Role of Nd^{3+} and Pb^{2+} on the RNA Cleavage Reaction by a Small Ribozyme†

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ABSTRACT: Leadzyme is a ribozyme that requires Pb^{2+}. We have previously shown that the addition of Nd^{3+} in the presence of Pb^{2+} increased significantly the yield of the RNA cleavage reaction by a leadzyme, although other rare earth ions or divalent ions except Pb^{2+} did not promote the reaction [Sugimoto, N., & Ohmichi, T. (1996) *FEBS Lett. 393*, 97−100]. To investigate the combined effects of Nd^{3+} and Pb^{2+} on the binding and cleavage steps of a leadzyme, CUGGGAGUCC, with a substrate, GGACCGAGCCAG, kinetics for the leadzyme reaction have been measured at various concentration ratios of Nd^{3+} and Pb^{2+}. At low concentration ratios of Nd^{3+} under a constant total concentration of metal ions, Nd^{3+} increased the stability of the complex between the leadzyme and the substrate. In contrast, at high concentration ratios of Nd^{3+}, the addition of Nd^{3+} decreased the stability of the complex. The rate constant of the cleavage step was maximized when the ratio of Nd^{3+} to Pb^{2+} was 1:1. These results suggest that the complex between the leadzyme and the substrate has binding sites for Nd^{3+} ion that influence complex stability and catalyze directly the cleavage reaction. On the basis of the results, we propose a two-metal-ion mechanism in which Pb^{2+} and Nd^{3+} play the roles of base and acid catalyst, respectively.

A ribozyme is an RNA enzyme that can catalyze a biochemical reaction (Cech, 1993; Pan et al., 1993). Known ribozymes are derived from self-splicing group I and II introns, an RNA subunit of ribonuclease P, satellite RNAs of tobacco ringspot virus and human hepatitis δ virus, and so on (Guerrier-Takada., 1983; Prody et al., 1986; Wu et al., 1989; Symons, 1992; Cech, 1993; Pan et al., 1993). Ribozyme reactions are very sensitive to species and concentration of metal ions (Pyle, 1993; Long et al., 1995; Bevilacqua et al., 1996), suggesting that divalent metal ions may play important roles for the chemical reaction and structural stabilization of the ribozyme (Grosshans & Cech, 1989; Pyle, 1993). Thus, the secondary and tertiary structures of ribozyme−substrate complexes are important for ribozyme activities. In order to understand the structures, NMR and X-ray measurements were extensively done (Chastain & Tinoco, 1992, 1993; Pley et al., 1994; Scott et al., 1995; Nowakowski & Tinoco, 1996). Also, on the basis of the nearest-neighbor model and parameters (Freier et al., 1986; Turner et al., 1988; Sugimoto et al., 1995), the predictions of the secondary structure were done (Sugimoto et al., 1993). For example, secondary and tertiary structures of a hammerhead ribozyme, which can cleave substrate RNAs with high sequence specificity by using recognition of Watson−Crick base pairs (Uhlenbeck, 1987; Symons, 1992), were revealed by X-ray measurements and the prediction of the secondary structures (Sugimoto et al., 1993; Pley et al., 1994; Scott et al., 1995). In many studies of ribozyme mechanisms, the chemical steps as well as the active structures have been discussed (Sugimoto et al., 1988, 1989; Piccirilli et al., 1993; Pyle & Green, 1994; Dahm et al., 1993; Hendry & McCall, 1995; Long et al., 1995; Jankowsky & Schwenzer., 1996). For example, it was suggested that the reverse-cyclization reaction of the group I intron from *Tetrahymena thermophila* requires two Mg^{2+} ions in the cleavage step (Sugimoto et al., 1988).

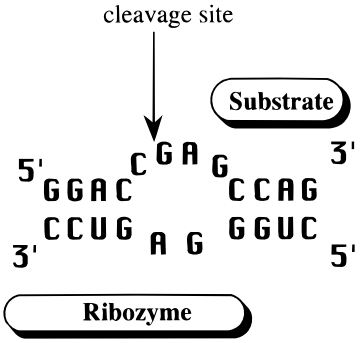

FIGURE 1: Secondary structure of the complex between the leadzyme and the substrate RNA.

The leadzyme is an RNA originally isolated from a library of $tRNA^{Phe}$ with 9−10 randomized positions by using *in vitro* selection. The sequences required for cleavage form a smaller secondary structure than hammerhead and hairpin ribozymes (Pan & Uhlenbeck, 1992a,b). Cleavage of an RNA substrate by the ribozyme requires at least two steps: binding and cleavage. Recently, we reported that the yield of the RNA cleavage catalyzed by a lead-dependent ribozyme (leadzyme) increased significantly when Nd^{3+} was present in addition to Pb^{2+} (Ohmichi et al., 1995; Sugimoto & Ohmichi, 1996). The combined effect of Pb^{2+} and Nd^{3+} on the leadzyme reaction provides an important view to the role of different metal ions on the ribozyme mechanism. In this paper, the combined effects of Nd^{3+} and Pb^{2+} on both the structural stabilization of the complex between a leadzyme, CUGGGAGUCC, and a substrate, GGACCGAGCCAG, and the cleavage step in the active center of the complex are investigated kinetically at various concentration ratios of Nd^{3+} and Pb^{2+}. The kinetic results suggest that Nd^{3+} as well

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[1] Abbreviations: HPLC, high-performance liquid chromatography; Na_2EDTA, ethylenediaminetetraacetate; MOPS, 3-(*N*-morpholino)-propanesulfonic acid.

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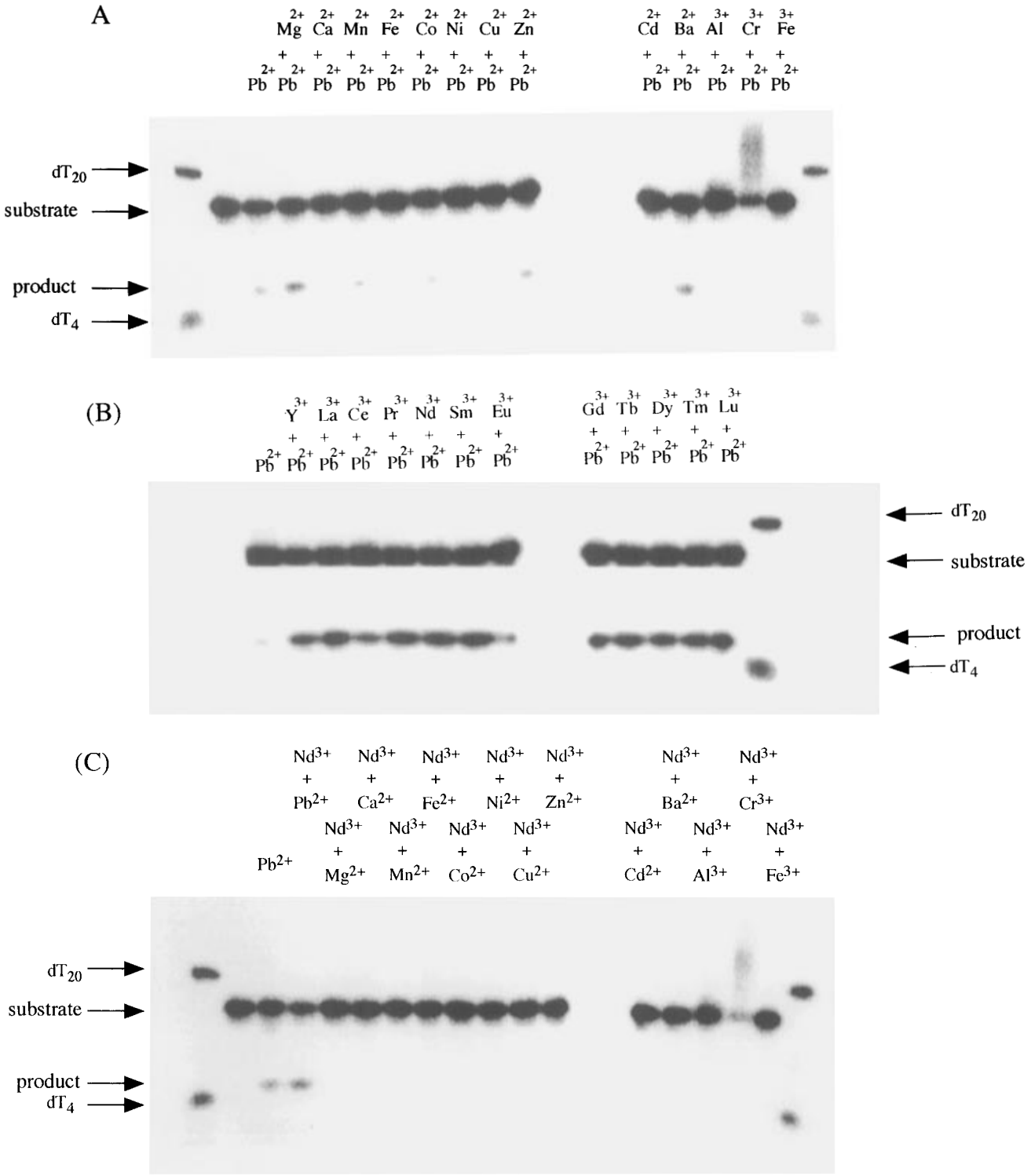

FIGURE 2: Autoradiograms of denaturing 20% polyacrylamide gels showing the cleavage of 250 nM substrate RNA by 2 μM ribozyme in 15 mM NaMOPS (pH 7.5) solution containing (A) 25 μM Pb^{2+} and 25 μM divalent or trivalent ion, (B) 25 μM Pb^{2+} and 25 μM rare earth ion, and (C) 25 μM Nd^{3+} and 25 μM divalent or trivalent ion at 25 °C for 20 min.

as Pb^{2+} directly takes part in the cleavage step and also influences the stability of the leadzyme−substrate complex.

MATERIALS AND METHODS

Preparation of Oligonucleotides. The RNA substrate and the leadzyme were synthesized on solid support with a phosphoramidite method on an Applied Biosystems model 391 DNA/RNA synthesizer (Kierzek et al., 1986). The synthesized oligomers were removed from 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 equiv of tetrabutylammonium fluoride (TBAF) per equivalent of silyl, and the mixtures were incubated overnight in the dark at room temperature (Campbell & Cech, 1995). Then, the samples were passed through a C18 Sep-Pak cartridge (Waters) to be desalted 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

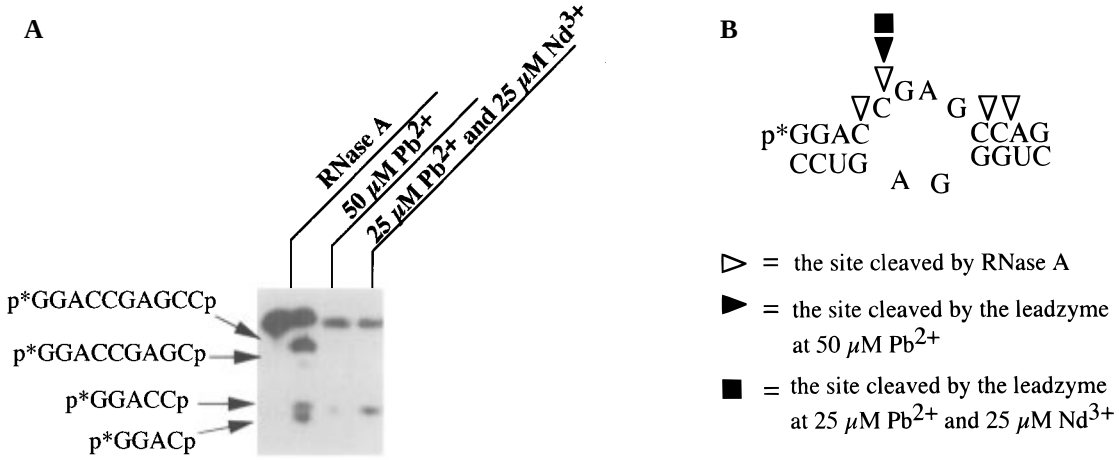

FIGURE 3: (A) Autoradiogram of denaturing 20% polyacrylamide gel showing the substrate cleavage by the ribozyme or RNase A. The substrate cleavage by the ribozyme was done in a solution containing 125 nM substrate RNA, 15 μM leadzyme, 15 mM NaMOPS (pH 7.5), and 50 μM Pb^{2+}, or both 25 μM Pb^{2+} and 25 μM Nd^{3+} at 25 °C. Reaction time was 20 min. (B) Secondary structure of the complex between the leadzyme and the substrate RNA. Solid square, solid triangle, and open triangle show the sites cleaved by the leadzyme at 25 μM Nd^{3+} and Pb^{2+}, the leadzyme at 50 μM Pb^{2+}, and RNase A, respectively.

purification by HPLC, the oligomers were desalted again with a C18 Sep-Pak cartridge. Final purities of the oligomers were checked by HPLC and were greater than at least 98%. Concentrations of the purified oligonucleotides were determined spectrophotometrically with a Hitachi U-3210 spectrophotometer. The RNA substrate with 5′-OH was 5′ end-labeled in 25-μL reaction mixture containing 25 pmol of substrate RNA, 150 μCi [γ-^{32}P]ATP (6000 Ci/mol, New England Nuclear), 10 units of T4 polynucleotide kinase (Pharmacia Biotech), 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 5 mM dithiothreitol.

Cleavage Buffer. Cleavage was initiated by the addition of 7 μL of 15 mM NaMOPS buffer containing metal ions and rare earth ions. The cleavage buffer was made by the additions of $Pb(OAc)_2$, $MgCl_2$, $BaCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $CaCl_2$, $FeCl_2$, $NiCl_2$, $CuCl_2$, $CdCl_2$, $AlCl_3$, $CrCl_3$, $FeCl_3$, $LaCl_3$, $CeCl_3$, $Pr(OAc)_3$, $Nd(OAc)_3$, $SmCl_3$, $EuCl_3$, $GdCl_3$, $Tb(OAc)_3$, $DyCl_3$, $TmCl_3$, or $Lu(OAc)_3$ to 15 mM NaMOPS buffer (pH 7.5).

Kinetics. The rate constants for cleavage reactions by the ribozyme were determined under single-turnover conditions. Single-turnover experiments with the ribozyme in excess over the substrate RNA were carried out in 15 mM NaMOPS (pH 7.5) at 25 °C. The ribozyme (2.5−8.0 μM) and the 5′ end-labeled substrate RNA (250 nM) 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 NaMOPS, pH 7.5. Cleavage was initiated by the addition of 7 μL of 15 mM NaMOPS buffer containing Nd^{3+} and Pb^{2+}. Reactions were terminated by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of 200 mM Na_2EDTA, 8 M urea, 0.02% bromophenol blue, and 0.02% xylene cyanol. The labeled product and substrate were separated by denaturing 20% polyacrylamide gel electrophoresis. The radioactivity of the substrate and the product was analyzed with a Bio-Image Analyzer (BAS 2000; Fuji Film, Tokyo). The observed rate constants were obtained by plotting the natural log of the product vs time. When the amount of product after 24 h was chosen as the amount at the endpoint of the reaction, all the plots were linear. Nonlinear least-squares fits of plots of observed rate constants vs ribozyme concentration to kinetic equations were done with a software Igor (Wava Metrics) to obtain separately the binding constant and the cleavage rate constants.

UV Melting Measurements and Melting Temperature Prediction. Absorbance measurements in UV region were made with Hitachi U-3200 and U-3210 spectrophotometers. Melting curves (absorbance vs temperature curves) were measured at 260 nm with these spectrophotometers connected to Hitachi SPR-7 and SPR-10 thermoprogrammers and melting temperatures (T_m) were obtained from these curves. The heating rate was 0.5 or 1.0 °C/min. The water condensation on the cuvette exterior at the low temperature range was avoided by flushing with a constant stream of dry N_2 gas. The predicted melting temperature for the leadzyme−substrate complex was calculated with a nearest-neighbor thermodynamic calculation (Turner et al., 1988, 1990) by using enthalpy and entropy numbers for Watson−Crick pairs, a helix initiation (Freier et al., 1986), and an asymmetric internal loop (Peritz et al., 1991).

RESULTS

Combined Effect of Metal Ions on RNA Cleavage Yield. The structure of the complex between the leadzyme and the RNA substrate used in our experiments is shown in Figure 1. This leadzyme, CUGGGAGUCC, binds to the RNA substrate, GGACCGAGCCAG, and acts as a ribozyme in the presence of 25 μM Pb^{2+} at 15 mM NaMOPS (pH 7.5) (Sugimoto & Ohmichi, 1996) so that it cleaves the substrate RNA at one site as shown in Figure 1. The cleavage reactions were initiated by mixing the leadzyme−substrate complex with Pb^{2+} under single-turnover conditions. This Pb^{2+}-dependent reaction was not observed with other divalent ions (Mg^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{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+}, Lu^{3+}, Pr^{3+}, La^{3+}, Sm^{3+}, and Nd^{3+}) at 25 μM metal ion concentration (Sugimoto & Ohmichi, 1996). These results are consistent with those reported for the similar Pb^{2+} self-cleavage RNA (Pan & Uhlenbeck, 1992b; Pan et al., 1994).

Substrate cleavage was not observed in the presence of Pb^{2+} but absence of the leadzyme. This result indicates that formation of the leadzyme−substrate complex is necessary for substrate cleavage. The melting temperature (T_m) for 8

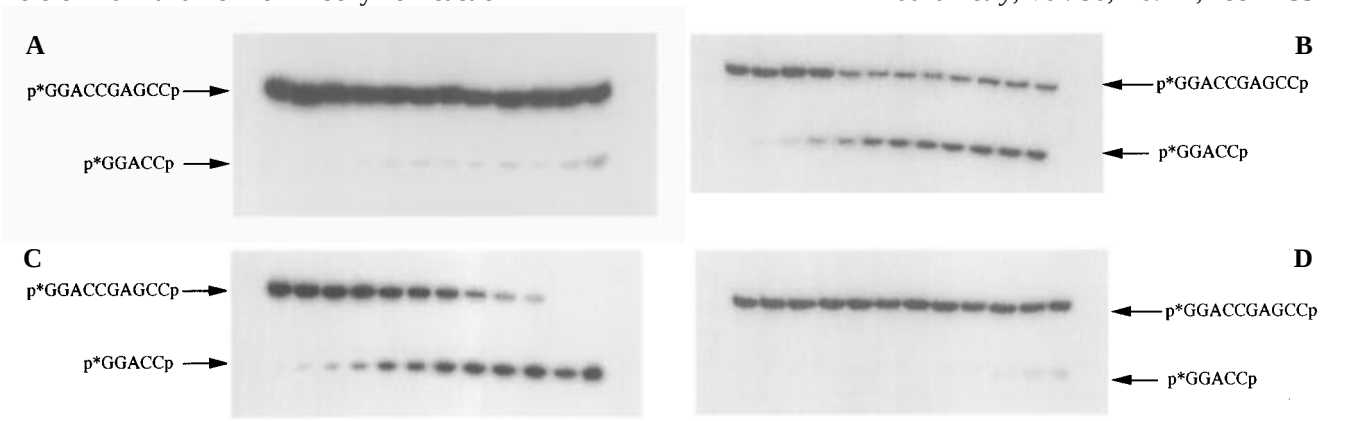

FIGURE 4: Autoradiograms of denaturing 20% polyacrylamide gels showing time dependence of the substrate cleavage by the leadzyme at (A) 50 μM Pb^{2+}, (B) 37.5 μM Pb^{2+} and 12.5 μM Nd^{3+}, (C) 25 μM Pb^{2+} and 25 μM Nd^{3+}, and (D) 12.5 μM Pb^{2+} and 37.5 μM Nd^{3+}. The substrate (125 nM) was incubated at 25 °C with 15 μM ribozyme in 15 mM NaMOPS (pH 7.5) solution. From left to right in the gels, incubation times are 0, 1, 2, 4, 6, 8, 12, 16, 20, 30, 60, and 90 min, respectively.

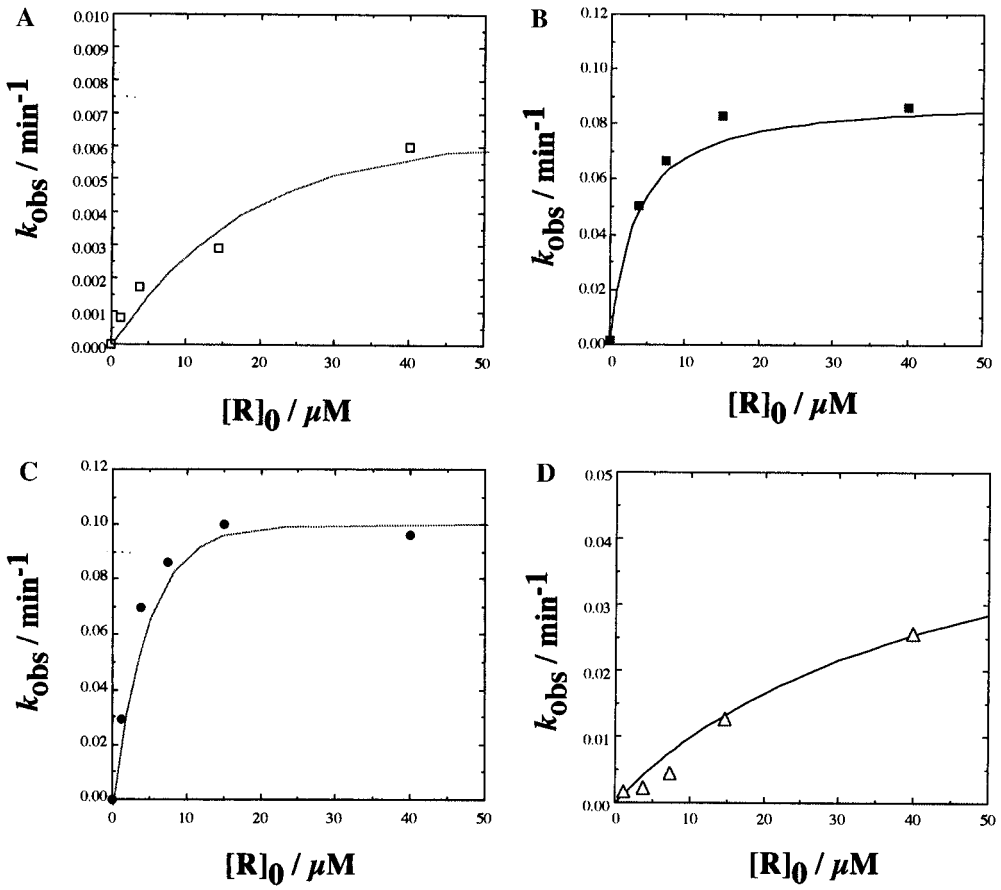

FIGURE 5: Plots of k_{obs} at 25 °C vs the concentration of ribozyme in 15 mM NaMOPS (pH 7.5) solution containing (A) 50 μM Pb^{2+}, (B) 37.5 μM Pb^{2+} and 12.5 μM Nd^{3+}, (C) 25 μM Pb^{2+} and 25 μM Nd^{3+}, and (D) 12.5 μM Pb^{2+} and 37.5 μM Nd^{3+}, respectively. Solid curves were obtained with the nonlinear least-square fits to eq 2.

μM leadzyme−substrate (CUGGGAGUCC/GGACCGAGCCAG) duplex in 1 M NaCl−phosphate buffer (pH 7.0) was 40.1 °C. The value of T_m in 15 mM NaMOPS buffer (pH 7.5) containing 25 μM Mg^{2+} at which the substrate was not cleaved was 33.2 °C. As the predicted T_m value for 8 μM at 1 M NaCl was calculated to be 33 °C, these measured values were slightly higher than the predicted value. The stable complex formation at 1 M NaCl or even at the very low concentration of divalent ion is probably due to the high stability of the G·A tandem mismatches in the asymmetric internal loop of the complex since the G·A tandem mismatches are known to be very stable (SantaLucia et al., 1991a,b).

It is known that metal ions participate both in the folding of ribozymes and in the catalytic mechanism (Grosshans & Cech, 1989, Pyle, 1993). Figure 2 shows the combined effects of 25 μM metal ions and 25 μM Pb^{2+} on the reaction yield for a 20 min incubation. The concentration of 25 μM is the lowest concentration of Pb^{2+} at which the cleavage reaction of the substrate by the leadzyme was observed after 20 min in the presence of Pb^{2+} only (Sugimoto & Ohmichi, 1996). As shown in Figure 2, the cleavage site was not changed by the addition of the divalent or trivalent ion. The cleavage yield in the presence of both 25 μM Mg^{2+} and 25 μM Pb^{2+} was 14.0%. Although the value is two times larger than the 7.0% observed at 25 μM Pb^{2+} only, this was almost

Table 1: Kinetic Parameters for Cleavage[a]

ion condition	$K_1 \times 10^{-5}$ (M^{-1})	$k_2 \times 10$ (min^{-1})	$\Delta G°_{25,complex}$[b] (kcal/mol)
50.0 μM Pb^{2+}	0.93 ± 0.27	0.07 ± 0.01	−6.77 ± 0.21
37.5 μM Pb^{2+} and 12.5 μM Nd^{3+}	3.03 ± 0.48	0.90 ± 0.02	−7.48 ± 0.04
25.0 μM Pb^{2+} and 25.0 μM Nd^{3+}	3.03 ± 0.80	1.26 ± 0.02	−7.48 ± 0.02
12.5 μM Pb^{2+} and 37.5 μM Nd^{3+}	0.22 ± 0.02	0.54 ± 0.03	−5.92 ± 0.06

[a] All experiments were done in ribozyme excess condition with ribozyme concentration ranging from 1.25 to 40 μM and [5′−^{32}P] substrate concentration of less than 0.125 μM. [b] The value calculated from K_1.

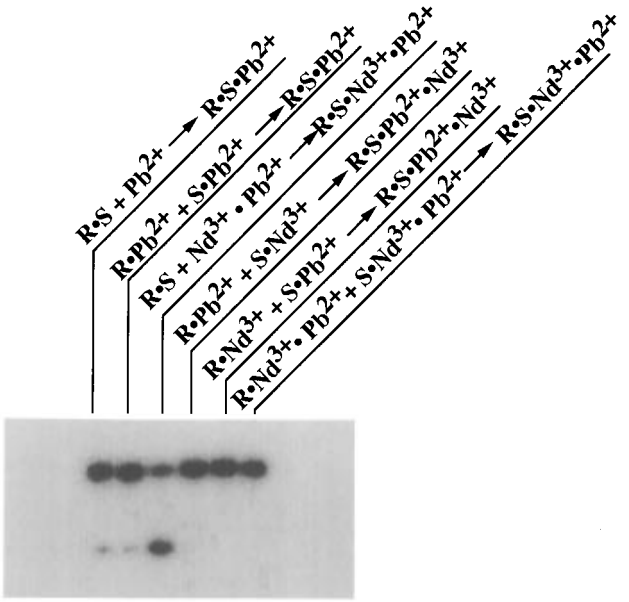

FIGURE 6: Autoradiogram of denaturing 20% polyacrylamide gel showing the effect of different initiations on the cleavage reaction.

equal to the 13.5% observed at 50 μM Pb^{2+} (Sugimoto & Ohmichi, 1996). Thus, Mg^{2+} addition shows an effect similar to addition of Pb^{2+} beyond 25 μM. When Ba^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, or Ca^{2+} was added, the reaction yield was 13.0, 10.0, 9.1, 7.0, or 3.1%, respectively. Other divalent ions (Fe^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}) or the trivalent ions (Al^{3+}, Cr^{3+}, and Fe^{3+}) did not increase the reaction yield for a 20 min incubation even in the presence of 25 μM Pb^{2+}.

On the other hand, surprisingly, the addition of rare earth ions in the presence of 25 μM Pb^{2+} lead to a significant increase in yield as shown in Figure 2B. The cleavage yield with the addition of 25 μM Nd^{3+} was 40.0% after 20 minutes. This yield is about six times larger than that for 25 μM Pb^{2+}. When Gd^{3+}, Tm^{3+}, or La^{3+} was added, the yield was 25.0%, 32.0%, or 35.0%, respectively. The combinations of Nd^{3+}, which showed the highest reaction yield within the rare earth ions, and divalent/trivalent ions except Pb^{2+} showed no cleavage of the substrate as shown in Figure 2C. Therefore, the increase of the cleavage yield is due to the combined effect of Pb^{2+} and the rare earth ions, especially Nd^{3+}.

Cleavage Site of the Substrate RNA. To determine the cleavage site of the substrate in the presence of Nd^{3+} and Pb^{2+}, the site cleaved by the leadzyme was compared with the cleavage sites generated by partial enzymatic digestion. Figure 3A shows the autoradiogram of the cleavage reaction of the substrate by the leadzyme at 50 μM Pb^{2+} or both 25 μM Nd^{3+} and 25 μM Pb^{2+} and by RNase A. Because RNase A cleaves to the 3′ side of C or U, the substrate GGACCGAGCCAG has four cleavage sites as shown in Figure 3B. Figure 3A shows that the cleavage product of the leadzyme in the presence of Pb^{2+} or both Nd^{3+} and Pb^{2+} is consistent with the product cleaved between CpG at the asymmetric internal loop of the substrate RNA by RNase A. This cleavage site in the presence of Nd^{3+} and Pb^{2+} is the same cleavage CpG site of the Pb^{2+} self-cleavage RNA in the presence of excess Mg^{2+} over Pb^{2+} (Pan & Uhlenbeck, 1992b).

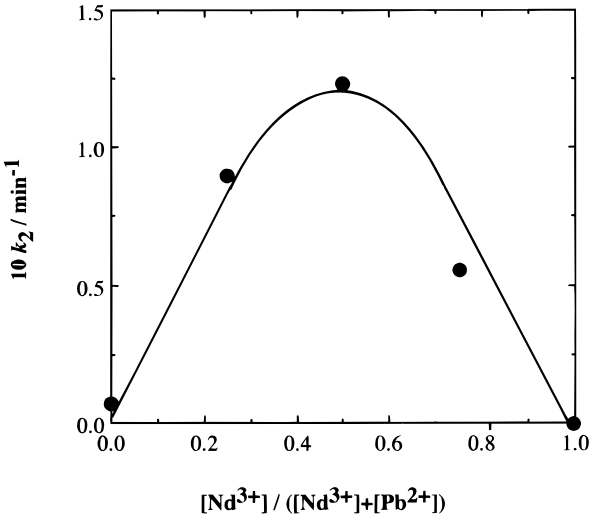

FIGURE 7: Plot of k_2 vs ratio of Pb^{2+} and Nd^{3+}. The substrate (125 nM) was incubated at 25 °C with 15 μM ribozyme in 15 mM NaMOPS (pH 7.5) solution. Total concentration of Pb^{2+} and Nd^{3+} was 50 μM.

Effect of the Concentration Ratio of Nd^{3+} with Pb^{2+} on the Ribozyme Reaction. To investigate the mechanism of the cleavage reaction, we measured the time courses of the cleavage reaction at 25 °C in various concentration ratios of Nd^{3+} and Pb^{2+}, and 15 mM NaMOPS, pH 7.5, as shown in Figure 4. The total concentration of Nd^{3+} and Pb^{2+} was kept 50 μM and these experiments were done under single-turnover conditions with the ribozyme in excess over the substrate RNA, that is, 125 nM substrate RNA and 15 μM leadzyme. At the various ratios of Nd^{3+} and Pb^{2+}, only one product band was observed as shown in Figure 4. In the absence of the leadzyme, the cleavage product was not observed in all the ratios of Nd^{3+} and Pb^{2+} (data not shown). Thus, the leadzyme plays the role of catalyst at all the ratios of Nd^{3+} and Pb^{2+} studied.

In the presence of 25 μM Pb^{2+} and 25 μM Nd^{3+}, the cleavage reaction finished in 60 min at 25 °C, as shown in Figure 4. But, in the case of 37.5 μM Pb^{2+} and 12.5 μM Nd^{3+}, the cleavage reaction did not finish in 60 min. Thus, the ratios of Pb^{2+} and Nd^{3+} influence the reaction rate for substrate cleavage. We determined the rate constants by plotting the natural log of product concentration vs time. When the ratios of Pb^{2+} and Nd^{3+} were 1:0, 3:1, 1:1, and 1:3, the values of the rate constant, k_{obs}, were 2.7×10^{-3}, 8.3×10^{-2}, 1.2×10^{-1}, and 1.2×10^{-2} min^{-1}, respectively.

Effect of Nd^{3+} on the Complex-Formation Step and the Chemical Step. Because the kinetic measurements were done under conditions where the substrate was expected to form a Michaelis−Menten complex with excess and sufficiently high concentrations of the leadzyme, and the reverse ligation

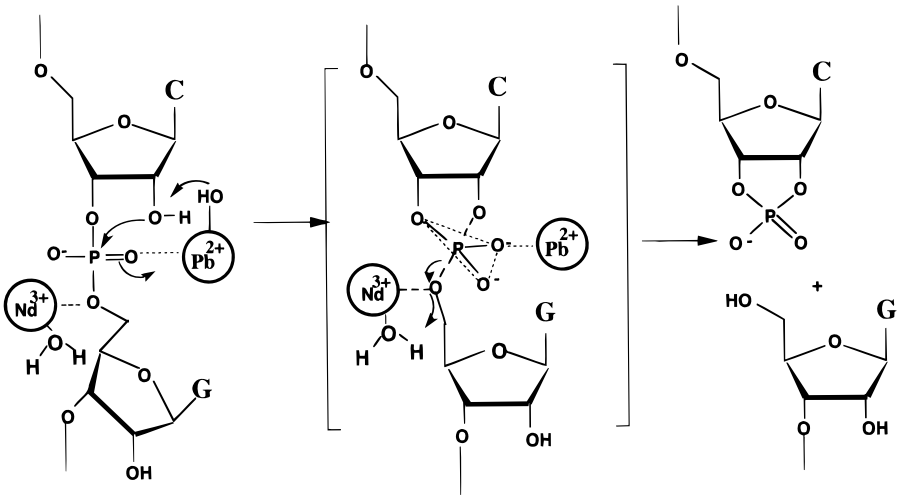

FIGURE 8: Schematic illustration of the postulated mechanism for the cleavage reaction of the substrate RNA by Pb^{2+} and Nd^{3+}.

reaction was not observed under these conditions (data not shown), the cleavage reaction is shown in eq 1

$$R + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R{\cdot}S \xrightarrow{k_2} R{\cdot}P_1{\cdot}P_2 \qquad (1)$$

where R is the leadzyme, S is the substrate RNA, R·S is a complex of the leadzyme and the substrate, and $R{\cdot}P_1{\cdot}P_2$ is the complex between the leadzyme and the products. Plots of k_{obs} vs the initial concentration of the leadzyme $[R]_0$ at various ratios of Nd^{3+} with Pb^{2+} are shown in Figure 5. When the first step in eq 1 is much faster than the second step and $[R]_0 \gg [S]_0$, the equilibrium constant K_1 ($= k_1/k_{-1}$) in the first step and the cleavage rate constant k_2 in the second step can be obtained by eq 2.

$$k_{obs} = \{k_2[R]_0/([R]_0 + 1/K_1)\} \qquad (2)$$

Nonlinear least-squares fits of the data to eq 2 provided values of K_1 and k_2. The values are listed in Table 1 and the fitting curves are shown in Figure 5. The stabilization energies of the complex formation at 25 °C, $\Delta G°_{25,complex}$ were calculated from the value of K_1, and are also listed in Table 1. Since the slopes of the log of saturating rate vs pH were almost equal to 1.0 from pH 7.0 to 7.5 (data not shown), k_2 probably corresponds to the chemical step.

Effect of Different Initiations on the Cleavage Reaction. Table 1 indicates that the addition of Nd^{3+} affects not only the cleavage step but also the stability of the R·S complex. The binding of Nd^{3+} to the R·S complex would be considered to induce the conformation change of the complex at different mixing initiations. To investigate this hypothesis, we tried different mixing methods to initiate the cleavage reaction. Figure 6 shows the effect of the different mixing methods on the cleavage reaction. The expressions such as R·S, R·-Pb^{2+}, and $R{\cdot}Nd^{3+}{\cdot}Pb^{2+}$ etc. indicate the different states of incubation at 25 °C for 30 min before the mixing of the leadzyme, the substrate, and the metal ions. In the case of Pb^{2+} only, the yield of the reaction initiated by combining $R{\cdot}Pb^{2+}$ and $S{\cdot}Pb^{2+}$ was the same as that by combining R·S and Pb^{2+}. On the other hand, in the case of Pb^{2+} and Nd^{3+}, the cleavage reaction was observed only by the initiation of R·S and $Nd^{3+}{\cdot}Pb^{2+}$ mixing. The initiation by other mixing treatments induced no cleavage reaction. These results suggest that Nd^{3+} and Pb^{2+} would stabilize the R·S complex, induce a conformational change to cleavage competent conformation, or destabilize a inactive self-folding structure like hairpin of the substrate or the leadzyme.

DISCUSSION

The cleavage reaction of the substrate RNA by the ribozyme consists of at least two steps, binding and cleavage, as shown in eq 1. The results reported here provide a new view into the roles of different cations on these two steps of the substrate cleavage reaction by the leadzyme.

No Change of the Cleavage Site by Addition of Nd^{3+}. The effects of different metal ions on the hammerhead ribozyme have been investigated (Dahm et al., 1991, 1993; Long et al., 1995). The substrate cleavage rate by the hammerhead ribozyme in 10 mM Mg^{2+} is approximately 15-fold faster than that in 10 mM Ca^{2+} at every pH (Dahm et al., 1993). Our results show that the substrate cleavage rate of leadzyme is also very sensitive to the identity of the metal ions. The cleavage site of the substrate, however, is not dependent on the identity of the metal ions in the hammerhead ribozyme and leadzyme systems.

Role of Nd^{3+} in the Formation of the Leadzyme−Substrate Complex. The dependency of K_1 on the metal ions suggests that Nd^{3+} and Pb^{2+} directly influence the conformation of the leadzyme−substrate complex. At 25 °C and 50 μM Pb^{2+}, the stabilization energy for the formation of the duplex between the leadzyme and the substrate ($\Delta G°_{25,complex}$) calculated from the value of K_1 was −6.8 kcal/mol. When the concentration ratios of Nd^{3+} to Pb^{2+} were 1:1 and 1:3, both of $\Delta G°_{25,complex}$ values were −7.5 kcal/mol. When the concentration ratio of Nd^{3+} to Pb^{2+} was 3:1, the value of $\Delta G°_{25,complex}$ was −6.0 kcal/mol which was the most unstable of all the ratios of Nd^{3+} and Pb^{2+}. Since all the cleavage reactions were carried out with 15 mM NaMOPS, the charge difference between divalent and trivalent ions of 50 μM has little effect on the total ionic strength. Thus, the effect of Nd^{3+} on the stability of the leadzyme−substrate complex is not due to changes of the total ionic strength of the solution but the stability change of the leadzyme−substrate complex induced by the decrease of Pb^{2+} and the increase of Nd^{3+}.

Different Roles of Pb^{2+} and Nd^{3+} on the Chemical Step. Figure 7 shows a plot of the cleavage rate constant k_2 vs the concentration ratio of Nd^{3+} and Pb^{2+}. The value of k_2 is maximum at 25 μM Nd^{3+} and 25 μM Pb^{2+}, and the plot is asymmetric. Since k_2 is probably due to the chemical step

as mentioned above, this result suggests that one Nd^{3+} and one Pb^{2+} may catalyze the cleavage reaction at the active site of the leadzyme−substrate complex. In the case of the mechanism of a phosphoryl transfer reaction catalyzed by the 3′,5′-exonuclease of DNA polymerase I, the cleavage reaction of nucleic acids is facilitated by two ions (Joyce et al., 1994). This mechanism is called the two-metal-ion mechanism. Although the cleavage mechanism of the ribozyme is also facilitated by metals ions, the number of the metal ions are still unknown. In the hammerhead ribozyme, a single-metal-ion model and a two-metal ion model have been proposed (Kimelis & McLaughlin., 1996; Steitz et al., 1993; Sawata et al., 1995; Zhou et al., 1996). Our combinative effect of Nd^{3+} and Pb^{2+} on the cleavage mechanism can not be understood by the proposed single-metal model, since the contribution of Nd^{3+} and Pb^{2+} on the cleavage mechanism is not independent. If the two-metal-ion mechanism applies to our combinative effect of Nd^{3+} and Pb^{2+}, the increase of k_2 can be understood reasonably: One metal ion activates the attacking water or sugar hydroxyl, while the other ion coordinates and stabilizes the oxygen leaving group. Although it is known that Pb^{2+} may act as a general base for cleavage of phosphodiester linkages in nucleic acid substrates using transesterification or hydrolytic mechanisms, a directly coordinating ion which stabilizes the leaving 5′-oxygen has been unknown (Pan et al., 1994). Molecular orbital studies indicate that a rate-limiting step in the cleavage of RNA by metal ions is the cleavage of the bond between phosphorus and the 5′-oxygen (Mizukami et al., 1991; Storer et al., 1991). Thus, in the presence of Pb^{2+} only, where Pb^{2+} acts as both acid and base, the cleavage rate is slow because Pb^{2+} is not a good acid.

In the presence of both Nd^{3+} and Pb^{2+}, however, Pb^{2+} can act as a general base and Nd^{3+} as a general acid. Pb^{2+} can activate abstraction of a proton from the 2′-OH and Nd^{3+} can act as an acid to coordinate with the leaving 5′-oxygen at the cleavage site as shown in Figure 8. The activity of metal ions as an acid is proportional to the ionic potential (Z/r). Here, the values of Z and r express the charge and the ionic radius, respectively. The value of Z/r for Pb^{2+} is 1.5, and that for Nd^{3+} is 3.0. Thus, the properties of the ions show that the ability of Nd^{3+} as the acid catalyst would be superior to that of Pb^{2+}. On the other hand, a Pb^{2+}-bound water molecule has pK_a of 7.2 and the pK_a value of Nd^{3+} is 8.6 (Burgess, 1978). At neutral pH, about 50% of Pb^{2+} would be present in hydroxide form. Thus, the fraction of Pb^{2+} coordinated by a hydroxyl group is much more than that of Nd^{3+} at pH 7.5. Since the enzyme activity depends on the concentration of a catalyst (Fersht, 1985), the difference of the pK_a indicates that the ability of Pb^{2+} as the base catalyst would be superior to that of Nd^{3+}. These properties of Nd^{3+} and Pb^{2+} are in agreement with the roles of both ions as shown in Figure 8. These properties of Nd^{3+} and Pb^{2+} also correspond to the asymmetric profile of k_2 versus the ratio of Pb^{2+} and Nd^{3+} as shown in Figure 7, because the result shows that Nd^{3+} does not have the same abilities for acid and base catalysis on the cleavage reaction and the ability of Nd^{3+} as the acid catalyst would be superior to that as the base catalyst. On the basis of these results, we propose a two-metal-ion mechanism in which Pb^{2+} is the base catalyst and Nd^{3+} is the acid catalyst for the cleavage reaction. Note, however, the Nd^{3+} that serves as the acid catalyst may or may not be identical with the Nd^{3+} which induces the conformational change of the leadzyme−substrate complex.

Comparison to Other RNA Enzymes. The combined effect of metal ions was studied for endoribonuclease activities of the *Tetrahymena* ribozyme and RNase P (Guerrier-Takada et al., 1986; Grosshans & Cech, 1989). The results indicated that the complex between the ribozyme and the substrate had two classes of the metal ion binding sites. The two classes of the metal ion binding site were defined as the sites required for specific structural roles or directly active-site chemistry and the sites for the stability of the complex. However, in the large ribozymes, the combined effect of different metal ions for active-site chemistry reported here was not observed. Recently, it was reported that some ribozymes isolated from randomized libraries by *in vitro* selection require two metal ions for activity. For example, a DNA metalloribozyme with DNA ligase activity requires Mg^{2+} and Cu^{2+} or Zn^{2+} (Cuenoud & Szostak., 1995). The activity of aminoacyl-tRNA synthetase ribozyme also requires Mg^{2+} and Ca^{2+} (Illangasekara et al., 1995). Although the roles of different metal ions are not understood in detail, these metal ions could influence the chemical step at the active center and the recognition step between the enzyme and the substrate. Interestingly, the addition of Ca^{2+} in the presence of Mg^{2+} enhances phosphodiester hydrolysis by *Eco*RV endonuclease, and Ca^{2+} plays a role in the active site as well as the Nd^{3+} role reported in this paper (Vipond et al., 1995). Since enhancement of activity by the combined effect of metal ions has been seen in both ribozyme and protein enzyme systems, the combination of metal ions is a good method to enhance reaction yield and develop new ribozyme systems.

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