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Properties of a Hammerhead Ribozyme with Deletion of Stem II[1]

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The properties of a mutant hammerhead ribozyme system, which consists of two RNA oligomer strands and in which stem II is deleted (replaced with a UUUU loop), are described. The effects of temperature, pH, and metal ions on the cleavage reaction were similar to those for the parent ribozyme with stem II. The mutant ribozyme showed a much lower cleavage rate ($k_{cat}=0.04$ min^{-1}) in the presence of 10 mM $MgCl_2$, where the parent ribozyme showed full cleavage activity. However, increasing the concentration of $MgCl_2$ from 10 to 100 mM restored the cleavage activity of the mutant ribozyme to the original level ($k_{cat}=0.2$ min^{-1}). CD titration experiments with $MgCl_2$ using a noncleavable substrate were carried out. Deletion of stem II resulted in an about 20-fold reduction of the apparent Mg^{2+} binding affinity when the Mg^{2+} concentrations of half-saturation are compared. The results were analyzed by curve-fitting analysis and compared with those for the parent ribozyme. The analysis showed that the Mg^{2+} concentration dependence data in CD and cleavage experiments for the mutant enzyme can be explained by a two Mg^{2+} ion binding mechanism. These results suggest that stem II is important for maintaining the conformation of the catalytic core suitable for Mg^{2+} binding.

Key words: hammerhead, metal binding, oligonucleotide, ribozyme, RNA.

Among many types of ribozymes, the hammerhead ribozyme is one of the simplest systems and consists of about 40 nucleotides (Fig. 1) (*1-3*). This system contains three double helical stem regions (stems I-III), two internal loop regions (residues 3-9 and 12-14), and one bulged residue (residue 17) (*4*), and can be divided into two components, a substrate and an enzyme. The enzyme catalytically cleaves the substrate oligomer at the bulge-stem I junction (between residues 17 and 1.1) in the presence of Mg^{2+} through intramolecular transesterification of the 3′-5′ phosphate diester producing a 2′,3′-cyclic phosphate diester. The cleavage reaction was examined by modification of the base (*2, 3, 5-9*), sugar (*6, 10-14*), and phosphate residues (*15, 16*) in the conserved internal loop regions. Several 2′-hydroxyl groups and exocyclic amino groups were shown to be important for the cleavage.

^{1}H NMR studies have also been performed on hammerhead ribozymes (*3, 17-21*). Our NMR studies on hammerhead ribozyme complexes, which consist of three RNA oligomer strands (Fig. 1a), suggested the presence of A:G base pairs (*17*). A similar suggestion was also made by other groups (*8, 18, 22*). However, the structure of the cleavage site has not yet been fully elucidated, although the crystal structures of some hammerhead ribozymes have recently been reported (*23, 24*).

Ribozymes, in which stem II is deleted and replaced by a loop (*25-28*) or non-nucleotidic linker (*29, 30*), are examined. For the structural study of hammerhead ribozymes by NMR, a small system is preferable to simplify the spectrum. Thus, we designed a ribozyme (Fig. 1b) in which stem II of the parent ribozyme (Fig. 1a) is replaced by a UUUU loop, and examined the properties of the mutant ribozyme to determine whether the essential properties observed for the parent ribozyme are retained by it.

MATERIALS AND METHODS

Preparation of RNA—Except for Tris-HCl buffer, all solutions were treated with diethyl pyrocarbonate, to inactivate a trace of ribonucleases, and autoclaved. The enzyme component and the substrate were synthesized by *in vitro* transcription with T7 RNA polymerase using fully double-stranded promoter-template DNAs. An extra G residue was added at the 5′-terminus of the substrate to improve the efficiency of transcription. The DNA oligomers were synthesized using a DNA synthesizer (model 392, Applied Biosystems). T7 RNA polymerase was purified from *Escherichia coli* BL21 cells carrying the plasmid, pAR1219 (*31*). The transcription reaction was carried out at 37°C for 1 h according to the procedure of Milligan *et al.* (*32*) with some modifications. The reaction mixture com-

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Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; NTP, ribonucleoside triphosphate; PEG, polyethylene glycol; Rz, ribozyme; $tRNA^{Phe}$, phenylalanine transfer RNA.

prised 2 μM DNA template, 0.1 mg/ml T7 RNA polymerase, 7.5 mM each NTP, 35 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.1), 5 mM DTT, 70 mM spermidine, 0.1% Triton X-100 (v/v), and 80 mg/ml polyethylene glycol (PEG 8000). After transcription, the crude RNA was subjected to preparative denaturing (7 M urea) polyacrylamide gel electrophoresis. The transcription products were located by UV shadowing. The gel containing the products was crushed and soaked overnight in 0.3 M sodium acetate with shaking at room temperature. The extracted RNA was isolated by two rounds of ethanol precipitation at −25°C for 3 h. For sequence analysis, the RNA was labeled by kination of the 5′-terminal OH with T4 polynucleotide kinase (Takara Shuzo, Kyoto) and [γ-^{32}P]ATP (~6,000 Ci/mmol), after dephosphorylation of the 5′-terminal phosphate with alkaline phosphatase (Takara Shuzo). The labeled RNA was subjected to partial alkaline hydrolysis (*33*), and partial digestion with ribonuclease T_1, ribonuclease U_2, ribonuclease *phy*M, and *Bacillus cereus* ribonuclease (Pharmacia). The digestion products were separated by 20% denaturing polyacrylamide gel electrophoresis and identified by comparison of the product bands. The yields and concentrations of the RNAs were calculated from the UV absorbance at 260 nm using the molar absorption coefficients of the component nucleotides.

Synthesis of the Non-Cleavable Substrate Containing 2′-O-Methylcytidine Instead of C17—N^4-Benzoyl-2′-*O*-methylcytidine was prepared by the published procedure (*34*). The oligoribonucleotide was synthesized by the solid- phase phosphoramidite method using *o*-nitrobenzyl groups for 2′-OH protection and purified as described previously (*19*).

Measurement of CD Spectra—CD spectra were measured with a J-720 spectropolarimeter (JASCO, Tokyo). Oligonucleotides, the enzyme and the non-cleavable substrate (8.8 μM each), were dissolved in 10 mM sodium phosphate buffer (pH 7.5) and 100 mM NaCl, and used for the Mg^{2+} titration experiment. Mg^{2+} titration was performed in a 1-mm cell by the addition of a concentrated $MgCl_2$ solution. CD-temperature profiles were obtained with 1 μM complex in a 10-mm cell.

Cleavage Reactions—The substrate was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP after dephosphorylation. To examine the effects of the $MgCl_2$ concentration, temperature and pH on the cleavage reaction, cleavage reactions were carried out with 1 μM enzyme and 1 μM substrate for 1 h, the other conditions being varied. The conditions for each experiment are shown in the corresponding figure legends. To examine the effects of $MnCl_2$, $CaCl_2$, and $CoCl_2$ instead of $MgCl_2$, the reactions were carried with 10 mM metal chlorides in 50 mM Tris-HCl (pH 7.0) at 37°C for 1 h. To determine K_m and k_{cat}, cleavage reactions with 0.1 μM ribozyme and 0.5-10 μM substrate in 50 mM Tris-HCl (pH 8.0) were performed with 10 and 100 mM $MgCl_2$. To compare the activity of the mutant ribozyme with that of the parent ribozyme, the cleavage reaction was performed under the same conditions as reported for the parent ribozyme (*35*): 50 mM Tris-HCl (pH 8.0), 1 μM enzyme, and 1 μM substrate with 10 and 100 mM $MgCl_2$. The substrate and the enzyme dissolved in 50 mM Tris-HCl (pH 8.0) were heated at 90°C for 3 min separately. After preincubation at 37°C for 5 min, the cleavage reaction was started by mixing the substrate, the enzyme and $MgCl_2$. The reaction was stopped by the addition of 2 volumes of 80% (v/v) aqueous formamide, and 20 mM EDTA. The substrate and the cleaved product were separated by denaturing 20% polyacrylamide gel electrophoresis, and the radioactivities were quantitated with a Bio-Image Analyzer BAS 1000 (Fuji Film, Tokyo).

Data were fitted with the KaleidaGraph (Abelbeck Software) curve-fitting program using equations given in the text.

RESULTS AND DISCUSSION

We designed a mutant ribozyme system (Fig. 1b), with replacement of stem II in the parent ribozyme (Fig. 1a) by a UUUU loop. The substrate used for cleavage reactions contained an extra G residue at the 5′-terminus for convenience.

The substrate and enzyme RNAs were synthesized with T7 RNA polymerase. A 200 μl reaction yielded 3 nmol of the enzyme RNA (24-mer) and 9 nmol of the substrate (12-mer), respectively. These RNAs were identified by RNA sequencing (data not shown). The non-cleavable substrate (11-mer) was chemically synthesized.

It has been reported that mutant ribozymes with deletion of stem II retain cleavage activity, but their activities are much lower than those of the parent ribozymes (*25-27, 29, 30*). In this study, the mutant ribozyme also showed lower activity under the standard conditions with respect to that of the parent ribozyme. Thus, we examined the cleavage activity of the mutant ribozyme under various conditions to investigate the role of stem II and to improve the cleavage efficiency.

Effect of the Magnesium Ion Concentration on the Activity of the Mutant Ribozyme—The effect of the Mg^{2+} concentration (0-100 mM) was examined under the standard conditions (Fig. 2). The cleavage yield increased with increasing $MgCl_2$ concentration and reached a plateau at around 60 mM, while the activity of the parent ribozyme reached a plateau at around 3 mM $MgCl_2$ (*35*). K_m and k_{cat} were determined from Eadie-Hoffstee plots of the cleavage

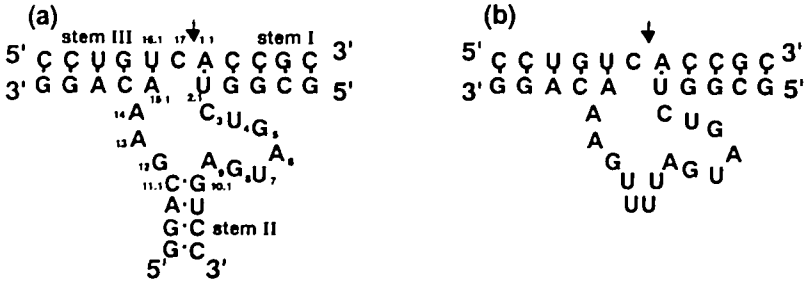

Fig. 1. **(a) Structure of the parent hammerhead ribozyme complex. (b) Structure of the mutant ribozyme complex with deletion of the stem II.** The arrows indicate the cleavage site.

reaction data at 10 or 100 mM $MgCl_2$. K_m and k_{cat} were 0.6 μM and 0.04 min^{-1}, respectively, at 10 mM $MgCl_2$. At 100 mM $MgCl_2$, K_m and k_{cat} were 0.2 μM and 0.2 min^{-1}, respectively. K_m and k_{cat} of the parent ribozyme are about 1 μM and 0.4 min^{-1}, respectively, at 10 mM $MgCl_2$ (*35*). The activity of the mutant ribozyme was further compared with that of the parent ribozyme directly, using the time course experiment data for which the substrate (1 μM) and the enzyme (1 μM) were employed. The substrate was cleaved by 4 and 17% with the mutant ribozyme in 2 and 20 min, respectively, at 37°C in the presence of 10 mM $MgCl_2$. In the presence of 100 mM $MgCl_2$, the substrate was cleaved by 16 and 56% with the mutant ribozyme in 2 and 20 min, respectively. On the other hand, the substrate was cleaved by 30 and 89% with the parent ribozyme in 2 and 20 min, respectively, in the presence of 10 mM $MgCl_2$. These data clearly suggest that the activity of the mutant ribozyme recovered to half the level of the parent ribozyme in the presence of 100 mM $MgCl_2$. These results show that an excess of Mg^{2+} ions can complement the reduced catalytic activity of the mutant ribozyme.

The single-turnover rate constants (k_{obs}) can be calculated from the time-course experiment data using the equation described by McCall *et al.* (*25*): $P(t)=P_{max}-(P_{max}-P_0)\exp(-k_{obs}t)$, where $P(t)$ is the cleavage yield at time t, P_{max} is the cleavage yield at infinite time, and P_0 corresponds to the extrapolated cleavage yield at $t=0$. The k_{obs} values obtained on curve fitting were 0.13, 0.013, and 0.089 min^{-1} for the parent ribozyme with 10 mM $MgCl_2$, the mutant ribozyme with 10 mM $MgCl_2$, and the mutant ribozyme with 100 mM $MgCl_2$, respectively. The experimental data well fitted the curve defined by the equation with parameters obtained by computer calculation; the P_{max} values were 0.6-0.8 for the mutant ribozyme, while those of the parent ribozyme were 0.9-0.95. The P_0/P_{max} values were usually 0.04-0.1. A similar k_{obs} value was obtained in a time-course experiment under excess enzyme conditions using the substrate (0.1 μM) and the mutant enzyme (0.6 μM), confirming that the k_{obs} values obtained in the experiments involving the substrate (1 μM) and the enzyme (1 μM) are indeed the pseudo first-order rate constants.

Effects of Other Metal Ions—The effects of metal ions other than Mg^{2+} on the cleavage activity were examined at pH 7.0, and the cleavage yields after 1 h were compared (Table I). The activity with 10 mM $MnCl_2$ was about the same as that with 10 mM $MgCl_2$. The cleavage activity with 10 mM $CaCl_2$ decreased about 10-fold. The cleavage activity with 10 mM $CoCl_2$ decreased about 2-fold. Similar effects of metal ion species have been reported previously (*1, 35-37*) for ordinary hammerhead ribozymes. The relative efficiency of metal ions does not change much on deletion of stem II, suggesting that the conformations of the metal binding sites are similar.

Effect of pH on the Activity of the Mutant Ribozyme—It is assumed that hydrated Mg^{2+} ions bind to the ribozyme and that one of the hydrating water molecule acts as a base removing a proton from the 2′-hydroxyl group of C17. The effect of pH on the cleavage activity supports the above idea (Fig. 3). The efficiency of the cleavage with 50 mM $MgCl_2$ for 1 h increased with increasing pH in the range of pH 6-8. A similar phenomenon was observed by Dahm *et al.* (*36*).

Effect of Temperature on the Activity of the Mutant Ribozyme—The effect of temperature on the cleavage activity was examined at 50 mM $MgCl_2$ (Fig. 4). The

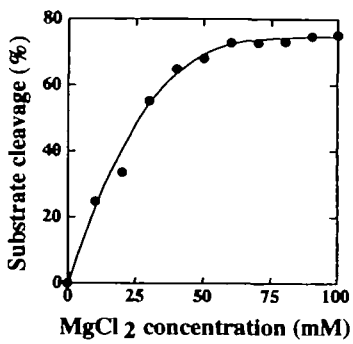

Fig. 2. **Effect of the $MgCl_2$ concentration on the cleavage reaction.** The cleavage reaction was performed with 1 μM substrate and 1 μM enzyme in 50 mM Tris-HCl (pH 8.0) containing 0-100 mM $MgCl_2$ at 37°C for 1 h.

TABLE I. **Effects of divalent metal ion species on the substrate cleavage yield.** The reaction was carried out at pH 7.0 and 37°C for 1 h in the presence of 10 mM metal chloride.

Cleavage yield (%)			
Mg^{2+}	Mn^{2+}	Ca^{2+}	Co^{2+}
7.0	6.4	0.42	3.1

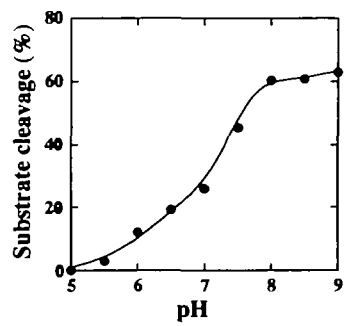

Fig. 3. **Effect of pH on the cleavage reaction.** The cleavage reaction was performed with 1 μM substrate and 1 μM enzyme in 50 mM $MgCl_2$, containing 50 mM buffer (MES for pH 5-6.5 and Tris-HCl for pH 7-9) at 37°C for 1 h.

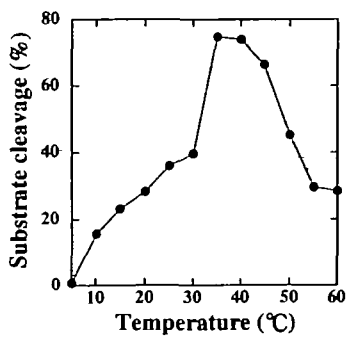

Fig. 4. **Effect of temperature on the cleavage reaction.** The cleavage reaction was performed with 1 μM substrate and 1 μM enzyme in 50 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) for 1 h at various temperatures (5-60°C).

highest cleavage was observed at around 35-40°C. Figure 5 shows the melting curve of the mutant ribozyme complex containing a non-cleavable substrate monitored as to CD ellipticity at 265 nm. In the absence of $MgCl_2$, T_m was observed to be around 22°C (Fig. 5). In the presence of 50 mM $MgCl_2$, the ribozyme shows T_ms of 24 and 46°C (Fig. 5). The melting point at 46°C is assumed to be due to melting of stems I and III stabilized by Mg^{2+} ions. These results suggest that the ribozyme is most active under conditions where the complex is partially melted. Similar phenomena have been observed previously (*35, 38, 39*). The other melting point at 24°C may be due to melting of tertiary interactions of the internal loop region. Modification studies and structural studies suggested the possibility that A:G base pairs may stabilize the catalytic core (*8, 17, 18, 21, 23, 24*).

Effect of the Magnesium Ion Concentration on CD Spectra of the Mutant Ribozyme—To examine the binding affinity of Mg^{2+} ions to the mutant ribozyme complex, CD spectra were measured at various $MgCl_2$ concentrations and 5°C using a non-cleavable substrate, which contains a 2′-*O*-methylcytidine residue at the cleavage site (Fig. 6). With increasing Mg^{2+} concentrations, the intensity of the positive band at 265 nm increased with a concomitant shift of λ_{max} to a shorter wavelength. The CD change is assumed to reflect a conformational change of the ribozyme caused by Mg^{2+} binding (*35, 40, 41*). Figure 7 shows a normalized Mg^{2+}-titration curve of the complex monitored as to $[\theta]_{265}$ values. The curve levels off at around 150 mM $MgCl_2$; the Mg^{2+} concentration for half-saturation ($[Mg^{2+}]_{1/2}$) is 19 mM. In the case of the parent ribozyme, the corresponding $[Mg^{2+}]_{1/2}$ is 1 mM (*35*). These results suggest that the apparent affinity to Mg^{2+} ions is reduced by about 20-fold, in terms of $[Mg^{2+}]_{1/2}$, upon deletion of stem II. The reduction in the affinity can be overcome by increasing the Mg^{2+} concentration, and the activity of the mutant ribozyme recovers to the original level. Thus, stem II seems to be important for maintaining the proper conformation of the Mg^{2+} binding site.

It should be noted that the fraction of Mg^{2+}-bound ribozyme *vs.* Mg^{2+} concentration curve (Fig. 7) is similar to

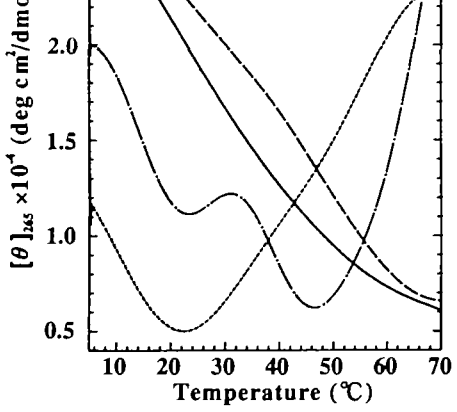

Fig. 5. **CD melting curves measured at 265 nm for the ribozyme complex (1 μM) containing the non-cleavable substrate in the absence of $MgCl_2$ (—; the derivative curve, ·····), and in the presence of 50 mM $MgCl_2$ (- - -; the derivative curve, -·-·-) in 100 mM NaCl and 10 mM sodium phosphate buffer (pH 7.5).**

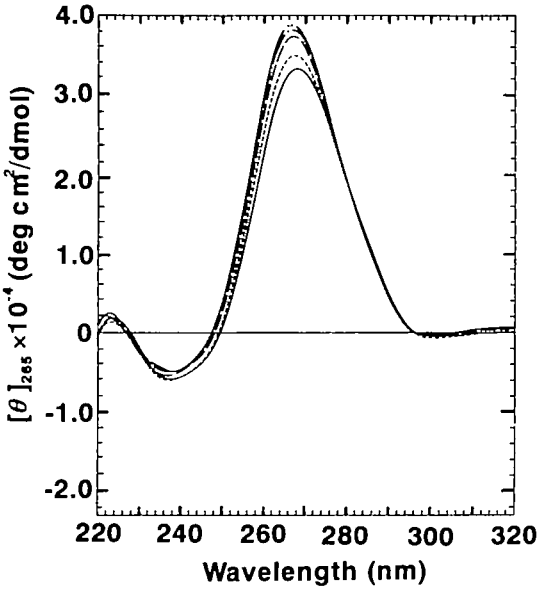

Fig. 6. **CD spectra of the mutant ribozyme complex (8.8 μM) containing the non-cleavable substrate in 0.1 M NaCl and 10 mM sodium phosphate buffer (pH 7.5) containing 0 mM (—), 10 mM (····), 40 mM (- - -), 80 mM (-·-·-), and 150 mM $MgCl_2$ (-···-) at 5°C.**

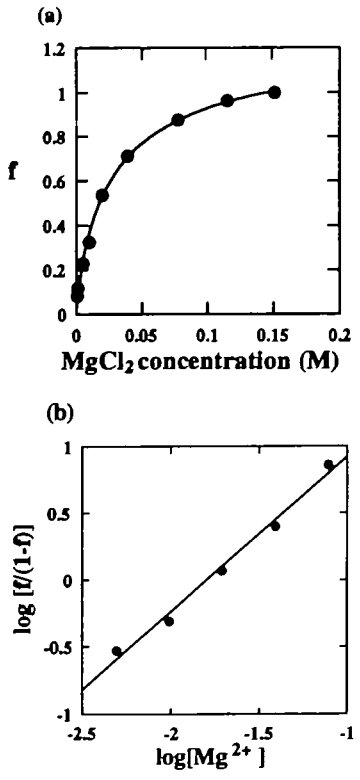

Fig. 7. **(a) Profiles of the fraction (*f*) of Mg^{2+}-bound ribozyme, which was calculated from the change in $[\theta]$ at 265 nm, *vs.* the $MgCl_2$ concentration. (b) Hill plot analysis of the data.**

that of the substrate cleavage *vs.* Mg^{2+} concentration curve (Fig. 2), where the midpoint concentration, when normalized with respect to the maximum cleavage, is around 18 mM. Coincidence between the corresponding midpoint concentrations (about 1 mM) was also observed in the case of the parent ribozyme (*35*). The similarity in the midpoint concentrations is coincidental since the fraction of the Mg^{2+}-bound complex should be correlated with the cleavage rate, and the cleavage yield is not a good measure of the cleavage rate except for in a very low yield region. However, the parallel shifts of the midpoint concentrations of Mg^{2+} for conformational and activity changes clearly indicate that the reduced activity of the mutant ribozyme is due to its reduced binding affinity toward Mg^{2+} ions. These results also confirm that the CD change induced by $MgCl_2$ addition is indeed relevant to Mg^{2+} binding to the active site.

The CD titration data were further examined by Hill plot analysis, a simple equilibrium between metal ions and a ligand being assumed: $Rz + nMg^{2+} \rightleftarrows Rz\text{-}nMg^{2+}$ (*42, 43*); $K_d = [Rz][Mg^{2+}]^n/[Rz\text{-}nMg^{2+}]$; Rz: ribozyme. The plot is based on the linear relation, $\log(f/(1-f)) = n\log[Mg^{2+}] - \log K_d$. The slope of the line in the region of 50% saturation is described as the Hill coefficient (n), which provides a measure of cooperativity as well as a lower limit of the number of metal ions involved in the binding (*42*). Analysis of the mutant ribozyme data (Fig. 7b) gave n, 1.16 ($[Mg^{2+}]_{1/2} = 16$ mM), while the value for the parent ribozyme is 1.46 ($[Mg^{2+}]_{1/2} = 1$ mM) (*35*). These n values larger than one suggest that there is some positive cooperativity in the binding and that the number of Mg^{2+} involved is at least two. This point will be discussed below.

Analysis of the Number of Mg^{2+} Ions Bound to a Ribozyme—The Hill coefficient (n) is a measure of the cooperativity of binding if two or more metal ions are involved in the binding. The values for the ribozymes turned out to be larger than one, as described above, suggesting that at least two Mg^{2+} ions may be involved. For interactions involving a ribozyme and two Mg^{2+} ions, the following equations can be written:

$$K_{d1} = [Rz][Mg^{2+}]/[Rz\text{-}Mg^{2+}]$$

$$K_{d2} = [Rz\text{-}Mg^{2+}][Mg^{2+}]/[Rz\text{-}2Mg^{2+}]$$

$$F_1 = [Rz\text{-}Mg^{2+}]/([Rz] + [Rz\text{-}Mg^{2+}] + [Rz\text{-}2Mg^{2+}])$$

$$F_2 = [Rz\text{-}2Mg^{2+}]/([Rz] + [Rz\text{-}Mg^{2+}] + [Rz\text{-}2Mg^{2+}])$$

From these equations, we can derive the following relations.

$$F_1 = 1/(K_{d1}/[Mg^{2+}] + [Mg^{2+}]/K_{d2} + 1)$$

$$F_2 = 1/(K_{d1} \cdot K_{d2}/[Mg^{2+}]^2 + K_{d2}/[Mg^{2+}] + 1)$$

When we consider the equilibrium at $[Mg^{2+}] = K_{d1}$, if $K_{d2} \gg K_{d1}$, F_1 will be about 0.5 and F_2 will be close to 0. This means that the Mg^{2+} binding is not cooperative. On the other hand, if $K_{d2} \ll K_{d1}$, F_1 will be very small and F_2 will be close to 1. This means that the Mg^{2+} binding is completely cooperative.

When the contribution of $[\theta]$ from the $Rz\text{-}Mg^{2+}$ species relative to that from $Rz\text{-}2Mg^{2+}$ is r ($0 \leq r \leq 1$), the normalized CD intensity, F_{cd}, can be described as follows:

$$F_{cd} = a(r \cdot F_1 + F_2)$$

The factor, a, is added to correct for the error in the estimate of $[\theta]$ at saturation. With any given r value between 0.1 and 1.0, curve fitting analysis gave a set of parameters which fitted the experimental data well. A similar relation may be applied to the activity of the ribozyme:

$$k_{obs} = a'(r' \cdot F_1 + F_2)$$

If we assume that the activity of $Rz\text{-}Mg^{2+}$ is very low relative to that of $Rz\text{-}2Mg^{2+}$ ($r' \approx 0$), $k_{obs}(100\text{ mM }MgCl_2)/k_{obs}(10\text{ mM }MgCl_2) = F_2(100\text{ mM }MgCl_2)/F_2(10\text{ mM }MgCl_2)$. The ratio of k_{obs} determined was 6.8. The parameter set, which gave a F_2 ratio (6.9) close to this value, was $K_{d1} = 7.3$ mM, $K_{d2} = 59$ mM, $r = 0.4$ and $a = 1.23$.

When the CD data of the parent ribozyme were analyzed in the same way for the two metal binding mode, the best-fit curve was obtained with the parameters, $K_{d1} = 1.9$ mM, $K_{d2} = 1.0$ mM, $r = 1.0$ and $a = 1.0$. These data suggest that the reduced affinity toward Mg^{2+} ions of the mutant ribozyme is mainly due to a change in K_{d2}. In the case of the parent ribozyme, the parameter set shown above or other well-fit parameter sets with $r < 1$ did not give a satisfactory F_2 ratio [F_2 (10 mM $MgCl_2$)/F_2 (1 mM $MgCl_2$) = 3.4 for the former and smaller than that for the latter] for the activity data; the k_{obs} (10 mM $MgCl_2$) determined earlier was 0.13 min^{-1}, and k_{obs} (1 mM $MgCl_2$) estimated from P (30 min) using appropriate P_{max} (0.91) and P_0/P_{max} (0.1) values (*35*) was 0.015 min^{-1}. These results suggest that more than three Mg^{2+} ions may be involved for the efficient catalysis by the parent ribozyme.

CONCLUSIONS

The mutant ribozyme, in which stem II was replaced with a tetranucleotide (UUUU) loop, showed properties (dependence on metal ions, pH, and temperature of the cleavage activity) very similar to those of the parent ribozyme, although its RNA cleavage activity is much lower at a low Mg^{2+} concentration, at which the activity of hammerhead ribozymes is usually assayed. It turned out that the activity can recover almost to the original level with increasing Mg^{2+} concentration. Recently, similar results were reported by Hendry *et al.* for other mutant ribozymes with a stem II deletion (*44*). These results reveal that the ribozyme system with a stem II deletion could be a good one for studying the properties and structure of hammerhead ribozymes because of their smaller size. The fact that the deletion mutant still retains catalytic activity implies that the internal loop region itself, which is assumed to form the catalytic core, has the ability to form secondary and/or tertiary structure. The two loop strands may primarily associate with each other through the formation of two tandem G:A pairs (G12:A9 and G8:A13), as postulated in structural studies involving NMR (*17, 18, 45*). G:A pairs were recently found in the crystal structures of hammerhead ribozymes (*23, 24*).

A Mg^{2+} titration study of the uncleavable mutant ribozyme complex monitored as to CD revealed that the Mg^{2+} binding affinity of the ribozyme is reduced about 20-fold and that the reduction in the cleavage activity parallels the reduction in the Mg^{2+} binding affinity. The results of Hill plot analysis of the CD-$[Mg^{2+}]$ profile data for the mutant

and parent ribozymes suggest that at least two Mg^{2+} ions are involved in the binding. Curve-fitting analysis of the same data revealed that the CD data can be explained by a two-metal binding mode for both ribozymes. Taking the activity data into account, K_{d1} and K_{d2} (7.3 and 59 mM) were estimated for the mutant ribozyme. The best-fit curve for the parent ribozyme suggested K_{d1} and K_{d2} to be about 2 and 1 mM, although three or more Mg^{2+} ions seem to be required for efficient catalysis when the activity data are also taken into account. The involvement of two or more Mg^{2+} ions in the catalysis was also suggested by kinetic studies on the cleavage reaction (*46, 47*). Hendry *et al.* recently reported that the kinetic dependence on the Mg^{2+} concentration is biphasic (*44, 48*). In a crystal structure of a ribozyme complex containing an all-RNA substrate analogue, several Mg^{2+} binding sites including a site close to the cleavage site were found (*24*).

The present results suggest that stem II is important for maintaining the conformation of the Mg^{2+} binding site(s) with the hydrated Mg^{2+} ion(s) in the right place and in the right orientation for catalysis. Minimization of the hammerhead ribozyme retaining the original activity will facilitate structural studies involving NMR.

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