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Ribonuclease P (RNase P) RNA is converted to a Cd^{2+}-ribozyme by a single Rp-phosphorothioate modification in the precursor tRNA at the RNase P cleavage site

JENS M. WARNECKE*, JENS PETER FÜRSTE†, WOLF-DIETRICH HARDT‡, VOLKER A. ERDMANN†, AND ROLAND K. HARTMANN*§

*Medizinische Universität zu Lübeck, Institut für Biochemie, Ratzeburger Allee 160, D-23538 Lübeck, Germany; †Institut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany; and ‡School of Medicine, Department of Microbiology, State University of New York, Stony Brook, NY 11794-5222

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ABSTRACT **To study the cleavage mechanism of bacterial Nase P RNA, we have synthesized precursor tRNA substrates carrying a single Rp- or Sp-phosphorothioate modification at the RNase P cleavage site. Both the Sp- and the Rp-diastereomer reduced the rate of processing by *Escherichia coli* RNase P RNA at least 1000-fold under conditions where the chemical step is rate-limiting. The Rp-modification had no effect and the Sp-modification had a moderate effect on precursor tRNA ground state binding to RNase P RNA. Processing of the Rp-diastereomeric substrate was largely restored in the presence of the "thiophilic" Cd^{2+} as the only divalent metal ion, demonstrating direct metal ion coordination to the (*pro*)-Rp substituent at the cleavage site and arguing against a specific role for Mg^{2+}-ions at the *pro*-Sp oxygen. For the Rp-diastereomeric substrate, Hill plot analysis revealed a cooperative dependence upon $[Cd^{2+}]$ of n_H = 1.8, consistent with a two-metal ion mechanism. In the presence of the Sp-modification, neither Mn^{2+} nor Cd^{2+} was able to restore detectable cleavage at the canonical site. Instead, the ribozyme promotes cleavage at the neighboring unmodified phosphodiester with low efficiency. Dramatic inhibition of the chemical step by both the Rp- and Sp-phosphorothioate modification is unprecedented among known ribozymes and points to unique features of transition state geometry in the RNase P RNA-catalyzed reaction.**

RNase P is an essential structure-specific endoribonuclease that generates the mature 5′ ends of tRNAs. *In vitro*, RNA subunits of bacterial RNase P enzymes were shown to be catalytically active in the absence of the protein subunit (1). Processing of precursor tRNAs (ptRNAs) by RNase P is an essentially irreversible reaction yielding 3′-OH and 5′-phosphate termini. A solvent hydroxide is thought to act as the nucleophile in an S_N2 in-line displacement mechanism (2, 3).

To gain a deeper insight into the cleavage mechanism by *Escherichia coli* RNase P RNA, we have synthesized ptRNA substrates carrying a single Rp- or Sp-phosphorothioate modification at the RNase P cleavage site. The diastereomeric substrates were analyzed for gel-resolvable binding to RNase P RNA and were studied in single turnover experiments in the presence of different divalent metal ions, such as Mg^{2+}, Mn^{2+}, and Cd^{2+}. The following results were obtained: (*i*) the Sp-diastereomer moderately affected ptRNA ground state binding, while the Rp-diastereomer had no effect; (*ii*) cleavage by RNase P RNA involves direct metal ion coordination to the *pro*-Rp oxygen; (*iii*) there is no specific role for Mg^{2+} at the *pro*-Sp oxygen; and (*iv*) cleavage of the Rp-diastereomeric substrate has a lower cooperative dependence (n_H = 1.8) upon $[Cd^{2+}]$ than cleavage of the unmodified substrate upon $[Mg^{2+}]$ (n_H = 3.3). Implications for the unique RNase P RNA cleavage mechanism are discussed in the context of previously proposed mechanistic models (2–4).

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MATERIALS AND METHODS

Separation and Identification of Diastereomeric 13-Mers. Oligoribonucleotides of identical sequence (13-mers), either unmodified or carrying a single phosphorothiote modification (5′-CCCUUUC$_S$GCGGGA), were prepared by solid-phase synthesis essentially as described (5, 6). Oligoribonucleotides were purified by reversed-phase HPLC on an ODS C18 Beckman Ultrasphere column at 45°C (5). The material of the peak containing the two diastereomeric 13-mers was vacuum-dried, and the Rp- and Sp-diastereomers were separated by a second reversed-phase run at 4°C. The assignment of configuration of the two diastereomeric 13-mers was accomplished by digestion with the stereoselective enzymes snake venom phosphodiesterase I or nuclease P1 essentially as described (6). Phosphorothioate-specific iodine hydrolysis was performed essentially as described recently (7).

Assembly of the ptRNA^{Gly}. Chemically synthesized RNA oligomers were phosphorylated at their 5′ termini by T4 polynucleotide kinase; T7 transcripts were synthesized in the presence of excess 5′-GMP to obtain 5′-monophosphates (7). Modified or unmodified 13-mers, a second 11-nt RNA oligonucleotide (5′-GUAGCUCAGUC-3′, obtained either by chemical RNA synthesis or T7 transcription), and the 3′-portion of the tRNA (obtained by T7 transcription, starting at G+18; see Fig. 1) were annealed to a bridging DNA oligonucleotide (complementary to nucleotides +37 to −3 of the ptRNA; see Fig. 1) for one-step ligation by T4 DNA ligase (8). RNAs were purified by 8% (ptRNAs) or 6% (RNase P RNA) PAGE/8 M urea, essentially as described (7).

Kinetics. Single turnover (pre-steady state) experiments (9) were performed with 1–10 nM 5′-^{32}P-end-labeled ptRNA and 5 μM *E. coli* RNase P RNA in 50 mM Mes, pH 6.0 (at 37°C), 0.1 mM EDTA, 1 M NH_4OAc, and 15 mM divalent cations, if not stated otherwise. In the case of transition metal ions, the metal ion acetate was freshly dissolved at 37°C in this buffer immediately before preincubations were started. RNase P RNA was preincubated at 37°C for 1 h and ptRNAs were incubated at 55°C for 10 min and at 37°C for 30 min under cleavage assay conditions before starting the reactions by combining prewarmed enzyme and substrate mixtures. Reactions were stopped as described (9) and analyzed by 25% PAGE/8 M urea, and bands corresponding to ptRNA and 5′-flank were excised from gels and quantified by Cerenkov counting. Pseudo-first-order rate constants of cleavage (k_{obs}) were calculated by the slope of a least-squares linear regression as described (9).

Abbreviation: ptRNA, precursor tRNA.

§To whom reprint requests should be addressed.

Gel Retardation. Binding of 5′-end-labeled ptRNA to RNase P RNA was analyzed under cleavage assay conditions (see above) but containing 50 mM Ca^{2+} as divalent metal ion, as described recently (9).

RESULTS

ptRNA Binding to RNase P RNA. By combining chemical and enzymatic techniques, we have synthesized a bacterial $ptRNA^{Gly}$ carrying a 7-nt 5′-flank and a single Rp- or Sp-phosphorothioate modification at the RNase P cleavage site (Fig. 1). Unmodified $ptRNA^{Gly}$ synthesized according to the same protocol served as the control. For this substrate, we determined a pre-steady state $K_{m(pss)}$ of 0.15 μM and a single turnover rate of cleavage (k_{react}) of about 3 min^{-1}. We then analyzed whether the phosphorothioate modifications had an effect on specific binding of the $ptRNA^{Gly}$ to *E. coli* RNase P RNA. This was accomplished by gel retardation performed in the presence of Ca^{2+}, which is able to promote ptRNA binding as efficiently as Mg^{2+} (3, 10), while strongly reducing ptRNA cleavage (3, 11). Since the 5′-^{32}P label remained essentially on the ptRNA during gel retardation experiments, we could exclude significant ptRNA maturation. Apparent K_d values (Table 1) demonstrate that the unmodified ptRNA and the Rp-diastereomeric variant bind with identical affinities to *E. coli* RNase P RNA, while the Sp-phosphorothioate-modified ptRNA showed impaired ground state binding. The loss in binding energy (≈2 kcal/mol) would be consistent with perturbation of one hydrogen bond.

Single Turnover Kinetics. Single turnover kinetic experiments were performed in the presence of trace amounts of 5′-^{32}P-end-labeled ptRNAs and 5 μM *E. coli* RNase P RNA. Since the Rp-phosphorothioate modification had no effect and the Sp-modification had only a moderate effect on ptRNA binding (Table 1), the ribozyme concentration was inferred to largely exceed the pre-steady state $K_{m(pss)}$ (0.15 μM for the unmodified $ptRNA^{Gly}$) in single turnover cleavage reactions. This assumption was verified by the observation that cleavage rates of the Sp-diastereomeric ptRNA were essentially identical when the ribozyme concentration was varied in the range of 2–10 μM (data not shown). Processing assays were performed at pH 6.0 in the presence of 1 M NH_4OAc. The high monovalent salt concentration was applied to minimize structural effects of divalent metal ions (3, 12). At pH 6.0, the chemical step is rate-limiting (3, 9), as confirmed by the linear dependence of cleavage rates on the OH^- concentration in the pH range of 5.0–6.5 (Fig. 2*A*). In addition, formation of insoluble metal hydroxides and/or oxides is avoided at pH 6.0 (13).

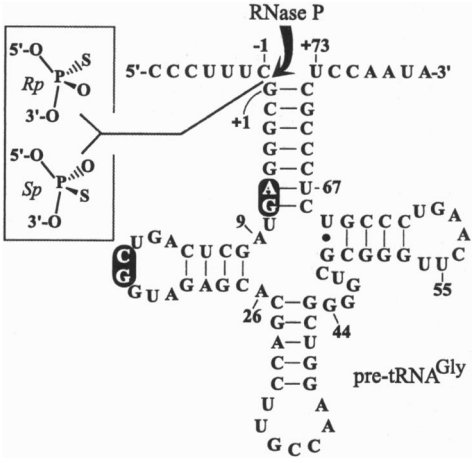

FIG. 1. $ptRNA^{Gly}$ variants carrying a single Rp- or Sp-phosphorothioate modification at the RNase P cleavage site. Highlighted nucleotides mark the sites of ligation. The arrow indicates the canonical RNase P cleavage site.

Table 1. $ptRNA^{Gly}$ binding to *E. coli* RNase P RNA

$ptRNA^{Gly}$ species	Apparent K_d, nM
Unmodified	1.3
All-Rp	4.9
Single Rp diastereomer	1.4
Single Sp diastereomer	41

Apparent K_d values were calculated using the following equation: Apparent K_d = [RNase P $RNA_{50\%}$] − 0.5 × [ptRNA], where [RNase P $RNA_{50\%}$] is the concentration at which 50% of the ptRNA was shifted into the complex. ptRNA concentrations were <1 nM.

Typical cleavage assays performed in the presence of 15 mM Mg^{2+} are illustrated in Fig. 3*A*. Processing of the Rp-diastereomeric ptRNA occurred with low efficiency, which is in line with a previous study (14), generating the expected 7-nt 5′ cleavage product. With the Sp-diastereomer, a 6-nt 5′ cleavage product accumulated at a slow rate, showing that this sulfur substitution shifts the cleavage site to the next phosphodiester in the 5′ direction.

Direct metal ion coordination can be deduced from rescue experiments in those cases, where inhibition of cleavage due to a phosphorothioate modification is strongly relieved in the presence of more "thiophilic" cations such as Mn^{2+} or Cd^{2+} (15, 16). We tested several transition metal ions, which have ionic radii similar to Mg^{2+} in their hexacoordinated forms, for their capability to partially restore cleavage of the phosphorothioate-modified substrates. In such assays, cleavage rates at 15 mM Mg^{2+} were compared with those at 10 mM Mg^{2+} plus 5 mM Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+}. Stimulation of cleavage relative to that at 15 mM Mg^{2+} was only observed with the Rp-diastereomeric substrate in the presence of Cd^{2+} and, to a minor extent, with Mn^{2+} (Fig. 3*B*).

The 5′ cleavage product generated in the presence of Cd^{2+} comigrated with that obtained by cleavage of the unmodified

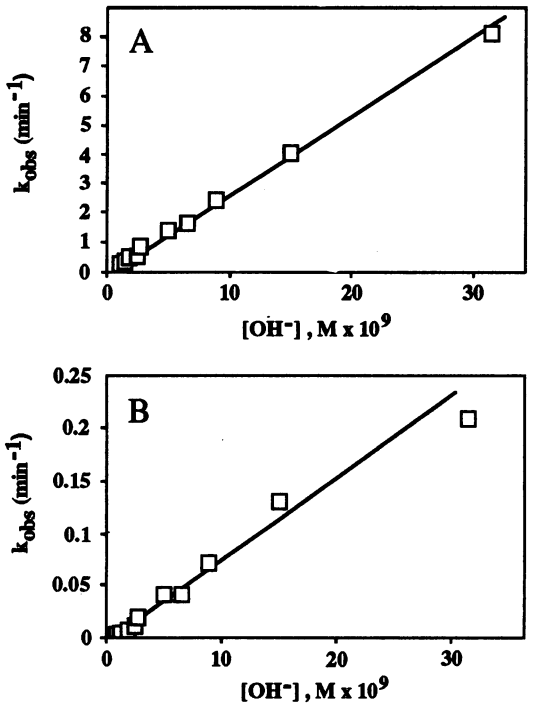

FIG. 2. Dependence of cleavage rate on [OH^-] (calculated as $10^{(pH-14)}$ M) in the range of pH 5.0–6.5 under conditions of $E \gg S$. (*A*) Unmodified $ptRNA^{Gly}$ in the presence of 15 mM Mg^{2+} and (*B*) Rp-diastereomeric $ptRNA^{Gly}$ in the presence of 15 mM Cd^{2+}.

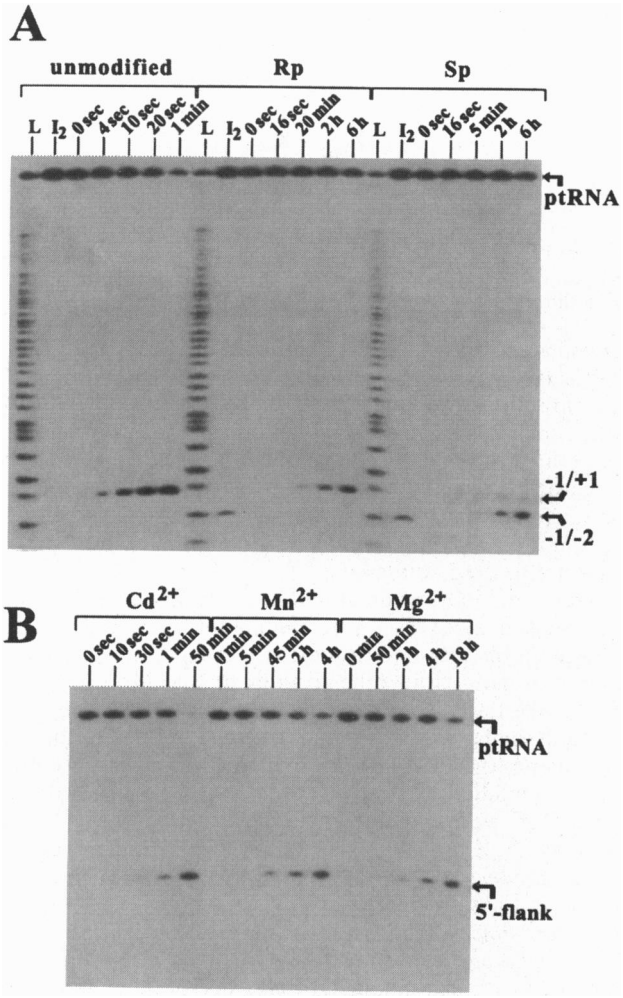

FIG. 3. Processing by *E. coli* RNase P RNA of (*A*) 5′-^{32}P-end-labeled unmodified, Rp- or Sp-diastereomeric ptRNA^{Gly} in the presence of 15 mM Mg^{2+}, or (*B*) Rp-diastereomeric ptRNA^{Gly} in the presence of 15 mM either Mg^{2+}, Cd^{2+}, or Mn^{2+}. Aliquots withdrawn at indicated times were analyzed by 25% PAGE in the presence of 8 M urea. In *A*, the corresponding sites of canonical (−1/+1) and aberrant (−1/−2) cleavage are indicated at the right according to the ptRNA numbering system shown in Fig. 1. Lanes in *A*: I_2, iodine treatment (see legend to Fig. 4); and L, alkaline hydrolysis ladders. The smallest alkaline hydrolysis bands included in the shown section of the gel are 7 nt (unmodified ptRNA) and 6 nt (Rp- and Sp-diastereomeric ptRNAs). Note that alkaline and iodine hydrolysis bands migrate considerably faster than 5′ cleavage products generated by RNase P RNA due to their additional 2′,3′-cyclic phosphate group at the 3′ terminus.

ptRNA in the presence of Mg^{2+} (Fig. 4, lanes 5 and 6). Both 5′ cleavage products migrated at a different position relative to an RNA fragment of identical sequence but carrying a terminal 2′,3′-cyclic phosphate (Fig. 4, lane 2). Thus, cleavage in the presence of Mg^{2+} as well as Cd^{2+} yields the same products—i.e., 3′-OH and 5′-phosphate (or phosphorothioate) termini. This observation and very similar dependences of the Mg^{2+}- and Cd^{2+}-catalyzed reactions upon [OH^-] (Fig. 2) are consistent with an identical cleavage mechanism. To largely exclude that Mg^{2+} ions inherently bound to substrate and enzyme RNAs might have influenced the reaction in the presence of Cd^{2+}, RNAs were purified in the presence of 50 mM instead of 1 mM EDTA (see *Materials and Methods*). No effect on the rate of cleavage of the Rp-diastereomeric ptRNA was observed, making it unlikely that small amounts of Mg^{2+} ions may have assisted the Cd^{2+}-catalyzed reaction.

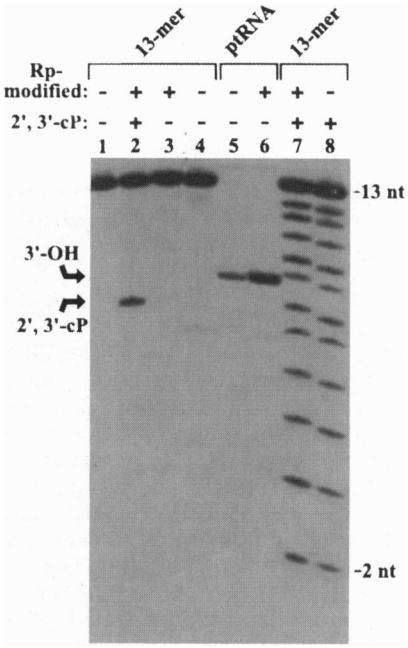

FIG. 4. Analysis of the 3′ termini generated by RNase P RNA processing of 5′-end-labeled unmodified (lane 5) or Rp-diastereomeric ptRNA^{Gly} (lane 6) in the presence of 15 mM Mg^{2+} (lane 5) or Cd^{2+} (lane 6). Analysis was performed by 25% PAGE in the presence of 8 M urea. Lanes 1–4, 7, and 8: 5′-^{32}P-end-labeled unmodified (lanes 1, 4, and 8) and Rp-diastereomeric (lanes 2, 3, and 7) RNA 13-mers, which are identical to the 5′-terminal 13 nt of the respective assembled ptRNAs. Lanes 1 and 2, phosphorothioate-specific iodine treatment; iodine hydrolysis in lane 2 ocurred at the same phosphodiester as RNase P RNA cleavage (lanes 5 and 6) but yielded a terminal 2′,3′-cyclic phosphate (24). Lanes 3 and 4 were as lanes 2 and 1, but without iodine treatment. Lanes 7 and 8, partial alkaline hydrolysis of the Rp-diastereomeric (lane 7) and unmodified (lane 8) 13-mer, which also generates 2′,3′-cyclic phosphate termini; the smallest alkaline hydrolysis band (1 nt) migrated below the shown section of the gel.

Metal ion-dependent cleavage rates (k_{obs}) have been summarized in Table 2. The k_{obs} for the cleavage of unmodified ptRNA was 3.3 min^{-1} in the presence of 15 mM Mg^{2+} and only slightly reduced at 12.5 mM Mg^{2+} and either 2.5 mM Cd^{2+} or 2.5 mM Mn^{2+} (k_{obs} of 2.2 and 1.5 min^{-1}). However, at 15 mM Cd^{2+}, the rate of cleavage was dramatically reduced ($\leq 10^{-4}$ min^{-1}). The Rp-diastereomeric ptRNA was processed at a rate of $\approx 10^{-3}$ min^{-1} at 15 mM Mg^{2+}, which corresponds to a $\geq$1000-fold reduction in the reaction rate compared with the unmodified ptRNA. This effect is on the chemical step, since cleavage was analyzed at saturating ribozyme and pH 6.0, where the chemical step is rate-limiting (see above). Cleavage in the presence of 12.5 mM Mg^{2+}/2.5 mM Mn^{2+} suggested a slight rescue of inhibition, which was enhanced at 15 mM Mn^{2+}, resulting in a $\approx$7-fold enhanced rate compared with that obtained at 15 mM Mg^{2+}. This suggested direct coordination of a metal ion to the *pro*-Rp oxygen at the scissile phosphodiester. At 15 mM Mn^{2+}, the cleavage rate of the Rp-diastereomer (8×10^{-3} min^{-1}) was $\approx$100-fold slower than the rate obtained for the unmodified substrate (0.75 min^{-1}). This is consistent with the preferred coordination of Mn^{2+} to oxygen over sulfur (17).

When Cd^{2+} was partially substituted for Mg^{2+}, cleavage of the Rp-diastereomeric ptRNA was $\approx$100-fold faster than at 15 mM Mg^{2+}, with a further increase in the presence of 15 mM Cd^{2+} as the only divalent cation, reaching $\approx$5% of the rate obtained for the unmodified ptRNA at 15 mM Mg^{2+}. Very similar results were obtained with the same tRNA carrying Rp-phosphorothioate modifications 5′ of every G residue, including the canonical cleavage site (all-Rp ptRNA; Table 2),

Table 2. Cleavage of modified and unmodified ptRNA^{Gly} by *E. coli* RNase P RNA

ptRNA^{Gly} species	Single turnover rate of cleavage k_{obs}, min^{-1}				
	15 mM Mg^{2+}	15 mM Cd^{2+}	15 mM Mn^{2+}	12.5 mM Mg^{2+}/ 2.5 mM Cd^{2+}	12.5 mM Mg^{2+}/ 2.5 mM Mn^{2+}
Unmodified	3.3 ± 1	$\leq 10^{-4}$	0.75 ± 0.25	2.2 ± 1.1	1.5 ± 0.4
All-Rp	$3 (\pm 1.5) \times 10^{-4}$	0.2 ± 0.1	$2 (\pm 1) \times 10^{-2}$	0.09 ± 0.02	$4 (\pm 2) \times 10^{-3}$
Single Rp modific.	$1.5 (\pm 0.5) \times 10^{-3}$	0.14 ± 0.03	$8 (\pm 3) \times 10^{-3}$	0.1 ± 0.05	$4 (\pm 2) \times 10^{-3}$
Single Sp modific.					
Cleavage at nt +1/−1	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$	$\leq 10^{-4}$
Cleavage at nt −1/−2	$3 (\pm 2.5) \times 10^{-3}$	$\leq 10^{-4}$	$5 (\pm 4) \times 10^{-3}$	$\leq 10^{-4}$	$2 (\pm 1) \times 10^{-3}$

Cleavage rates represent mean values derived from at least three independent experiments. Very low activities have been categorized as $\leq 10^{-4}$, since nonspecific degradation became significant in this activity range.

demonstrating that a substitution of the *pro*-Rp oxygen at other positions than the cleavage site has little effect on cleavage efficiency as observed in a previous study (14).

Hill Plot Analysis. The Mg^{2+}-dependent cooperativity of the cleavage reaction catalyzed by *E. coli* RNase P RNA has been analyzed in a previous study (3) under very similar conditions (single turnover, pH 6.0, 1 M NaCl) but using a different ptRNA^{Asp}. A Hill coefficient of ≈3.2 was determined in the range of 2–20 mM Mg^{2+} (3), which is similar to the value of ≈3.3 determined in our study in the same Mg^{2+} concentration range using the ptRNA^{Gly} as the substrate (data not shown). However, a Hill coefficient of 1.8 was obtained for cleavage of the Rp-diastereomeric ptRNA^{Gly} in the presence of Cd^{2+} as the only divalent metal ion (Fig. 5), which is more similar to the value of 1.4 observed for ptRNA^{Asp} cleavage in the presence of Ca^{2+} (3).

DISCUSSION

The strong rescue of cleavage of the Rp-diastereomeric ptRNA in the presence of Cd^{2+} provides the most convincing evidence that direct metal ion coordination occurs to the *pro*-Rp oxygen at the scissile phosphodiester. This result verifies one assumption made in previous transition state models (2–4). The rate of cleavage of the Rp-diastereomeric ptRNA in the presence of 120 mM Cd^{2+} was only ≈8-fold reduced compared with processing of the unmodified ptRNA at saturating Mg^{2+} (Fig. 5). This difference can be largely accounted for by the lower intrinsic reactivity (4- to 11-fold reduction) of phosphorothioate versus phosphate diesters (18).

Steitz and Steitz (4) have proposed a general two-metal ion mechanism for catalytic RNAs, which has been inferred from mechanisms used by protein metalloenzymes. The mechanism implies simultaneous direct coordination of two metal ions to one of the nonbridging oxygens at the scissile phosphodiester. The authors suggested a similar coordination geometry for the RNase P RNA hydrolysis reaction. Smith and Pace (3) have studied the influence of [Mg^{2+}] on the cleavage rate by Hill plot analysis. Based on a Hill coefficient of ≈3, the authors suggested that three metal ions may be required for catalysis. Two Mg^{2+} ions were proposed to coordinate simultaneously to the *pro*-Sp oxygen. The third Mg^{2+} was proposed to directly coordinate to the *pro*-Rp oxygen.

A Hill coefficient of 1.8 (Fig. 5) obtained for Cd^{2+}-dependent cleavage of the Rp-diastereomeric ptRNA would be consistent with simultaneous direct coordination of two Cd^{2+} ions to the Rp-diastereomeric sulfur, thus being in line with the proposed two-metal ion mechanism proposed by Steitz and Steitz (ref. 4; see Fig. 6). Alternatively and consistent with the model of Smith and Pace (3), only one metal ion may directly coordinate to the *pro*-Rp oxygen or Rp-diastereomeric sulfur. One or more metal ion hydrates may additionally support transition state formation—e.g., by stabilizing the 3′-oxyanion leaving group. Mg^{2+} cooperativity ($n_H \geq 3$) was significantly higher (ref 3; this study) compared with

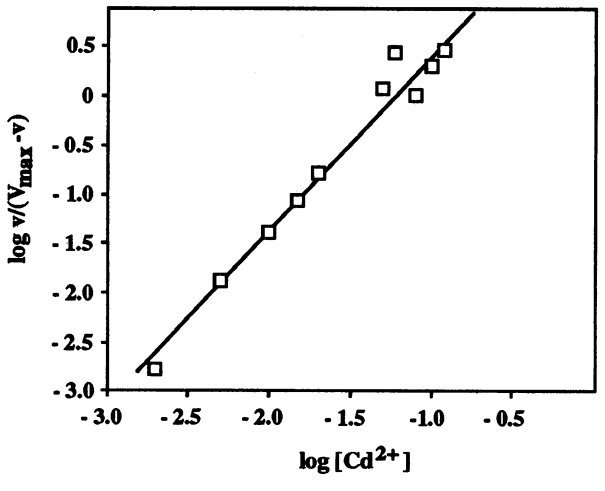

FIG. 5. Cd^{2+} dependence of RNase P RNA-catalyzed cleavage of the Rp-diastereomeric ptRNA^{Gly} (Hill analysis). The Cd^{2+} concentration was varied in the range of 2–120 mM in single turnover reactions; higher Cd^{2+} concentrations than 120 mM started to inhibit cleavage. The slope of the plot yields a Hill coefficient (n_H) of 1.8. The cleavage rate at 120 mM Cd^{2+} (representing V_{max}) was 1.2 min^{-1}; in comparison, cleavage of the unmodified ptRNA^{Gly} at saturating Mg^{2+} (e.g., 120 mM) occurred at a rate of 10 min^{-1}. It should be noted that Cd^{2+} concentrations as high as 120 mM did not cause substantial degradation of substrate and enzyme RNAs.

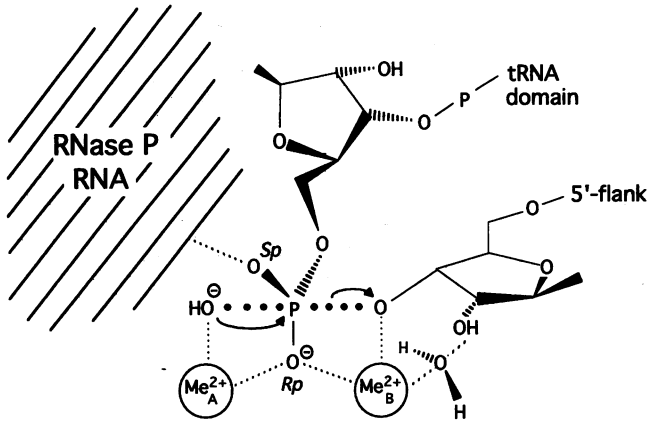

FIG. 6. Possible mechanism for ptRNA cleavage by RNase P RNA. The model includes main features of the general two-metal ion mechanism proposed for protein- and RNA-catalyzed hydrolytic and phosphoryl transfer reactions (4). Based on the observation that the 2′-OH group at the scissile phosphodiester (nt −1) is required for high cleavage rates, Smith and Pace (3) have proposed that this 2′-OH would form a hydrogen bond to a metal ion-bound water molecule, thereby facilitating proton transfer from this water to the 3′-oxyanion leaving group.

that observed for the Rp-diastereomeric ptRNA cleavage reaction in the presence of Cd^{2+} (n_H = 1.8; Fig. 5). One explanation may be that the Hill coefficient overestimates the number of Mg^{2+} ions directly involved in the cleavage mechanism. This may be related to the unique properties of Mg^{2+} ions in RNA folding and in stabilizing the ptRNA/RNase P RNA complex in addition to being efficient in promoting catalysis (2, 10, 19). For example, folding of the *Bacillus subtilis* RNase P RNA was shown to be cooperative with respect to the Mg^{2+} concentration in the lower mM range [n_H = 3.2] (19).

When sulfur was substituted for the nonbridging *pro*-Sp oxygen at the canonical cleavage site, the ribozyme was unable to restore cleavage at the modified phosphodiester in the presence of Mn^{2+} or Cd^{2+} (Table 2). This result indicates a crucial role of the *pro*-Sp oxygen in the cleavage mechanism but provides no evidence for direct metal ion coordination to this oxygen. What might be the specific role of the *pro*-Sp oxygen? The involvement of the *pro*-Sp oxygen in a tertiary contact required for docking the correct internucleotide bond in the active site would provide a plausible interpretation of the results obtained here (Tables 1 and 2; Fig. 3*A*). As a result, exact positioning of the scissile phosphodiester would be prevented in the presence of the Sp-phosphorothioate, which then favors docking of the adjacent internucleotide linkage in the active site. Alternatively, the role of the *pro*-Sp oxygen may be primarily in transition state stabilization. The negative charge developing on the *pro*-Sp oxygen in the pentacoordinate transition state (or intermediate; ref. 20) may be neutralized by direct interaction with one or more functional groups of the ribozyme or indirectly by interacting with a metal ion hydrate bound to the ribozyme-substrate complex. Yet another possibility is that a single metal ion directly coordinates to the *pro*-Rp oxygen and forms a hydrogen bond to the *pro*-Sp oxygen of the same phosphodiester via one of its bound water molecules, as observed in crystals of yeast $tRNA^{Phe}$ (21). Assuming the *pro*-Sp oxygen at the RNase P cleavage site may be involved in this type of interaction, the Sp-diastereomer might prevent binding of a metal ion-coordinated water, thereby precluding direct coordination of this metal ion to the *pro*-Rp oxygen. This could explain the complete inhibition of cleavage at the canonical site by the Sp-phosphorothioate modification, irrespective of the type of metal ion used. Also, Cd^{2+}, by virtue of its higher affinity to sulfur versus oxygen, may coordinate directly to the Sp-diastereomeric sulfur instead of coordinating to the *pro*-Rp oxygen. This would result in the wrong stereochemistry, being consistent with the failure of Cd^{2+} to restore detectable cleavage of the Sp-diastereomer. It should also be noted that phosphorothioates have an asymmetric charge distribution, with the negative charge mainly localized on the sulfur (22). As a consequence, replacement of the *pro*-Sp oxygen with sulfur may lead to electrostatic repulsion between the sulfur and functional groups of the ribozyme and/or reduced negative charge density at the Rp oxygen may impair direct coordination to Mg^{2+}.

What is the relation of the RNase P RNA-catalyzed cleavage mechanism to that of other ribozymes? In contrast to RNase P RNA, which is strongly inhibited by both diastereomers, the endonuclease reaction catalyzed by the *Tetrahymena* ribozyme is hardly affected by the Rp-diastereomer. An Rp-phosphorothioate at the cleavage site showed only a moderate "thio effect" under conditions of rate-limiting chemistry, 2.3-fold for the reaction with guanosine and 7-fold with a solvent hydroxyl acting as the nucleophile, which is attributable to the lower intrinsic reactivity of phosphate versus phosphorothioate diesters (18). The first step of the transesterification reaction catalyzed by the αI5γ self-splicing group II intron may represent an intermediate type between the *Tetrahymena* ribozyme and RNase P RNA with respect to the stereochemical requirements at the scissile phosphodiester. Here, the first step catalyzed by this group II intron is essentially abolished with the Rp-diastereomer and proceeds at an ≈30-fold reduced rate in the presence of the Sp-diastereomer (23).

A single Rp-phosphorothioate modification at the RNase P cleavage site had no effect on RNase P RNA–ptRNA complex formation (Table 1) and the sulfur substitution, although more bulky than oxygen, allowed efficient coordination to Cd^{2+}, which has a larger ionic radius than Mg^{2+} and Mn^{2+}. This indicates a low degree of structural hindrance in the vicinity of the *pro*-Rp oxygen. In contrast, the *pro*-Sp oxygen seems to be in close contact to the ribozyme (see discussion above and Fig. 6), as inferred from impaired ground state binding of the ptRNA when this oxygen is replaced with sulfur (Table 1). Taking into account the results obtained here and in a previous study (3) and following the mechanism proposed for the exonuclease reaction of DNA polymerase I (4), a model for the RNase P RNA cleavage reaction may incorporate the following characteristics (Fig. 6): (*i*) direct coordination of two metal ions to the *pro*-Rp oxygen at the cleavage site; (*ii*) hydrogen bonding of the *pro*-Sp oxygen to the ribozyme; (*iii*) stabilization of the negative charge of the 3′-oxyanion leaving group by direct metal ion coordination; (*iv*) proton transfer to the 3′-oxyanion leaving group by a metal ion-bound water molecule that simultaneously interacts with the 2′-OH group at the scissile phosphodiester; and (*v*) nucleophilic attack by a metal ion-bound hydroxide ion.

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