Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E., & Lodish, H. F. (1985) *Science 229*, 941-945.
Orci, L., Thorens, B., Ravazzola, M., & Lodish, H. F. (1989) *Science 245*, 295-297.
Permutt, M. A., Koranyi, L., Keller, K., Lacy, P. E., Scharp, D. W., & Mueckler, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8688-8692.
Sener, A., Giroix, M.-H., Malaisse, W. J. (1984) *Eur. J. Biochem. 144*, 223-226.
Suzuki, K. (1988) *J. Biol. Chem. 263*, 12247-12252.
Thorens, B., Sarkar, H. K., Kaback, H. R., & Lodish, H. F. (1988) *Cell 55*, 281-290.
Vera, J. C., & Rosen, O. M. (1989) *Mol. Cell. Biol. 9*, 4187-4195.
Whitesell, R. R., Regen, D. M., & Abumrad, N. A. (1989) *Biochemistry 28*, 6937-6943.
Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinski, B., Ruoho, A. E., & Pilch, P. F. (1989) *J. Biol. Chem. 264*, 12358-12363.

Effects of Phosphorothioate and 2-Amino Groups in Hammerhead Ribozymes on Cleavage Rates and Mg^{2+} Binding†

Makoto Koizumi and Eiko Ohtsuka*
Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan
Received October 31, 1990; Revised Manuscript Received February 8, 1991

ABSTRACT: Mg^{2+} is important for the RNase activity of the hammerhead ribozyme. To investigate the binding properties of Mg^{2+} to the hammerhead ribozyme, cleavage rates and CD spectra for substrates containing inosine or guanosine at the cleavage site were measured. The 2-amino group of this guanosine interfered with the rate of the cleavage reaction and did not affect the amount of Mg^{2+} bound to the hammerhead RNA. The kinetics and CD spectra for chemically synthesized oligoribonucleotides with a S_p or R_p phosphorothioate diester bond at the cleavage site indicated that 1 mol of Mg^{2+} binds to the *pro-R* oxygen of phosphate. The binding constant for Mg^{2+} was about 10^4 M^{-1}, which represents outer-sphere complexation. The hammerhead ribozyme catalyzes the cleavage reaction via an *in-line* pathway. This mechanism has been proved for RNA cleavage by RNase A by using a modified oligonucleotide that has an S_p phosphorothioate bond at the cleavage site. From these results, we present the reaction pathway and a model for Mg^{2+} binding to the hammerhead ribozyme.

Hammerhead ribozymes, which contain 3 stems and 13 conserved nucleotides, have been found in avocado sunblotch viroid (Hutchins et al., 1986), the plus strand of tobacco ringspot virus (Buzayan et al., 1986; Prody et al., 1986) the virusoid of lucerne transient streak virus (vLTSV; Forster & Symons, 1987a,b) and transcripts of satellite DNA of the newt (Epstein & Gall, 1987). During a rolling circle replication of these RNAs, the hammerhead ribozymes seem to serve as the catalyst that cleaves multimeric replicated RNA into monomeric RNA in the presence of Mg^{2+}. It has been shown that a hammerhead domain was essential for this reaction in short transcribed RNA (Uhlenbeck, 1987). We have also found some crucial base pairs by synthesizing several mutants (Koizumi et al., 1988a). From the results of these mutagenesis experiments, we were able to design riboyzmes that cleavaged targeted RNA by recognizing a sequence of 9-15 nucleotides and hydrolyzed a mutant c-Ha-*ras* mRNA without cleaving the wild-type mRNA (Koizumi et al., 1988b, 1989). Haseloff and Gerlach (1988) reported that RNA enzymes could cleave transcripts of the chloramphenicol acetyltransferase gene. Sarver et al. (1990) showed that a designed hammerhead ribozyme reacted as an anti-HIV-1 therapeutic agent.

Mg^{2+} is necessary for reactions of the other ribozymes that were found in self-splicing RNAs (Grosshans & Cech, 1989) and an RNA component of RNase P (Guerrier-Takada et al., 1986). Sugimoto et al. (1988, 1989) showed that the intervening sequence (IVS) of *Tetrahymena* rRNA bound to 2 mol of Mg^{2+} to act as a catalyst for linearization of IVS with cytidylyluridine. Uhlenbeck (1987) showed that cleavage rates of the hammerhead ribozyme depend on the Mg^{2+} concentration. However, the Mg^{2+} binding properties and mechanisms of participation of Mg^{2+} in the hammerhead ribozyme reaction are not clear.

For this paper, we investigated a mode of Mg^{2+} binding in a hammerhead ribozyme by measuring cleavage rates and CD spectra for substrates containing inosine or guanosine at the cleavage site and concluded that the 2-amino group of guanosine interferes with the rate of cleavage reaction and did not affect the amount of Mg^{2+} bound to the hammerhead RNA. The kinetics and CD spectra for chemically synthesized oligoribonucleotides with an S_p or R_p phosphorothioate diester bond at the cleavage site indicated that 1 mol of Mg^{2+} bound to the *pro-R* oxygen of phosphate at this site. Using a modified oligonucleotide that has an S_p phosphorothionate bond at the cleavage site, we showed that the hammerhead ribozyme catalyzed the cleavage reaction via an *in-line* pathway, which has been shown to be a mechanism of RNA cleavage by RNase A (Usher et al., 1972; Eckstein, 1985).

Materials and Methods

Oligonucleotides. Oligoribonucleotides were synthesized by the phosphoramidite method using 2′-*O*-(tetrahydropyranyl) and 5′-*O*-(dimethoxytrityl) protecting groups as described previously (Koizumi et al., 1989). Oligoribonucleotides con-

† This research was supported by a grant-in-aid from the Ministry of Education, Science, and Culture of Japan.
* To whom correspondence should be addressed.

0006-2960/91/0430-5145$02.50/0

taining 2′-*O*-methylcytidine (Inoue et al., 1987) or inosine (Kawase et al., 1989) were prepared as described. These oligoribonucleotides were synthesized on a DNA synthesizer, Applied Biosystems 380A.

Oligoribonucleotides containing a phosphorothioate bond were synthesized as described (Ott & Eckstein, 1987; Stein et al., 1988). In the sulfur oxidation step, 5% S_8 in pyridine–CS_2–triethylamine (95:95:10 v/v/v) was allowed to react for 450 s. S_p and R_p phosphorothioate bond containing deblocked oligonucleotides were separated and purified by reverse-phase HPLC with a linear gradient of 7–10% CH_3CN in 0.1 M triethylammonium acetate (pH 7.0) during 20 min. The retention times of substrates in complexes **7** and **8** were 10.7 min (R_p) and 11.8 min (S_p), respectively.

Cytidine 3′-O-Phosphorothioate Methyl Ester. 4-*N*-Benzoyl-5′-(dimethoxytrityl)-2′-*O*-(tetrahydrdopyranyl)cytidine 3′-*O*-(*O*-methyl *N*,*N*-diisopropylphosphoramidite) (0.84 g, 0.94 mmol) was added to tetrazole (0.22 g, 3 mmol) and ethylene cyanohydrin (96 μL, 1.41 mmol) in dry CH_3CN (6 mL). After stirring for 1 h at room temperature, sulfur (300 mg, 9.4 mmol) in pyridine (30 mL) was added and the solution was stirred for 1 h. The solution was evaporated to dryness. Methanol was added, the residual sulfur was removed by filtration, and the filtrate was evaporated. The residue was chromatographed on a column of a silica gel (20 g). The eluate gave 0.70 g (83%) of the S_p and R_p isomers (^{31}P NMR; $CDCl_3$/trimethyl phosphate; δ = 67.11 and 66.97 ppm). These isomers (0.14 g) were dissolved in pyridine (1 mL) and concentrated NH_4OH (9 mL) and were incubated for 5 h at 60 °C. The reaction mixture was evaporated to dryness, and the residue was chromatographed on a C18 reverse-phase column with a linear gradient of CH_3CN in 50 mM triethylammonium bicarbonate. The fractions containing the 5′-dimethoxytritylated product were evaporated and repeatedly evaporated with H_2O. HCl (0.01 N; pH 2.0) was added, the solution was stirred for 16 h at room temperature, and it was neutralized with 0.1 N NH_4OH. The solution was washed with AcOEt, and then it was evaporated. Two isomers of cytidine 3′-*O*-phosphorothioate methyl ester could be separated by reverse-phase HPLC with a linear gradient of 2.5–10% CH_3CN in 0.1 M triethylammonium acetate (pH 7.0) during 20 min. The faster eluting product, whose retention time was 14.5 min, yielded 300 A_{260} units (^{31}P NMR; D_2O/85% H_3PO_4; δ = 57.41 ppm). The slower eluting products, whose retention time was 17.2 min, yielded 250 A_{260} units (^{31}P NMR; D_2O/85% H_3PO_4; δ 57.48 ppm).

Transesterification of Cytidine 3′-O-Phosphorothioate Methyl Ester by RNase A. To an isomer of cytidine 3′-*O*-phosphorothioate methyl ester (0.6 A_{260} unit) dissolved in 9 μL of 0.1 M Tris-HCl (pH 7.5) and 1 mM EDTA was added a solution of RNase A (1 μL, 10 mg/mL, Sigma). After 30 min at 37 °C, the solution was treated with phenol-chloroform. The product was analyzed by reverse-phase HPLC (Saenger et al., 1974; Ludwig & Ecstein, 1989).

RNA Cleavage Reaction in the Presence of Mg^{2+}. The substrate (CL-3) and the ribozyme (CL-2 and -4) were incubated in 25 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), and 20 mM NaCl at 37 °C. Samples were mixed with the loading solution for 20% polyacrylamide gel electrophoresis in the presence of 8 M urea. Cleavage rates were estimated by cutting the gel and counting the radioactivity with a liquid scintillation counter after autoradiography.

Measurement of CD Spectra. CD spectra were recorded with a Jasco J-500A spectropolarimeter. Oligonucleotides (1 A_{260} unit/mL) were dissolved in 40 mM Tris-HCl (pH 7.5) and 20 mM NaCl. The titration curves were obtained by addition of 35 mM or 350 mM $MgCl_2$.

FIGURE 1: Secondary structures of hammerhead ribozymes. (A) Self-cleaving RNA containing the sequence of the satellite RNA of the newt. (B) A substrate (CL-3) and two-strand ribozyme (CL-2 and -4). X = C and Y = G were conserved nucleotides. Sequences of mutated RNA are shown in Tables I and II.

Table I: Initial Cleavage Rates of Complexes **1–3**

complex	CL-3 X	CL-2 Y	v (nM/min)	relative act. (%)
1	C	C	36	100
2	I	C	5.9	17
3	G	C	0.040	0.11

Results and Discussion

Effects of the 2-Amino Group of Guanosine at the Cleavage Site on the Ribozyme Activity. Figure 1 shows secondary structures of hammerhead ribozymes based on the newt satellite RNA sequence. We have shown that an enzyme fragment (CL-2) with a substrate fragment (CL-1) formed the A-type hammerhead ribozyme and cleaved the substrate, to yield a 5′-hydroxyl and a 2′,3′-cyclic phosphate at the terminus of the cleaved product (Koizumi et al., 1988a). The B-type hammerhead ribozyme, which consisted of two catalytic fragments (CL-2 and -4) and a substrate fragment (CL-3), has been cleaved at C4 as expected (Koizumi et al., 1989). The $t_{1/2}$ values for the A- and B-type ribozyme reaction were 3.5 h and 12 min, respectively, in the presence of 25 mM Mg^{2+} at 37 °C. The cleavage rate of B-type was about 18 times faster than that of A-type. The B-type ribozyme had greater stability because CL-1 was separated into two strands between C and G in the unstable loop, which consists of two nucleotides. The B-type ribozyme, which was proved to be efficient, was used in the mutagenesis experiment described below.

We have reportd that C at the cleavage site could be replaced by A or U but not by G (Koizumi et al., 1988a). Sheldon and Symons (1989) have also showed that vLTSV RNA having G at this site had less activity than the wild type. Why could the hammerhead ribozyme not cleave the substrate with G at this site? It is known that the 2-amino group of guanosine is in the minor groove of an RNA duplex. We postulated that a hammerhead RNA having G at that point could not be cleaved due to steric hindrance of the 2-amino group. An irregular binding of Mg^{2+} to this G and/or a base pairing between this G and the C that was one of the conserved nucleotides in CUGA of CL-2 may inhibit the reaction. To resolve this problem, we synthesized a substrate in which the C at the cleavage site was replaced by inosine. Because inosine does not have the 2-amino group, unlike G, we could investigate the effects of the 2-amino group on the cleavage reaction of the hammerhead ribozyme.

Substrates containing inosine or guanosine at the cleavage site were synthesized, and these mutated substrates were incubated with the ribozyme fragments (CL-2 and -4) in the presence of 25 mM Mg^{2+} at 37 °C. The initial rate for cleavage was obtained by plotting the amount of the cleaved substrate versus time. These rates are shown in Tables I and

Table II: Initial Cleavage Rates of Complexes 4–6

complex	CL-3 X	CL-2 Y	v (nM/min)	relative act. (%)
4	C	U	0.47	100
5	I	U	0.063	14
6	G	U	ND[a]	

[a] ND = not detected.

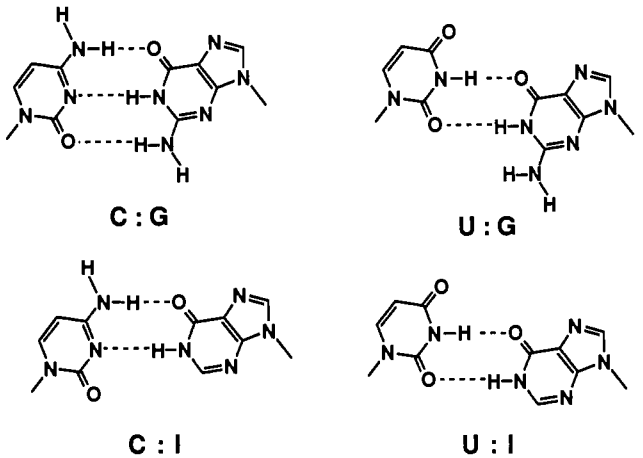
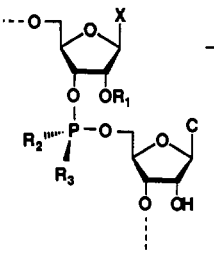


FIGURE 2: Formation of base pairing of I·C, G·C, I·U, and G·U.

II. The initial rate of complex **2** was one-sixth of that of complex **1**, which had a substrate-containing C at the cleavage site (Koizumi et al., 1988a). The substrate with G at the cleavage site (complex 3) was cleaved very slowly (0.11% of the cleavage rate of the wild type). In previous mutation experiments using the A-type ribozyme, the cleavage of the substrate with G at the cleavage site was not detected. This may be due to the fact that the rate in A-type ribozyme was slower than that in B-type as described above. G and I at the cleavage site form the Watson–Crick type and wobble base pairs with a nucleoside in position 7 of CL-2 as shown in Figure 2. These base pairs may affect the cleavage of the hammerhead ribozyme. We changed C7 in CL-2 into U7, so that G and I in mutated substrates form wobble base pairs with U7 of CL-2, and the 2-amino group of the guanosine does not affect the hydrogen bonds. The cleavage rate of complex **5** showed one-seventh activity with respect to complex **4**, which was much less active than the wild type (Table II). The cleavage of complex **6** was too slow to detect. We assume that the base pairing between the nucleotides of the cleavage site and position 7 in CL-2 gave rise to about a 6–7-fold decrease of the cleavage rate. From the comparison of the cleavage rate of complex **2** with that of complex **3**, the presence of 2-amino group of G at the cleavage site seems to result in about a 150-fold decrease of the cleavage rate.

Chemical Synthesis and Susceptibility of Substrates Containing a Phosphorothioate Linkage at the Cleavage Site. The phosphodiester bonds in the hammerhead ribozyme have been modified to phosphorothioate diesters of the R_p configuration using T7 RNA polymerase and NTPαS[1] (Buzayan et al., 1988; Uhlenbeck et al., 1989). It has been shown that modification of the phosphate to phosphorothioate at the cleavage site with R_p configuration strongly interfered with the cleavage reaction. We investigated the effects of the phosphorothioate diester of S_p configuration at the cleavage site on the reaction

[1] Abbreviations: NTPαS, nucleoside 5′-*O*-(1-thiotriphosphate); cCMPS, cytidine 2′,3′-cyclic phosphorothioate; >p, 2′,3′-cyclic phosphate; >pS, 2′,3′-cyclic phosphorothioate.



complex	R_1	R_2	R_3	configuration
1	H	O	O	
7	H	O	S	*Rp*
8	H	S	O	*Sp*
9	CH_3	O	O	
10	CH_3	O	S	*Rp*
11	CH_3	S	O	*Sp*

FIGURE 3: Configuration of the two isomers of phosphorothioate bond containing substrates (X = C).

Table III: Kinetic Data for the Phosphate or Phosphorothioate Bond Containing Substrate

complex	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})	relative act.
1	3.3	0.25	7.6×10^4	1.0
7	1.9	0.0045	2.4×10^3	0.032
8	2.7	0.70	2.6×10^5	3.5

of the hammerhead ribozyme. Oligoribonucleotides containing phosphorothioate diester bonds of S_p configuration cannot be enzymatically synthesized. However, when chemical synthesis methods are used, oligonucleotides containing both isomers can be obtained. Decaribonucleotides containing a phosphorothioate bond were synthesized by using 2′-*O*-(tetrahydropyranyl) and 5′-*O*-(dimethoxytrityl) protecting groups as described previously (Koizumi et al., 1988a), except for the oxidation step of the phosphate at the cleavage site. In this step, the mixture was treated with 5% S_8 in pyridine–CS_2–triethylamine (95:95:10 v/v/v) in place of I_2 in H_2O–pyridine. The fully deprotected product contained two isomers (S_p and R_p) derived from the chiral phosphorus of the phosphorothioate diester (Figure 3). The two isomers were separated by reverse-phase HPLC. The S_p and R_p isomers were characterized by digestion with snake venom phosphodiesterase (Burgers & Eckstein, 1979). Because the oligonucleotide that had a faster retention time was easily cleaved by snake venom phosphodiesterase, we characterized this isomer as the R_p isomer configuration.

Cleavage reactions of these substrates by the ribozyme (CL-2 and -4) were done and compared in the presence of 25 mM Mg^{2+} at 37 °C. Figure 4 shows the autoradiograms of cleavage mixtures in the reactions. The substrate containing the R_p phosphorothioate was cleaved very slowly even at pH 9.0. However, the cleavage rate of the S_p phosphorothioate containing substrate was larger than that of the wild-type substrate. Kinetic parameters for these three substrates obtained by Hanes–Woolf plots are shown in Table III. The K_m of the R_p phosphorothioate containing substrate was similar to that of the phosphate-containing substrate, but the k_{cat} differed by about 55-fold. Replacement of the oxygen by sulfur at the *pro-S* position caused a 3-fold increase of the k_{cat}. These results suggest that the *pro-R* oxygen of phosphate at the cleavage site is involved in the cleavage reaction. Saenger and Eckstein (1970) showed that a negative charge of a phosphorothioate diester localized at the oxygen atom in crystalline uridine 2′,3′-cyclic phosphorothioate. Jaffe and Cohn (1979) also showed that the localization of a negative charge of a phosphorothioate diester depended on the hardness or softness of the metal ion. The increased rate in the S_p isomer may be explained by an easy binding of the hard Mg^{2+} to the hard *pro-R* oxygen, which has a localized negative charge. In the presence of Mn^{2+}, the R_p phosphorothioate bond containing substrates at the cleavage site were cleaved efficiently (Uhlenbeck et al., 1989; van Tol et al., 1990), because Mn^{2+}, which is softer than Mg^{2+}, may be bound to

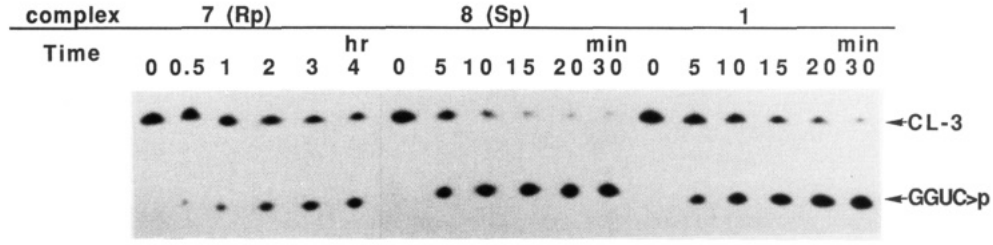


FIGURE 4: Autoradiograms of the cleavage products of complexes **1**, **7**, and **8**. The 5′-labeled substrate (15 pmol) was treated with two-strand ribozyme (22.5 pmol) in 13.8 μL of 40 mM Tris-HCl (pH 7.5 for complexes **1** and **8**, pH 9.0 for complex **7**), 25 mM $MgCl_2$, and 20 mM NaCl at 37 °C. Samples (2.5 μL) were taken at different times and mixed with loading solution for 20% polyacrylamide gel electrophoresis in the presence of 8 M urea.

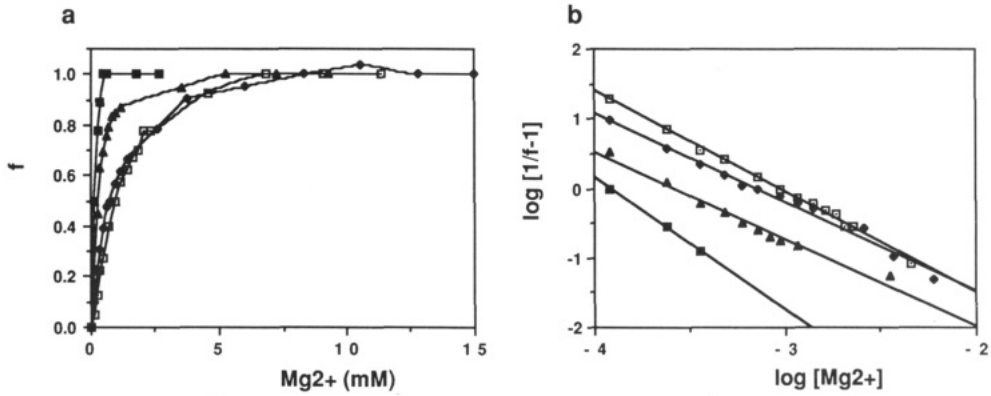


FIGURE 5: (a) Plots of the degree of Mg^{2+} binding to RNA complex vs concentration of Mg^{2+}. (b) Hill plots for calculating the number of Mg^{2+} binding sites (n) and the association constant (K_a) of complexes **3** (▲), **9** (⊡), **10** (■), and **11** (◆).

pro-R sulfur more easily than Mg^{2+}.

CD Spectra for Complexes Containing Modified Nucleotides. The magnitudes of peaks in CD spectra of tRNA increase when Mg^{2+} is added (Willick & Kay, 1971). The change of the ellipticity values shows a conformational change of RNA and Mg^{2+} binding to RNA. The Mg^{2+} binding constants to tRNA have been obtained from the change of $[\theta]$ values at various concentrations of Mg^{2+}.

We have shown that CD spectra for the hammerhead ribozymes containing 2′-*O*-methyl groups at the cleavage site (complex **9** in Figure 3) could be measured (Koizumi et al., 1989). This substrate was used for titration of Mg^{2+} binding to the hammerhead ribozyme. Figure 5 shows the Mg^{2+} titration curve of these RNAs from $[\theta]_{265}$ values of the CD spectra at 18 °C. When the Mg^{2+} concentration reached 7 mM, the ellipticity value at 265 nm did not change. This suggests that the maximum amount of Mg^{2+} binds to the hammerhead ribozyme at 7 mM. The number of Mg^{2+} binding sites (n) and the association constant (K_a) were calculated from a Hill plot using $[\theta]_{265}$ values on the concentration of Mg^{2+} varied from 0 to 7 mM (see Appendix). The n and K_a were 1.4×10^4 and 2.5×10^4 M^{-1}, respectively. The results described in the previous section and this section suggest that 1 mol of Mg^{2+} binds to a mole of the hammerhead ribozyme containing 2′-*O*-methylcytidine at the cleavage site. The order of the binding constant for Mg^{2+} was 10^4 M^{-1}, which represents outer-sphere complexation of Mg^{2+} (Sugimoto et al., 1988, 1989). This complexation indicates that the mode of Mg^{2+} binding may be Mg^{2+}–H_2O–RNA as shown in Figure 6. The Pb^{2+} and Mg^{2+}-indued cleavage have been found in yeast $tRNA^{Phe}$ and *Escherichia coli* $tRNA^{Phe}$ (Brown et al., 1985; Marciniec et al., 1989). The metal-bound hydroxyl group has been proposed to play a role of the nucleophile that removed a proton from the 2′-hydroxyl group of the cleavage site from the crystallographic study for the metal–tRNA complex. We assume from the above binding experiment that some hydroxyl

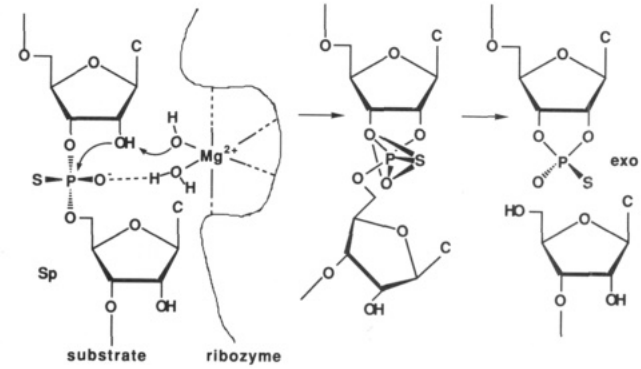


FIGURE 6: Proposed mode of Mg^{2+} binding and reaction pathway for the hammerhead ribozyme via the *in-line* mechanism.

groups were bound to 1 mol of Mg^{2+}, which was hexacoordinated, and that they supported the formation of the ternary structure in the hammerhead ribozyme. We postulated that one of the hydroxyl gorups might be the nucleophile that removed a proton from the 2′-hydroxyl group of the cleavage site (Figure 6), as found in the metal-induced cleavage in tRNA.

The CD spectra for the ribozyme with G at the cleavage site (complex **3**) were measured under the same conditions, since the substrate was stable under these conditions. The titration curve for complex **3** gave a K_a of 2.9×10^4 M^{-1} and an n of 1.2. Since these values for complex **3** were similar to those of the noncleavable ribozyme (complex **9**), the 2-amino group of G, which caused a decrease of the cleavage, did not affect the binding of Mg^{2+}. From these and the previous mutagenesis experiments, in which we have shown that the base at the cleavage site (C) could be replaced by A or U (Koizumi et al., 1988a), Mg^{2+} does not bind to the base of the nucleotide at the cleavage site. These results are consistent with a model of computational graphics, which showed that the base of the nucleotide at the cleavage site was at the surface

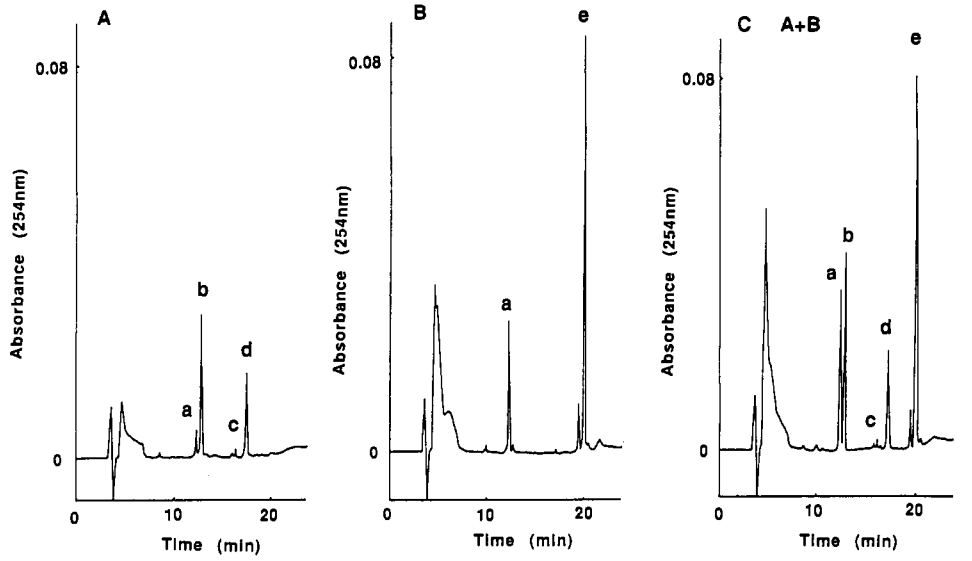


FIGURE 7: Reverse-phase HPLC analysis of RNase A digests of GGUC>pS and cytidine 3'-*O*-phosphorothioate methyl ester. (A) RNase A digestion of cytidine 3'-*O*-phosphorothioate methyl ester. Peak a, *exo*-cCMPS; peak b, *endo*-cCMPS; peak c, cytidine 3'-*O*-phosphorothioate methyl ester (R_p); peak d, cytidine 3'-*O*-phosphorothioate methyl ester (S_p). (B) RNase A digestion of GGUC>pS, which was the cleaved products of complex **8**. Peak a, *exo*-cCMPS; peak e, GGU>p. (C) Mixture of RNase A digests of cytidine 3'-*O*-phosphorothioate methyl ester (A) and digests of GGUC>pS (B).

Table IV: Number of Mg^{2+} Binding Sites (n) and Association Constant (K_a) from Hill Plots of the Data of CD Spectra

complex	n	K_a (M^{-1})
3	1.2	2.9×10^4
9	1.4	2.5×10^4
10	1.9	2.6×10^7
11	1.3	1.0×10^4

and free to move (Mei et al., 1989).

The CD spectra for complexes **7** and **8**, which contain a phosphorothioate bond at the cleavage site, could not be measured, because the substrate of these complexes cleaved under the measuring conditions. We synthesized a substrate containing both 2'-*O*-methylcytidine and a phosphorothioate diester bond at the cleavage site as shown in Figure 3. Two isomers (S_p and R_p configuration) derived from the chiral phosphorus as well as the substrates in complexes **7** and **8** were separated by reverse-phase HPLC. The CD spectra for complexes **10** and **11** could be measured without being cleaved. The n and K_a values of these complexes are shown in Figure 5 and Table IV. These values of complex **11** were similar to those of complex **9**. On the other hand, the n value of complex **10**, which had the R_p phosphorothioate bond containing substrate, was 1.9. This result showed that a molar excess of Mg^{2+} might bind to this complex. This irregular binding of Mg^{2+} may give rise to the less active complex **7** containing the R_p phosphorothioate diester bond at the cleavage site.

Stereochemical Pathway of Cleavage in Hammerhead Ribozyme. The hydrolysis of RNA occurs in two steps, a transesterification step and a hydrolysis step. Then a nucleoside 2',3'-cyclic phosphate is yielded as an intermediate. Usher et al. (1972) showed that the transesterification by bovine pancreatic RNase A followed the *in-line* mechanism, in which a 2'-hydroxyl group attacked the phosphorus in an S_N2 reaction. The reaction of *Tetrahymena* ribozyme proceeded with inversion of the configuration at phosphorus, and the reaction was proposed to occur by the *in-line* mechanism (McSwiggen & Cech, 1989; Rajagopoal et al., 1989). We investigated stereochemical pathways of cleavage in the hammerhead ribozyme by identifying the configuration of 2',3'-cyclic phosphorothioate on the 3'-termini from complex **8**. The complex containing the S_p phosphorothioate diester at the cleavage site was allowed to react in the presence of 25 mM Mg^{2+} at 37 °C. The cleavage products (GGUC>pS and CUAGGA) and two ribozyme fragments were separated by reverse-phase HPLC, and the tetramer GGUC>pS was isolated. Two peaks were observed by RNase A digestion of the GGUC>pS as shown in Figure 7B. The enzyme hydrolyzed the phosphodiester bond between U and C to give GGU>p (peak e) and cCMPS (peak a). Authentic *exo*- (peak a) and *endo*-cCMPS (peak b) were obtained by RNase A digestion of cytidine 3'-*O*-phosphorothioate methyl esters with S_p (peak d) and R_p configurations (peak c), respectively as shown in Figure 7A (Usher et al., 1972). The R_p phosphorothioate was cleaved faster than the S_p isomer (Burgers & Eckstein, 1979). The *exo*-cCMPS was eluted faster than the *endo*-cCMPS (Ludwig & Eckstein; 1989). The elution profile of the mixture containing both two isomers of cCMPS and digests of GGUC>pS is shown in Figure 7C. The digestion product of GGUC>pS (peak a in Figure 7B) was found to be the exo isomer of cCMPS. Therefore, the cleavage product from complex **8**, which has the S_p phosphorothioate diester, was found to have a 3'-terminal *exo*-2', 3'-cyclic phosphorothioate. These data suggest that the cleavage reaction of the hammerhead ribozyme occurs by the *in-line* mechanism as shown in Figure 6. This result is consistent with the pathway that has been shown in a report in which an enzymatically synthesized substrate containing an R_p phosphorothioate was used (van Tol et al., 1990). This result is also consistent with a proposed mechanism from ab initio molecular orbital calculations (Taira et al., 1989).

APPENDIX

The reaction of Mg^{2+} binding to the hammerhead RNA is presented below. When this RNA has n Mg^{2+} binding sites and the association constant K_a, the equations are

$$\text{RNA} + n\text{Mg}^{2+} \rightleftharpoons \text{RNA–Mg}^{2+}{}_n \quad (1)$$

and

$$K_a = [\text{RNA–Mg}^{2+}{}_n]/[\text{RNA}][\text{Mg}^{2+}]^n \quad (2)$$

The degree (f) of Mg^{2+} binding from the measured $[\theta_{265}]$

values of CD spectra is given by

$$f = [\text{RNA–Mg}^{2+}{}_n]/([\text{RNA}] + [\text{RNA–Mg}^{2+}{}_n]) = K_a[\text{Mg}^{2+}]/(1 + K_a[\text{Mg}^{2+}]) \quad (3)$$

$$f = ([\theta]_{265} - [\theta]_{\text{min}})/([\theta]_{\text{max}} - [\theta]_{\text{min}}) \quad (4)$$

Equation 3 may be rearranged to give

$$\log (1/f - 1) = -\log K_a - n \log [\text{Mg}^{2+}] \quad (5)$$

Equations 4 and 5 were used for plotting Figure 5.

References

Brown, R. S., Dewan, J. C., & Klug, A. (1985) *Biochemistry 24*, 4785=4801.

Burgers, P. M., & Eckstein, F. (1979) *Biochemistry 18*, 592–596.

Buzayan, J. M., Gerlach, W. L., & Bruening, G. (1986) *Nature 323*, 349–353.

Buzayan, J. M., Feldstein, P. A., Segrelles, C., & Bruening, G. (1988) *Nucleic Acids Res. 16*, 4009–4023.

Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367–402.

Epstein, L, M., & Gall, J. G. (1987) *Cell 48*, 535–543.

Forster, A. C., & Symons, R. H. (1987a) *Cell 49*, 211–220.

Forster, A. C., & Symons, R. H. (1987b) *Cell 50*, 9–16.

Grosshans, C. A., & Cech, T. R. (1989) *Biochemistry 28*, 6888–6894.

Guerrier-Takada, C., Haydock, K., Allen, L., & Altman, S. (1986) *Biochemistry 25*, 1509–1515.

Haseloff, J., & Gerlach, W. L. (1988) *Nature 334*, 585–591.

Hutchins, C. J., Rathjen, P. C., Forster, A. C., & Symons, R. H. (1986) *Nucleic Acids Res. 14*, 3627–3640.

Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K., & Ohtsuka, E. (1987) *Nucleic Acids Res. 15*, 6131–6148.

Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839–10845.

Kawase, Y., Koizumi, M., Iwai, S., & Ohtsuka, E. (1989) *Chem. Pharm. Bull. 37*, 2313–2317.

Koizumi, M., Iwai, S., & Ohtsuka, E. (1988a) *FEBS Lett. 228*, 228–230.

Koizumi, M., Iwai, S., & Ohtsuka, E. (1988b) *FEBS Lett. 239*, 285–288.

Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H., & Ohtsuka, E. (1989) *Nucleic Acids Res. 17*, 7059–7071.

Ludwig, J., & Eckstein, F. (1989) *J. Org. Chem. 54*, 631–635.

Marciniec, T., Ciesiolka, J., Wrzesinski, J., & Krzyzosiak, W. J. (1989) *FEBS Lett. 249*, 293–298.

McSwiggen, J. A., & Cech, T. R (1989) *Science 244*, 679–683.

Mei, H.-Y., Kaaret, T. W., & Bruice, T. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9727–9731.

Ott, J., & Eckstein, F. (1987) *Biochemistry 26*, 8237–8241.

Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., & Bruening, G. (1986) *Science 231*, 577–1580.

Rajagopal, J., Doudna, J. A., & Szostak, J. W. (1989) *Science 244*, 692–694.

Saenger, W., & Eckstein, F. (1970) *J. Am. Chem. Soc. 92*, 4712–4723.

Saenger, W., Suck, D., & Eckstein, F. (1974) *Eur. J. Biochem. 46*, 559–567.

Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stein, C. A., Subasinghe, C., Shinozuka, K., & Cohen, J. S. (1988) *Nucleic Acids Res. 16*, 3209–3221.

Sheldon, C. C., & Symons, R. H. (1989) *Nucleic Acids Res. 17*, 5679–5685.

Stephens, D. A., & Rossi, J. J. (1990) *Science 247*, 1222–1225.

Sugimoto, N., Kierzerk, R., & Turner, D. H. (1988) *Biochemistry 27*, 6384–6392.

Sugimoto, N., Tom, M., Kierzerk, R., Bevilacqua, P. C., & Turner, D. H. (1989) *Nucleic Acids Res. 17*, 355–371.

Taira, K., Uebayashi, M., & Furukawa, K. (1989) *Nucleic Acids Res. 17*, 3699–3708.

Uhlenbeck, O. C. (1987) *Nature 328*, 596–600.

Uhlenbeck, O. C., Dahm, S. C., Ruffner, D. E., & Fedor, M. J. (1989) *Nucleic Acids Symp. Ser. No. 21*, 95–96

Usher, D. A., Erenrich, E. S., & Eckstein, F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 115–119.

van Tol, H., Buzayan, J. M., Feldstein, P. A., Eckstein, F., & Bruening, G. (1990) *Nucleic Acids Res. 18*, 1971–1975.

Willick, G. E., & Kay, C. M. (1971) *Biochemistry 10*, 2216–2222.