2+} to site **1**: Pb^{2+} to site **2**: Mg^{2+} to site **2**: Mg^{2+} to site **1** = 100:20:20-10:2-1. It should be noted that Mg^{2+} binds to site **2** more strongly than to site **1**.

When the concentration of Pb^{2+} was increased in the presence of 10 mM Mg^{2+}, no cleavage at site **b** was observed (right panel of Fig. 2A). Even at the highest Pb^{2+} concentration (0.4 mM), there exists 25 times as much Mg^{2+} as Pb^{2+}. In that Pb^{2+} binds to site 2 only 1-2 times more strongly than Mg^{2+} does, the Mg^{2+} ion at site **2** may not be replaced by a Pb^{2+} ion under these conditions, and thus the cleavage at site **b** is not observed.

Effects of Pb^{2+} and Mg^{2+} on the Structure of the Lead Ribozyme, as Monitored by CD—Figure 5A shows CD spectra of the lead ribozyme under different metal conditions. As mentioned previously, in order to prevent cleavage of the substrate during the CD experiments, a non-cleavable substrate, in which C6 is replaced by 2′-*O*-methylcytidine, was used. No modification was introduced to the 2′-OH of G7 residue, because a preliminary gel electrophoresis experiment showed that the cleavage at site

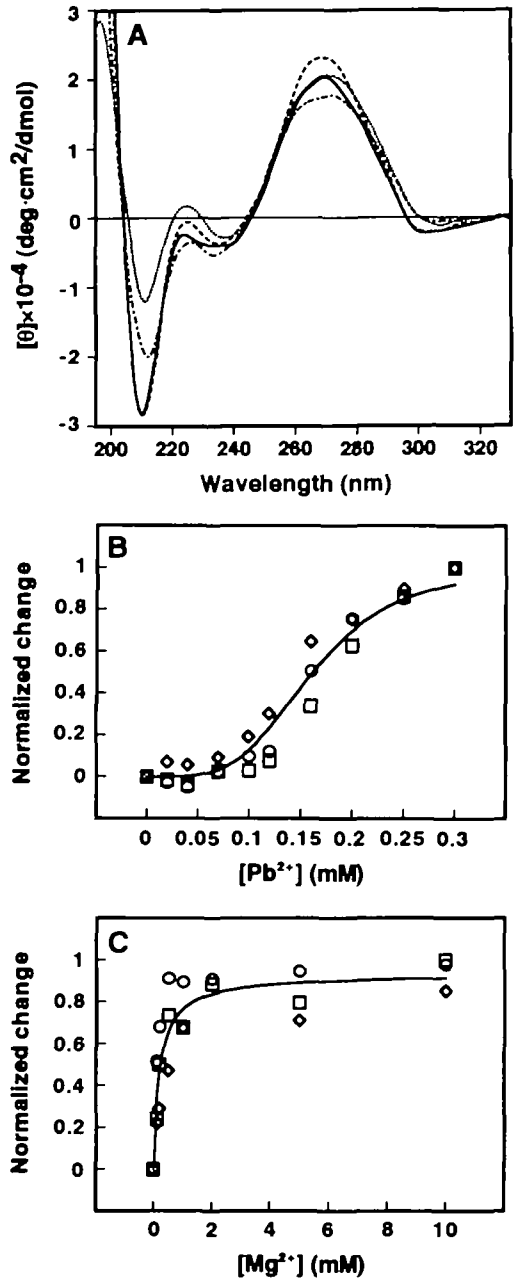

Fig. 5. **Changes of CD spectra by the addition of metals.** (A) CD spectra of the lead ribozyme (12 μM) in 15 mM MOPS buffer (pH 7.0) at 20°C without metal ions (dotted), with 0.2 mM Pb^{2+} (centered), with 10 mM Mg^{2+} (dashed), and with both 0.2 mM Pb^{2+} and 10 mM Mg^{2+} (solid). (B) Normalized changes of the ellipticity of the lead ribozyme (16 μM) in 15 mM MOPS buffer (pH 7.0) at 20°C upon the addition of Pb^{2+} at 210.5 nm (circles), 223 nm (squares), and 271 nm (diamonds). (C) Normalized changes of the ellipticity of the lead ribozyme (16 μM) in 15 mM MOPS buffer (pH 7.0) at 20°C upon the addition of Mg^{2+} at 210.5 nm (circles), 223 nm (squares), and 267 nm (diamonds).

b is repressed to a great extent by the modification of the C6. In fact, gel electrophoresis of the samples after CD measurement confirmed that the substrate was not cleaved during measurement of the CD spectra. On addition of Pb^{2+}, the ellipticity at 270 nm decreased, while on the addition of Mg^{2+} it increased with respect to that in the absence of the metals, suggesting that the two metals have different effects on the structure of the lead ribozyme. When both metals were added, the ellipticity at 270 nm was close to that in the absence of the metals, but the ellipticity at 210 nm was rather close to that in the presence of Mg^{2+}. This implies that the lead ribozyme assumes different structures in response to different metal conditions. The differences in the CD spectra in the presence of Pb^{2+} alone and in the presence of both Pb^{2+} and Mg^{2+} imply that the structural change may also be related to the enhancement of the cleavage at site a in step 4 in Fig. 3, in addition to the stabilization of the complex.

Binding Properties of the Two Ions—The binding properties of Pb^{2+} and Mg^{2+} to the lead ribozyme were examined by monitoring the changes in the ellipticity upon the addition of each metal. Figure 5, B and C, shows the normalized changes of the ellipticity at three different wavelengths. The two curves in Fig. 5, B and C, are quite different, the former being sigmoidal, the latter hyperbolic. These results indicate that the binding of Pb^{2+} to the lead ribozyme is cooperative, while the binding of Mg^{2+} is not. The Hill coefficient (*13, 15*) of the binding of Pb^{2+} was determined to be 3.9-4.4, about four, while that of Mg^{2+} was 0.7-0.9, depending on the wavelength employed. All four Pb^{2+} that bind to the lead ribozyme ions are not necessarily related to the expression of the activity. For simplicity, the binding of two Pb^{2+} ions is assumed to be directly related to the cleavage, because all data can be explained essentially by this idea as discussed above. The Hill coefficient of *ca.* 1 for Mg^{2+} does not mean that the number of Mg^{2+} ions bound to the lead ribozyme is one, rather it indicates a lack of cooperativity. Thus the CD results are not contradictory to the idea that Pb^{2+} and Mg^{2+} bind competitively to the two sites, **1** and **2**.

An NMR study of the lead ribozyme is now in progress in our laboratory to get further structural information.

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REFERENCES

1. Pan, T. and Uhlenbeck, O.C. (1992) A small metalloribozyme with a two-step mechanism. *Nature* **358**, 560-563
2. Pan, T. and Uhlenbeck, O.C. (1992) In vitro selection of RNAs that undergo autolytic cleavage with Pb^{2+}. *Biochemistry* **31**, 3887-3895
3. Perrotta, A.T. and Been, M.D. (1992) Cleavage of oligoribonucleotides by a ribozyme derived from the hepatitis d virus RNA sequence. *Biochemistry* **31**, 16-21
4. Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R., and Bruening, G. (1986) Autolytic processing of dimeric plant virus satellite RNA. *Science* **231**, 1577-1580
5. Uhlenbeck, O.C. (1987) A small catalytic oligoribonucleotide. *Nature* **328**, 596-600
6. Pan, T., Dichtl, B., and Uhlenbeck, O.C. (1994) Properties of an *in vitro* selected Pb^{2+} cleavage motif. *Biochemistry* **33**, 9561-9565
7. Sugimoto, N. and Ohmichi, T. (1996) Site-specific cleavage reaction catalyzed by leadzyme is enhanced by combined effect of lead and rare earth ions. *FEBS Lett.* **393**, 97-100
8. Ohmichi, T. and Sugimoto, N. (1997) Role of Nd^{3+} and Pb^{2+} on the RNA cleavage reaction by a small ribozyme. *Biochemistry* **36**, 3514-3521
9. Odai, O., Kodama, H., Hiroaki, H., Sataka, T., Tanaka, T., and Uesugi, S. (1990) Synthesis and NMR study of ribooligonucleotides forming a hammerhead-type RNA enzyme system. *Nucleic Acids Res.* **18**, 5955-5960
10. Inoue, T., Hayase, Y., Imura, A., Iwai, S., Miura, K., and Ohtsuka, E. (1987) Synthesis and hybridization studies on two complementary nona(2′-*O*-methyl)ribonucleotides. *Nucleic Acids Res.* **15**, 6131-6148
11. Tanaka, T., Tamatsukuri, S., and Ikehara, M. (1986) Solid phase synthesis of oligoribonucleotides using *o*-nitrobenzyl protection of 2′-hydroxyl via a phosphite triester approach. *Nucleic Acids Res.* **14**, 6265-6279
12. Sataka, T., Hiroaki, H., Oda, Y., Tanaka, T., Ikehara, M., and Uesugi, S. (1990) Studies on structure and stabilizing factor of the CUUCGG hairpin RNA using chemically synthesized oligonucleotides. *Nucleic Acids Res.* **18**, 3831-3839
13. Fersht, A. (1985) *Enzyme Structure and Mechanism*, Freeman & Co., New York
14. McCall, M.J., Hendry, P., and Jennings, P.A. (1992) Minimal sequence requirements for ribozyme activity. *Proc. Natl. Acad. Sci. USA* **89**, 5710-5714
15. Celander, D.W. and Cech, T.R. (1991) Visualizing the higher order folding of a catalytic RNA molecule. *Science* **251**, 401-407
16. Chartrand, P., Usman, N., and Cedergren, R. (1997) Effect of structure modifications on the activity of the leadyme. *Biochemistry* **36**, 3145-3150
17. Katahira, M., Sugiyama, T., Kanagawa, M., Kim, M.H., Uesugi, S., and Kohno, T. (1996) NMR studies of a lead ribozyme and its non-cleavage analogue. *Nucleosides Nucleotides* **15**, 489-503
18. Kim, M.H., Sugiyama, T., Kanagawa, M., Tanaka, Y., Nagaoka, M., Nishimura, Y., Kohno, T., Kurihara, Y., Katahira, M., and Uesugi, S. (1996) Effects of metal ions on activity and structure of a lead ribozyme. *Nucleic Acids Symp. Ser.* **35**, 133-134