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Analysis of the cleavage reaction of a *trans*-acting human hepatitis delta virus ribozyme

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ABSTRACT

The cleavage reaction catalyzed by the *trans*-acting genomic ribozyme of human hepatitis delta virus (HDV) was analyzed with a 13mer substrate (R13) and thio-substituted [SR13(*Rp*) and SR13(*Sp*)] substrates under single-turnover conditions. The cleavage of RNA by the *trans*-acting HDV ribozyme proceeded as a first order reaction. The logarithm of the rate of cleavage (k_{clv}) increased linearly (with a slope of ~1) between pH 4.0 and 6.0, an indication that a single deprotonation reaction occurred. This result suggests that k_{clv} reflects the rate of the chemical cleavage step, at least around pH 5. The amount of active complex with the SR13(*Sp*) substrate was almost as large as with R13 (60–80%), whereas the amount of the corresponding active complex formed with the SR13(*Rp*) substrate was, at most, 20% of this value (with 0.5–100 mM Mg^{2+} ions) at pH 5.0. Nonetheless, the value of k_{clv} for all substrates was almost the same (0.4–0.5 min^{-1}). Neither a 'thio effect' nor a 'Mn^{2+} rescue effect' were observed. These results suggest that Mg^{2+} ions do not interact with *pro-R* oxygen directly but are essential to the formation of the active complex of the ribozyme and its substrate.

INTRODUCTION

Human hepatitis delta virus (HDV) exists naturally as a satellite virus of the hepatitis B virus (1). The genome of HDV is a single-stranded circular RNA of ~1.7 kb (2) and the mechanism of replication of this small viral RNA genome resembles that of plant virusoids, which replicate by a rolling-circle mechanism, exploiting self-cleavage (ribozyme) activity (3,4). Ribozyme activity, which produces 5′-OH groups and 2′,3′-cyclic phosphates as do hammerhead or hairpin ribozymes, is associated with both the genomic and antigenomic strands of HDV RNA and a common secondary structure has been proposed for both ribozymes (5). Among several models proposed for the secondary structure of these ribozymes (6–9), the pseudoknot model (6) is well supported by results obtained after *in vitro* mutagenesis (10–16) and in chemical probing studies (17). Recent modification–interference analysis with thio-substitution (18) supports a model for the tertiary structure of the genomic HDV ribozyme (16) that is based on the pseudoknot secondary structure. A three-dimensional model of the antigenomic strand of HDV ribozyme was recently proposed with a folding similar to that of the genomic one (19). The HDV ribozyme can be separated into its substrate and *trans*-acting ribozyme strands in several different ways (7,8,20,21). Kinetic studies of the *trans*-acting HDV ribozyme derived from the antigenomic sequence have been performed and several kinetic parameters have been determined (22,23).

A major focus of discussions about the mechanism of catalysis by the ribozyme is the site of coordination of the prerequisite metal ion and the nature of the general base (and general acid) for the cleavage reaction. Many studies have been performed to determine the mechanism of the RNA-cleavage reactions that are catalyzed by hammerhead ribozymes, and the nucleophile that starts the reaction is considered to be a water molecule coordinated to a metal ion (24). The *pro-R* oxygen of the cleaving phosphate in hammerhead ribozymes is directly coordinated with a Mg^{2+} ion during the cleavage reaction (25–28). A recent study, however, has provided some evidence that fails to support this general conclusion (29). We identified important *pro-Rp* oxygens in the HDV ribozyme from the results of modification–interference experiments with phosphorothioate substitution (18), but we have no detailed information about the function of phosphorous oxygen at this site.

In the present study, we analyzed the cleavage reaction of the *trans*-acting ribozyme derived from the genomic sequence of HDV using several analogs of the natural substrate [Fig. 1; nucleotide numbering is that used by Makino *et al*. (30)]. The three substrates we used were the standard 13mer substrate (R13) and two phosphorothioate containing substrates, [SR13(*Rp*) and SR13(*Sp*)]. From studies with the two isomers of SR13, which have sulfur instead of the *pro-R* or *pro-S* oxygen of the cleaving phosphate (Fig. 1B), it is possible to clarify which phosphorous oxygen participates in coordination with a Mg^{2+} ion since this metal ion has a lower affinity for sulfur than for oxygen. Based on our analysis of pseudo-first order kinetics, we discuss the function of the metal ion and phosphorous oxygen.

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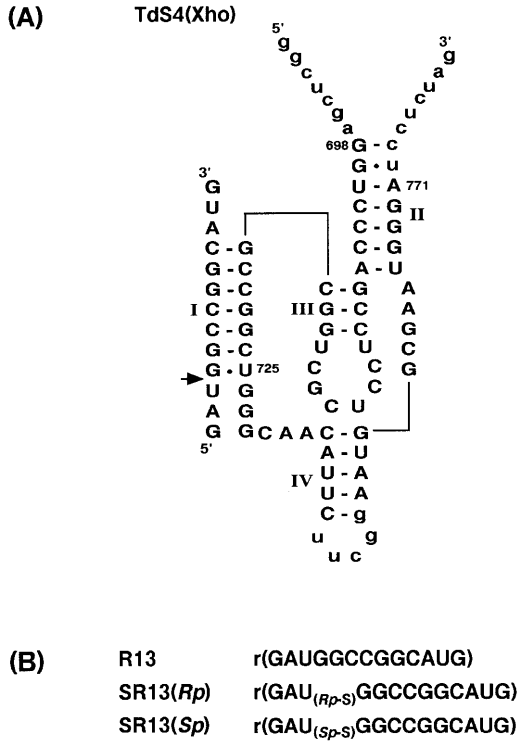

Figure 1. (**A**) *Trans*-acting genomic HDV ribozyme. The secondary structure is based on the pseudoknot model of the secondary structure proposed by Perotta *et al.* (6). Nucleotide numbers are those of Makino *et al.* (30). Lowercase letters at both ends indicate nucleotides that originated from the vector sequence. (**B**) Sequences of substrates for *trans*-cleavage reactions.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized by the phosphoramidite method with a DNA/RNA synthesizer (model 392 or 394; Applied Biosystems). All reagents necessary for the DNA and RNA synthesis were obtained from Applied Biosystems, American Bionetics Inc., and Glen Research. The substrate R13 [r(GAUGGCCGGCAUG)] was purified on a denaturing 20% polyacrylamide gel that contained 7 M urea and recovered by extraction and ethanol precipitation. Quantitative analysis was based on UV light absorption.

Thio-substituted oligoribonucleotides

The thio-substituted substrate designated SR13 [r(GAUsGGCCGGCAUG), where s indicates a phosphorothioate linkage], was synthesized as described above, using tetraethylthiuram disulfide at the sulfurization step instead of oxidation by I_2. After deprotection and desalting, *Rp* and *Sp* isomers were separated by reverse-phase column chromatography on a Shim-pack CLC-ODS column (6 mm i.d. × 15 cm; Shimadzu) using a gradient composed of buffer A [0.1 M triethyl ammonium acetate (TEAA, pH 7.0)/5% CH_3CN] and buffer B [0.1 M TEAA (pH 7.0)/25% CH_3CN]. The column was eluted with a gradient from 0 to 30% buffer B at a flow rate of 0.5 ml/min over the course of 180 min. According to the description by Slim and Gait (26), the isomer that eluted first (elution time = 112.9 min) was identified as the *Rp* isomer [SR13(*Rp*)] and the second isomer (elution time = 116.4 min) was the *Sp* isomer [SR13(*Sp*)]. After desalting by lyophilization, these substrates were labeled at their 5′ ends by T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol). The 5′-end-labeled substrates were purified on a denaturing 20% polyacrylamide gel and then recovered by extraction and ethanol precipitation. Amounts of the 5′-labeled substrates were calculated from the radioactivity of samples compared to the radioactivity of the original [γ-^{32}P]ATP (2 pmol/µl). To prevent slight contamination by SR13(*Sp*) of SR13(*Rp*), which was difficult to cleave, we carried out the cleavage reaction by the HDV ribozyme twice and the SR13(*Rp*) remaining after the first cleavage reaction was used as the *Rp* substrate.

Plasmid DNA

Vector pUCT7 was a modified version of pUC118; it included the promoter for T7 RNA polymerase and a *Xho*I site at the *Eco*RI–*Bam*HI site (15). All experiments were carried out with *Escherichia coli* MV1184 as the host. Plasmid DNA was prepared from an overnight culture by the alkaline lysis method and was purified using QIAGEN-Tip 5 or 20 (DIAGEN). DNA sequencing was performed with double-stranded DNA as a template using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and a DNA sequencer (model 373A, Applied Biosystems).

Preparation of *trans*-acting HDV ribozyme

The *trans*-acting HDV ribozyme TdS4(Xho) was prepared by transcription *in vitro* with an AmpliScribe T7-Specific Transcription Kit (Epicentre Technologies). The vector TransdS4 was linearized by cleavage with *Xba*I and used as a template for transcription *in vitro*. After the transcription reaction, an equal volume of stop solution, containing 50 mM EDTA and 9 M urea, was added to the reaction mixture to stop transcription. After denaturation at 90°C for 2 min and rapid chilling on ice, the transcript (73 nt) was isolated by electrophoresis on a denaturing 8% polyacrylamide gel that contained 7 M urea in the usual way.

Cleavage reactions

Mixtures for cleavage reactions mixture contained 5 µM ribozyme, 0.01 µM radiolabeled substrate, 50 mM Tris–HCl (pH 7.4) and 10 mM $MgCl_2$. All reactions were performed at 37°C. The standard protocol involved combining the ribozyme, substrate and buffer in one tube which was heated to 90°C for 2 min, then on ice for 10 min. This mixture was then incubated at 37°C for 10 min. Reactions were started by the addition of a prewarmed solution of $MgCl_2$ to the tube, and aliquots were removed at appropriate times. We compared several methods for initiating the cleavage reaction and the most efficient cleavage was observed when we used the above described procedures as explained in detail below. Reactions were terminated by placing each aliquot in an equal volume of stop solution (50 mM EDTA, 7 M urea and 0.02% bromophenol blue). Reaction products were separated by electrophoresis on a denaturing 20% polyacrylamide gel and quantified with a Bioimaging analyzer (BAS2000; Fuji Film). To study the dependence on pH, we used sodium acetate buffer instead of Tris for pH values between 4 and 5.5.

Cleavage activity was indicated by the rate of cleaved product formation. Kinetic analysis for substrate cleavage was determined from single-turnover reactions as described previously (31,32) by non-linear least-squares curve fitting to experimental data for the percent of cleaved product (P_t) versus time (t) to the simple pseudo-first order reaction equation:

$$P_t = [EP]\ (1 - \exp(-k_{clv} \times t))$$

where P_t is the percentage of the cleaved product at time t; EP is the end point (amount of active complex), which indicates the percentage of cleaved product at the plateau of the reaction ($t = \infty$); and k_{clv} is the rate constant for the reaction. This is based on the assumption that, under an excess ribozyme condition, substrates are saturated for the ribozyme at time zero. The reverse rate constants for the formation of active and inactive complexes are much smaller than the rate constant for cleavage. Thus, substrate cleavage would proceed as a first order reaction. The cleavage reaction consists of two steps, namely conformational change and chemical reaction.

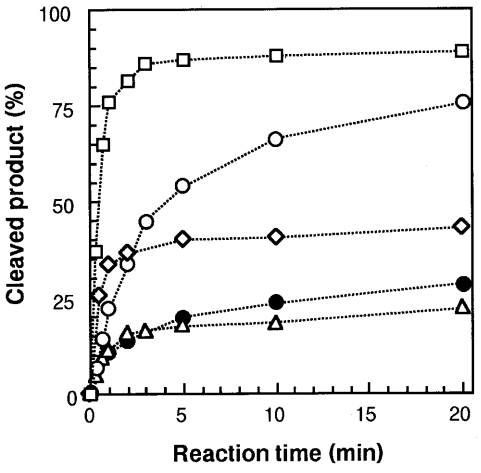

Figure 2. Effects of the order of addition to the reaction mixture of the ribozyme, the substrate and Mg^{2+} ions on the rate of reaction. The reaction was started by (i) mixing the ribozyme and substrate (●), (ii) the addition of the ribozyme (Δ), (iii) the addition of $MgCl_2$ without annealing of the ribozyme and substrate (◇), (iv) the addition of the substrate (○) and (v) the addition of $MgCl_2$ after annealing of the ribozyme and substrate (□).

RESULTS AND DISCUSSION

Trans-acting HDV ribozyme

Both antigenomic and genomic HDV ribozymes have a unique structure as compared to that of hammerhead ribozymes. The reaction mechanism of hammerhead ribozymes has been studied in detail. By contrast, data that might explain the reaction mechanism of the HDV ribozyme, and in particular of the *trans*-acting ribozyme, have not been reported. Several types of *trans*-acting HDV ribozyme were generated by Branch and Robertson (7), Perotta (20), Been (22) and Wu *et al*. (8,21). When we compared several combinations of substrate and ribozyme in an attempt to identify the most active *trans*-acting HDV ribozyme, only one of the ribozymes, which was separated into two fragments at the junction of stem I and stem II, had sufficient cleavage activity (33). Furthermore, the extension of stem II resulted in an increase in cleavage activity (33) and a reduction in size was possible by shortening stem IV, which is not essential for activity [Fig. 1, TdS4(Xho); 31].

In the present case, during the cleavage reaction, only the region on the 3′ side of the cleavage site of the substrate can hybridize to the binding site of the ribozyme portion (Fig. 1). Since the number of base pairs between the substrate and the genomic HDV ribozyme does not change during the cleavage reaction, the rate-limiting step in RNA cleavage by the genomic HDV ribozyme should be the product-release step under steady-state reaction conditions at 37°C as is the case for the antigenomic ribozyme (22). To avoid additional complexity, we chose reaction conditions that would give a single-turnover reaction using an excess of ribozyme, so that we could determine the cleavage rate directly.

Initiation of cleavage reaction

To select optimal conditions for initiating the reaction, we examined the following five sets of conditions: (i) the substrate and ribozyme were mixed independently with Mg^{2+} ions at 37°C and the reaction was started by mixing each component; (ii) the substrate was mixed with Mg^{2+} ions and denatured at 90°C for 2 min and then cooled to 37°C and the reaction was started by addition of the ribozyme; (iii) the ribozyme and substrate were mixed without annealing and the reaction was quickly started by addition of Mg^{2+} ions; (iv) the ribozyme was mixed with Mg^{2+} ions and denatured at 90°C for 2 min and then it was cooled to 37°C and the reaction was started by addition of the substrate; and (v) the ribozyme was mixed with the substrate and denatured at 90°C for 2 min and then the mixture was cooled to 37°C and the reaction was started by the addition of Mg^{2+} ions.

When we conducted the reaction with 10 mM Mg^{2+} ions at pH 7.4, condition (v) yielded the most efficient cleavage (k_{clv} = 1.9 min^{-1}, EP = 87% in Fig. 2). The rate constant under condition (iii) (k_{clv} = 1.8 min^{-1}) was the same as under condition (v), but the end point (the amount of active complex, EP = 40%) was lower. Under condition (i), both the rate constant and the amount of active complex were low (k_{clv} = 0.3 min^{-1}, EP = 28%). Nonetheless, the k_{clv} value of the reaction initiated by the addition of the substrate [condition (iv)], was the same as under condition (i), even though the amount of active complex (EP = 73%) was higher than in the latter case. Condition (ii) gave a higher k_{clv} value (1.0 min−1) but a lower EP value (18%) than condition (iv).

The results under conditions (iii) and (v) indicate that, in the absence of the Mg^{2+} ion, the ribozyme and substrate can interact to form a complex and the amount of complex is increased by further annealing. In hammerhead ribozymes, the result of the tertiary structure reveals that metal ions are not necessary for the formation of a ribozyme–substrate complex (28). In some cases, however, Mg^{2+} ions are necessary for the formation of the ribozyme–substrate complex even though the hammerhead ribozyme does not require a denaturation–renaturation prior to the measurement of kinetic parameters (34). In summary, therefore, in the case of the *trans*-acting HDV ribozyme, formation of the ribozyme–substrate complex is required for an efficient cleavage reaction before formation of the active complex that involves Mg^{2+} ions. We used optimal cleavage condition (v) in the experiments below.

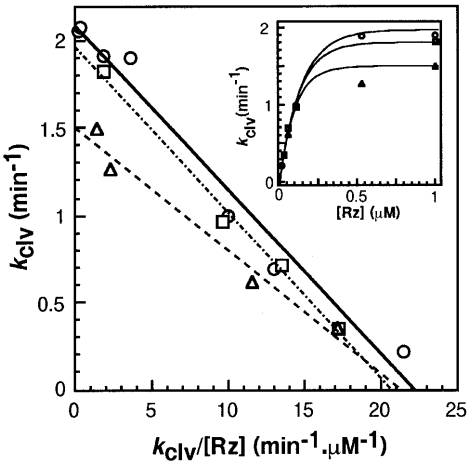

Figure 3. Kinetic parameters for cleavage by the *trans*-acting HDV ribozyme. The dependence on the concentration of the ribozyme of k_{clv} (insert) is show by an Eadie–Hofstee plot, ○ indicate R13, Δ SR13(*Rp*) and □ SR13(*Sp*). Reactions were carried out with 0.01–1 μM ribozyme, 0.01 μM substrate and 50 mM Tris–HCl (pH 7.4) at 37°C in the presence of 10 mM Mg^{2+} ions under standard conditions, as described in Materials and Methods.

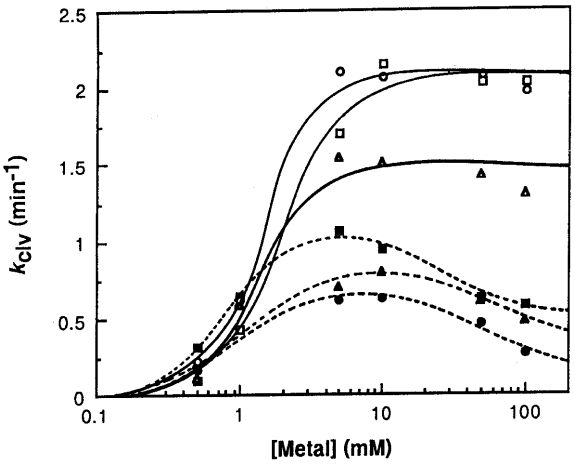

Figure 4. Analysis of cleavage time courses of unmodified and modified substrates (*Rp*- and *Sp*-isomer) at different concentrations of Mg^{2+} ions [○ indicate R13, Δ SR13(*Rp*) and □ SR13(*Sp*)] or Mn^{2+} ions [● indicate R13, ▲SR13(*Rp*) and ■ SR13(*Sp*)]. The cleavage reactions were carried out under standard conditions as described in Materials and Methods.

Ribozyme concentration

We conducted reactions under single turnover conditions with a *trans*-acting ribozyme at concentrations from 0.01 to 5 μM and 0.01 μM substrate in 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$ at 37°C. We confirmed that substrates were present at saturating levels for the concentrations of ribozyme <0.5 μM (Fig. 3, inset). The apparent K_s value, based on the plot of k_{clv} versus k_{clv}/[Rz], was 94 nM for R13 (Fig. 3). Because these reactions were carried out with the ribozyme in excess, rather than the substrate in excess, K_s is not a true Michaelis constant, but indicates the ribozyme concentration at which the reaction velocity is half-maximal. The K_M value of the *trans*-acting antigenomic HDV ribozyme was reported previously to be 0.5–0.7 μM at 55°C (20) and this value was not greatly changed by various mutations in stem II or in stem IV (22). This shows that our reaction condition uses sufficient ribozyme.

Mg^{2+} ion concentration

Cleavage by ribozymes requires divalent metal ions. We conducted cleavage reactions at different concentrations of Mg^{2+} ions (Fig. 4). In the presence of 0.1 mM Mg^{2+} ions, no significant cleavage of the substrate was observed, even after 6 h. At least 0.5 mM Mg^{2+} ions was required to cleave R13. With the increase in the concentration of Mg^{2+} ions from 1 to 10 mM, the cleavage rate increased and then plateaued. Mg^{2+} ions at 10 mM were sufficient under our reaction conditions.

pH dependence of cleavage reaction catalyzed by *trans*-acting HDV ribozyme

Under single turnover reaction conditions and in the presence of 10 mM Mg^{2+} ions, cleavage reactions were conducted at different pH values. TdS4(Xho) exhibited the highest activity at around pH 7.0–7.5, with a bell-shaped pH profile (Fig. 5). This pH profile may indicate a requirement for two groups to exist in particular ionic states. That is not the only possibility: under some circumstances, a single group that is required in different states for two steps of the reaction may give similar behavior. In other words, there is a change in the rate limiting step with pH. A linear correlation was observed between pH and logarithm of the rate constant over the range from pH 4.0 to 6.0. The gradient of linear portion of the slope was close to 1 (actually 0.75), indicating that a single deprotonation had occurred (35). The discrepancy between theoretical and observed values may be due to experimental error or to small differences in the ability of the ribozyme to bind Mg^{2+} ions at different pH values, as has been observed for hammerhead ribozymes and other metalloenzymes (36–38). The result indicates that k_{clv} reflects the rate of the chemical cleavage step between pH 4.0 and 6.0.

At around pH 7.0–7.5 the observed rate constant plateaued, then declined to low values. If the rate-limiting step becomes the association step between the ribozyme and substrate or a conformational change step in the ribozyme–substrate complex, the addition of the substrate or ribozyme should increase k_{clv} values. When we added 2- to 3-fold more substrate or ribozyme to the reaction mixtures at pH values >pH 7, we observed no change in k_{clv} (closed circles and triangles, Fig. 5). This result may suggest that the chemical step is still the rate-limiting step >pH 7. In the following experiments, we used two pH conditions, pH 5.0, which corresponds to the actual chemical step and pH 7.4, which is the optimum pH of the reaction. For our other variants of the *trans*-acting HDV ribozyme [e.g., HDV88–Trans, (33) etc.], the optimum pH for cleavage was ~6 (data not shown). This phenomenon is the same as reported in the case of the *cis*-acting HDV ribozyme (39).

Analysis of cleavage reaction of thio-substituted substrates

To analyze the relationship between divalent metal ions and phosphorous oxygens of the *trans*-acting HDV ribozyme, we

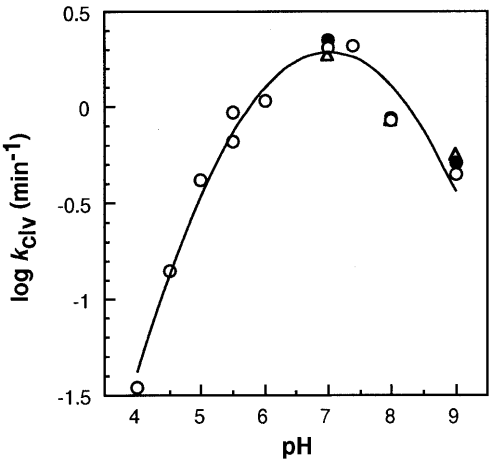

Figure 5. Logarithm of the rate constants of the *trans*-acting HDV ribozyme as a function of pH in the presence of 10 mM Mg^{2+} ions. Above pH 7.0, the cleavage reaction was repeated after a 2-fold increase in the concentration of the ribozyme (●) or a 5-fold increase in the concentration of the substrate (Δ) above standard conditions (○) as described in Materials and Methods.

used isomers with thio-substitution at the cleavage site, SR13(*Sp*) and SR13(*Rp*) (Fig. 1). Thio-substituted *Rp* and *Sp* isomers have often been used in attempts to clarify the function of *pro-R* oxygen of the cleaving phosphate in hammerhead ribozymes. In general, it is believed that an interaction between the metal ion and *pro-R* oxygen is required for catalysis (25–28), although this general conclusion was recently challenged (29). We conducted cleavage reactions at two different pH values under single turnover conditions with an excess of ribozyme to determine cleavage rates directly (time courses shown in Fig. 6). Both the wild type (R13) and *Sp* isomer gave similar amounts of product, whereas SR13(*Rp*) gave smaller amounts. These trends were the same at two different pH values, namely, at pH 5, which reflects the single-deprotonation step, and at pH 7.4, which was optimum for the cleavage reaction (Table 1).

The most important result from our analysis is that the values of k_{clv} were the same for all substrates with TdS4(Xho) as the ribozyme, namely, 0.4–0.5 min^{-1} at pH 5 and 1.5–2.2 min^{-1} at pH 7.4 (Table 1). The similarity in k_{clv} values obtained for the two isomers of SR13 led us to suspect slight contamination by SR13(*Sp*) of the preparation of SR13(*Rp*). We obtained the same result, however, when we repeated the cleavage reaction with the uncleaved SR13(*Rp*) substrate after its isolation from the gel, and thus we were able to exclude this possibility.

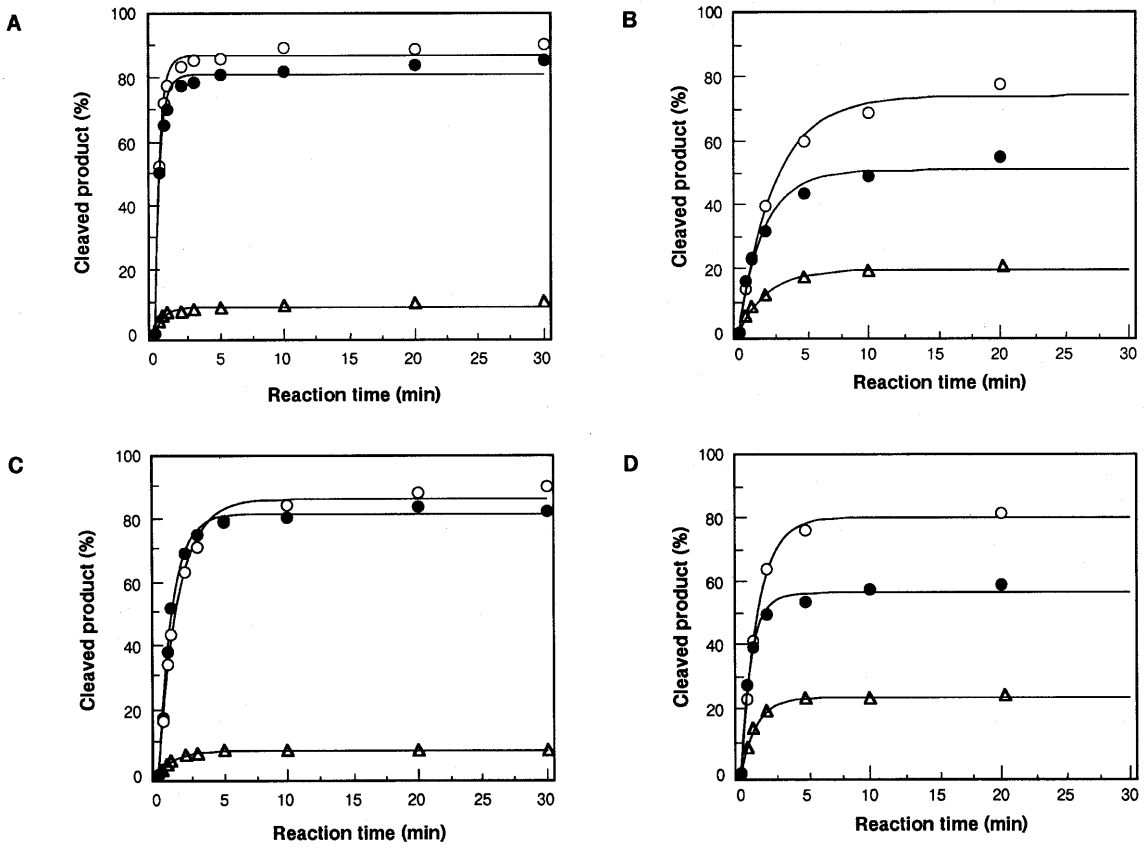

Figure 6. Determination of kinetic parameters of the *trans*-acting HDV ribozyme with R13 (○), SR13(*Rp*) (Δ) and SR13(*Sp*) (●) as substrates by non-linear curve-fitting, based on pseudo-first order analysis. Solid lines are curves fitted to data. Reactions were carried out in either the presence of 10 mM Mg^{2+} (**A** and **B**) or Mn^{2+} (**C** and **D**) at either pH 7.4 (A and C) or pH 5.0 (B and D) under standard conditions, as described in Materials and Methods.

Table 1. Summary of kinetic data at two different pH values

pH	Substrate	k_{clv} (min^{-1})		End point (%)	
		$[Mg^{2+}]$	$[Mn^{2+}]$	$[Mg^{2+}]$	$[Mn^{2+}]$
7.4	wild type (R13)	2.07 ± 0.23	0.64 ± 0.05	88 ± 1.0	86 ± 1.5
	SR13(*Rp*)	1.51 ± 0.40	0.80 ± 0.04	9 ± 0.4	8 ± 0.1
	SR13(*Sp*)	2.15 ± 0 18	0.95 ± 0.05	83 ± 1.2	82 ± 1.0
5.0	wild type (R13)	0.35 ± 0.04	0.74 ± 0.05	74 ± 2.2	80 ± 1.6
	SR13(*Rp*)	0.43 ± 0.04	0.84 ± 0.05	20 ± 0.4	23 ± 0.3
	SR13(*Sp*)	0.50 ± 0.10	1.21 ± 0.13	51 ± 2.4	56 ± 1.3

The effect on the rate of the reaction of sulfur substitution is commonly called the 'thio effect' (i.e., $k_{phosphate}/k_{phosphorothioate}$). The thio effect has been frequently used in the analysis of ribozyme reactions to probe the rate-limiting step of reactions and the coordination site of a metal ion with a phosphoryl oxygen (25–27,29,40). In our analysis, the thio effect value was 0.7–1.4-fold for *Rp*- and the *Sp*-substrate (Table 1). In other words, the substitution of sulfur for the *Rp*-oxygen and the *Sp*-oxygen at the cleavage site of the substrate had no effect on the rate constant (k_{clv}) of TdS4(Xho). Therefore, two possibilities can be considered to explain the absence of any significant difference among the values of k_{clv} for the three substrates. The rate-limiting step of the reaction may be the step at which a conformational change in the substrate–ribozyme complex occurs prior to the cleavage reaction, rather than the chemical cleavage step. Alternatively, the velocity of the chemical cleavage step may not change upon suppression of the metal ion exchange. In other words, the substitution of *pro-R* oxygen affects the coordination of the metal ion but does not influence the subsequent chemical cleavage step. From the pH profile (Fig. 5), in particular around pH 5, the values of k_{clv} clearly indicate the rate of the chemical step, and the second possibility is acceptable. Therefore, Mg^{2+} ions do not interact directly with the *pro-Rp* oxygen. This conclusion is supported by the absence of a 'Mn^{2+} rescue' effect (Fig. 6C and D and Table 1). At pH 5.0, the Mn^{2+} rescue value was 2.0 for SR13(*Rp*) and 2.4 for SR13(*Sp*), respectively. This result agrees with recent results obtained with a hammerhead ribozyme (29).

Our previous modification–interference analysis with thio-substitution revealed that *pro-R* oxygen is located in the catalytic core (18). Since the atomic radius of sulfur is larger than that of oxygen (the P-S bond length is 1.9 Å whereas the P-O bond length is 1.4 Å; 29,41) the substituion of *pro-Rp* sulfur for *pro-Rp* oxygen may effect the conformation of the ribozyme–substrate active complex. *Pro-Sp* oxygen originally faces outside helix I (Fig. 1A) and exchange to sulfur probably has little effect on conformation.

Function of metal ions at *pro-R* oxygen of phosphate moiety at cleavage site

The end points, which provide an indication of the amount of active complex, as described above, differed greatly among the three substrates (Table 1). One possible explanation for the differences is an alteration in the K_m value, in other words, there may have been insufficient ribozyme. This possibility can be excluded from the results of experiments with the ribozyme at several concentrations and unmodified and modified substrates (*Sp*- and *Rp*-isomer) (Fig. 3). The K_s value of SR13(*Rp*) was 70 nM and of SR13(*Sp*) 94 nM. They were similar to the wild type. Thus, substrates were saturated by the ribozyme at concentrations <1 μM. Our experiments were conducted with 5 μM of ribozyme, so such an effect can be excluded.

To investigate another possibility, namely, changes in K_{Mg} and K_{Mn}, we performed cleavage reactions with R13, SR13(*Rp*) and SR13(*Sp*) at different concentrations of Mg^{2+} ions and Mn^{2+} ions (Fig. 4). The rate constant of the cleavage reaction of TdS4(Xho) increased dramatically as the concentration of Mg^{2+} ions was raised from 0.5 to 5 mM, and then plateaued. Data points (Fig. 4) are fitted to the equation:

$$k_{clv} = \frac{(k_{clv})_{max} \cdot [Mg^{2+}]}{K_{Mg} + [Mg^{2+}]}$$

K_{Mg} values were deduced to be 1.7 ± 0.8 mM for R13, 2.0 ± 0.9 mM for SR13(*Rp*) and 2.5 ± 1.0 mM for SR13(*Sp*) and no significant difference was observed in K_{Mg}. Thus, the rate constant did change up to a concentration of 5 mM. At least in 1 mM, however, the end point did not strongly depend on the concentration of metal ions (data not shown).

From our results, we suggest that Mg^{2+} ions have two functions in the cleavage reaction of the HDV ribozyme, participating both in the rate-limiting chemical cleavage step (effect on the value of k_{clv} between 0.5 and 5 mM of Mg^{2+} ions) and in the formation of the active complex (effect on the end point as shown in Fig. 2). Thus, both *pro-R* oxygen and the metal ion participate in the formation of the active complex. In contrast, the rate constant increased slightly with increasing concentrations of Mn^{2+} ions up to 5 mM, and then decreased. We were, therefore unable to obtain the precise value of K_{Mn}. We observed similar results previously with the *cis*-acting HDV ribozyme and Mn^{2+} ions (42). Such results may have been due to an undesirable change in the conformation of the ribozyme at higher Mn^{2+} ions concentrations.

CONCLUSION

Metal ions appear to play at least two important roles in the cleavage of RNA by the *trans*-acting HDV ribozyme. When the reaction starts, metal ions function in the formation of the active complex of the substrate and ribozyme. Although we have evidence that *pro-R* oxygen of the cleaving phosphate also participates in this step, the nature of the interaction between the metal ion and *pro-R* oxygen is not yet known. After formation of the active complex, metal ions initiate the cleavage reaction and the phenomenon was clearly detectable at around pH 5.

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