

The naturally *trans*-acting ribozyme RNase P RNA has leadzyme properties

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ABSTRACT

Divalent metal ions promote hydrolysis of RNA backbones generating 5'OH and 2';3'P as cleavage products. In these reactions, the neighboring 2'OH act as the nucleophile. RNA catalyzed reactions also require divalent metal ions and a number of different metal ions function in RNA mediated cleavage of RNA. In one case, the LZV leadzyme, it was shown that this catalytic RNA requires lead for catalysis. So far, none of the naturally isolated ribozymes have been demonstrated to use lead to activate the nucleophile. Here we provide evidence that RNase P RNA, a naturally *trans*-acting ribozyme, has leadzyme properties. But, in contrast to LZV RNA, RNase P RNA mediated cleavage promoted by Pb²⁺ results in 5' phosphate and 3'OH as cleavage products. Based on our findings, we infer that Pb²⁺ activates H₂O to act as the nucleophile and we identified residues both in the substrate and RNase P RNA that most likely influenced the positioning of Pb²⁺ at the cleavage site. Our data suggest that Pb²⁺ can promote cleavage of RNA by activating either an inner sphere H₂O or a neighboring 2'OH to act as nucleophile.

INTRODUCTION

The interaction of divalent metal ions e.g. Mg²⁺ with the negatively charged backbone of RNA promotes proper folding and therefore plays an important role for RNA function/activity. Ribozymes or catalytic RNAs catalyze a large number of reactions including cleavage of other RNA molecules. In addition to promoting correct folding and facilitating the interaction with the RNA substrate, divalent metal ions are directly involved in the chemistry of RNA mediated cleavage of RNA. But note that certain RNAs e.g. the hammerhead and the

hairpin ribozymes function in the absence of divalent metal ions (1).

Binding of metal ions often results in hydrolysis of the RNA backbone, e.g. lead(II)-induced cleavage of RNA (2,3). Cleavage products in metal(II) ion-induced hydrolysis of RNA have 5'OH and 2';3' cyclic phosphate at their ends (Figure 1D). This is also the case when RNA is cleaved by small ribozymes e.g. the hammerhead RNA. For both hammerhead and metal(II) ion-induced cleavage it has been suggested that the 2'OH at the site of cleavage is the active nucleophile (2,4). For the naturally occurring *trans*-acting ribozyme RNase P RNA, and for other large ribozymes (originating from Group I and Group II introns) that generate 5'P and 3'OH as cleavage products, we have argued that the strategy must be to prevent the 2'OH at the cleavage site from acting as a nucleophile and instead facilitate nucleophilic attack from the other side of the phosphorous center to ensure correct cleavage products (5). Thus, positioning of metal ions in relation to the cleavage site is of fundamental importance to ensure correct cleavage and to suppress unwanted hydrolysis of the RNA.

RNase P RNA is the catalytic subunit of the endoribonuclease RNase P that is responsible for generating the mature 5' termini of tRNA (6,7). Cleavage requires the presence of divalent metal ions, with Mg²⁺ as the preferred divalent metal ion. However, the presence of other divalent metal ions such as Mn²⁺ and Ca²⁺ can also promote cleavage [(5,8) and references therein, see also below]. Also, combinations of divalent metal ions that do not promote cleavage (or do so poorly) when present alone resulted in increased cleavage activity when present in combination, e.g. mixing Sr²⁺ with Mn²⁺ or Zn²⁺ (9,10). This suggests that there is metal ion cooperativity in RNase P RNA mediated cleavage (9). One of the metal ions involved in this cooperativity was suggested to be positioned in the vicinity of the interaction between the 3' end of the substrate and RNase P RNA, the RCCA-RNase P RNA interaction [(5,9,11); interacting residues underlined]. According to our model, the other metal ion(s) would be positioned at or in the vicinity of the cleavage site and be involved in generating the nucleophile (Figure 1C). Recently, we presented functional

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changes at the cleavage site. Moreover, based on our previous data demonstrating that Pb^{2+} promotes hydrolysis of model RNA hairpin substrates near the RNase P cleavage site in the absence of RNase P RNA (21) and that combinations of metal(II) ions that do not promote cleavage (or do so poorly) when present alone can result in increased cleavage activity, we were also interested to investigate whether Pb^{2+} in combination with other divalent metal ions can promote RNase P RNA mediated cleavage.

Here we present data suggesting that the presence of a purine at the +1 position in the substrate suppresses Pb^{2+} -induced cleavage at this position. However, a guanosine at +1 resulted in M1 RNA (RNase P RNA derived from *Escherichia coli*) mediated cleavage at the correct position in the presence of Pb^{2+} and Sr^{2+} or $Co(NH_3)_6^{3+}$ as the only divalent metal ions. Thus, RNase P RNA has leadzyme properties. The observation that Sr^{2+} or $Co(NH_3)_6^{3+}$ did not promote cleavage alone is in keeping with a model that Pb^{2+} is responsible for activating the nucleophile and we identified residues in both RNase P RNA and in the substrate that influence cleavage in the presence of Pb^{2+} . Our findings provide experimental evidence for the model suggesting that the establishment of the RCCA-RNase P RNA interaction results in a re-coordination of Mg^{2+} positioned in the vicinity of the cleavage site (25).

MATERIALS AND METHODS

Preparation of substrates and RNase P RNA

The various pATSer derivatives were purchased from Dharmacon, USA, purified, labeled at the 5' end and gel purified according to standard procedures as described elsewhere (11,26,27). The RNase P RNA variants were generated as run-off transcripts using T7 DNA-dependent RNA polymerase (27,28).

Assay conditions and determination of the kinetic constants under single turnover conditions

The assays were performed under single turnover conditions at pH 6.1 or 7.2 (as indicated) in Buffer B: 50 mM Bis-Tris Propane, 5% (w/v) PEG 6000, 100 mM NH_4Cl and $MgCl_2/SrCl_2/Co(NH_3)_6^{3+}/PbOAc$ at different concentrations as indicated. All reactions were performed at 37°C. Reaction products were separated on denaturing 20–22% (w/v) polyacrylamide gels and quantified using Phosphorimager (Molecular Dynamics 400S) as described elsewhere (5). The final concentrations of RNase P RNA and substrate were <20 μM and 3.2 μM , respectively.

The kinetic constants k_{obs} and k_{obs}/K^{sto} ($= k_{cat}/K_m$) were determined under saturating single turnover conditions at pH 6.1 in Buffer B as described previously (5,12) at: $[Mg^{2+}] = 40$ mM; $[Sr^{2+}] = 40$ mM and $[Mg^{2+}] = 8$ mM; $[Sr^{2+}] = 40$ mM and $[Pb^{2+}] = 8$ mM; $[Sr^{2+}] = 160$ mM and $[Pb^{2+}] = 8$ mM. The final concentration of substrate was <20 nM whereas the concentration of RNase P RNA was varied between 0.025 and 6.4 μM dependent on substrate and RNase P RNA variant. For the calculations we used the 5' cleavage fragments and the time of cleavage was adjusted to ensure that we were in the linear part of the curve of kinetics. The kinetic constants were obtained by linear regression from Eadie–Hofstee plots.

Binding assay conditions

Spin columns were used to determine the apparent equilibrium dissociation constant ($appK_d$) as described elsewhere (5,12) in Buffer C: 50 mM MES at pH 6.0, 0.8 M NH_4OAc , 0.05 % (w/v) Nonidet P40, 0.1% (w/v) SDS and different concentration of $CaCl_2$ or $SrCl_2$ as indicated. The final concentration of substrate was <20 nM and [RNase P RNA] was varied between 0.025 to 6.4 μM (concentration range depended on RNase P RNA variant). The $appK_d$ was determined by using OriginPro 7.0 software and the equation $f_c = f_t \times [RNase P RNA]_{free} / (K_d + [RNase P RNA]_{free})$, where f_c represent fraction of substrate in complex with RNase P RNA and f_t = maximum fraction of substrate able to bind RNase P RNA, i.e. end point.

Lead(II)-induced cleavage conditions

The different 5' end labeled pATSer derivatives were subjected to Pb^{2+} -induced cleavage in Buffer A: 50 mM Tris–HCl pH 7.2, 5% (w/v) PEG 6000, 100 mM NH_4Cl , 40 mM $MgCl_2$ (or $SrCl_2$) and $PbOAc$ at a final concentration of 4 mM. The substrates were preincubated in Buffer A in the absence of $PbOAc$ for 10 min at 37°C, incubated for 5 min after addition of $PbOAc$ and the cleavage products were analyzed by gel-electrophoresis as described elsewhere (21,27).

Determination of the site of cleavage

Cleavage at +1 was inferred by comparing the mobility of the 5' cleavage fragments generated in the presence of Pb^{2+}/Sr^{2+} and Mg^{2+} (see above). To verify the presence of 5' phosphate at the 5' termini of the cleaved product (internally labeled with [α - ^{32}P]GTP), the large cleavage product was gel purified and subjected to digestion with RNase T1, RNase T2 and pancreatic RNase A as described previously (29). Thin-layer chromatography according to Nishimura (30) was used to detect the 5' phosphate-labeled nucleotide i.e. pGp.

RESULTS

A purine at +1 suppresses metal induced cleavage at this position

The pATSerCG substrate is a well-characterized RNase P substrate that is cleaved at several positions in the 5' leader in the presence of Pb^{2+} and absence of RNase P RNA (21). A similar type of substrate was also used elsewhere and it was demonstrated that Mg^{2+} induced cleavage in the 5' leader the vicinity of the RNase P cleavage site (15). Hence, to investigate the structural requirements for positioning of a metal(II) ion at the RNase P cleavage site, we introduced changes at positions –1, +1 and/or +72 in pATSer (Figure 1A; the residue that pairs with residue +1 corresponds to residue +72 in tRNA and will therefore be referred to residue +72). These pATSer variants were subjected to Pb^{2+} -induced cleavage. As shown in Figure 2, introduction of a U or C at +1 resulted in significant cleavage at +1 irrespective of identity of the residue at +72 or –1. By contrast, a significant reduction in Pb^{2+} -induced cleavage at +1 was observed due to the presence of purine derivatives at this position. Moreover, our data suggested that base pairing between U_{+1} and the base at +72 did not influence Pb^{2+} -induced cleavage at +1 (Figure 2, compare cleavage of

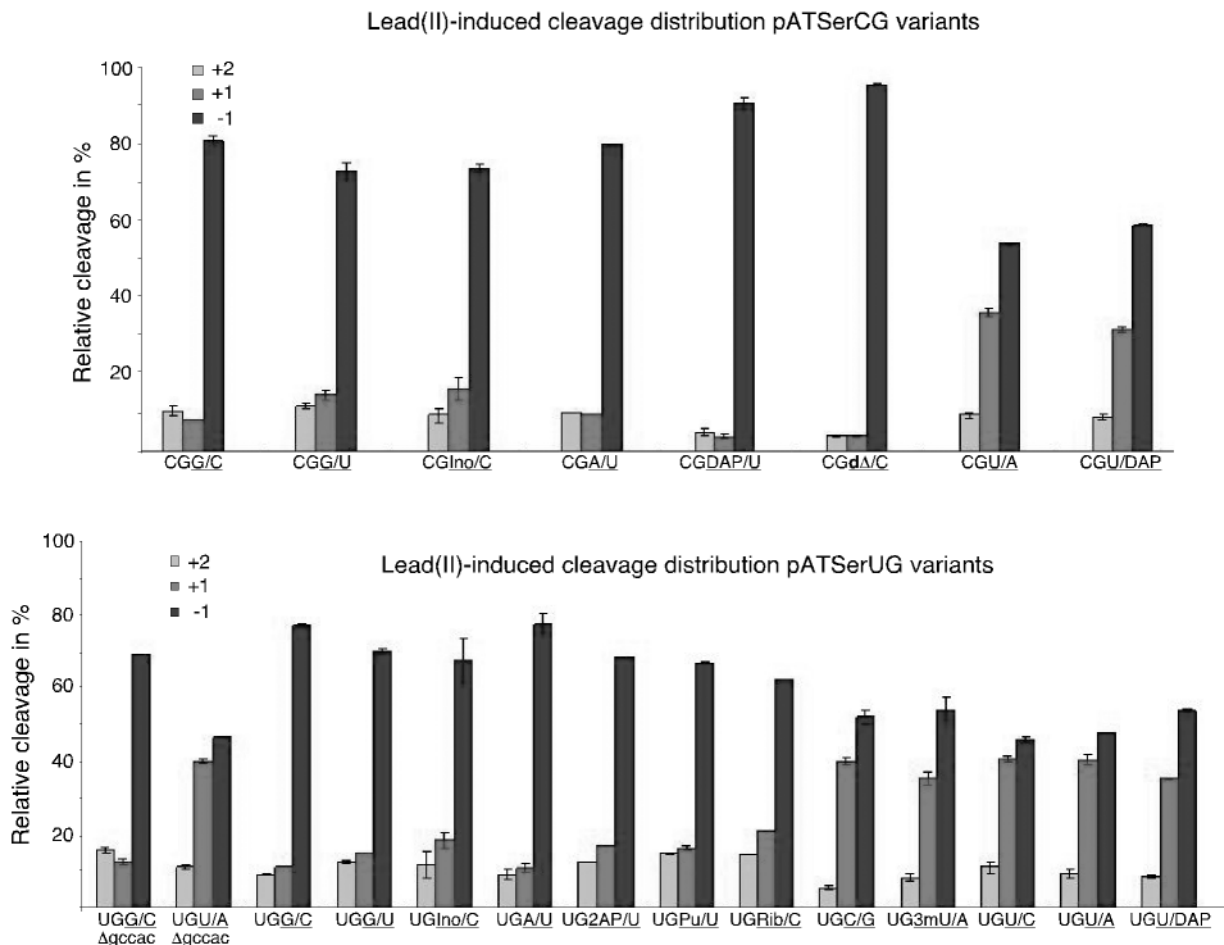


Figure 2. Histograms showing Pb^{2+} -induced cleavage of the different substrates in the absence of RNase P RNA as indicated. The distribution of the frequency of cleavage at -1 , $+1$ and $+2$ were calculated and the relative distribution in percent was plotted as indicated. $\Delta gcccac$ indicate residues that were deleted in these pATSerUG derivatives as illustrated in Figure 1.

U_{+1}/A_{+72} , U_{+1}/DAP_{+72} , U_{+1}/C_{+72} and $3mU_{+1}/A_{+72}$ variants). Neither did substitution of N7 of G_{+1} with $c7$ as was evident when Pb^{2+} cleavage of pATSerUGG/C, pATSerUGdG/C and pATSerUG(c7dG)/C was compared (data not shown; underlined residues corresponds to residues G_{+1} and C_{+72}) or in the absence of the 3' terminal sequence $G_{+73}CCAC$ (Figure 1A). A significant Mg^{2+} -induced cleavage at $+1$ was also observed due to replacement of G_{+1}/C_{+72} with U_{+1}/A_{+72} (data not shown). Surprisingly, introduction of ribavirin at $+1$ resulted in a similar suppression of Pb^{2+} -induced cleavage at $+1$ compared to when the substrate carried a purine at this position.

The 5' cleavage products generated as a result of addition of Pb^{2+} migrated slightly slower compared to the migration of the 5' cleavage fragments produced in the M1 RNA mediated cleavage reaction (see below Figure 3A and data not shown). In addition, the Pb^{2+} induced 5' cleavage products could not be labeled at their 3' ends with $[^{32}P]pCp$ (data not shown). These findings are expected given that metal(II)-induced cleavage generates cleavage product ends with 5'OH and 2';3' cyclic phosphates (2,3). Analysis of the 5' end of the larger cleavage product by thin-layer chromatography (i.e. the presence of nucleotides with a 5' phosphate could not be detected; Figure 3B upper panel) is in agreement with these findings.

We conclude that the local structural architecture around the $+1$ residue influence the positioning of Pb^{2+} (or Mg^{2+}) in its vicinity. More specifically we suggest that the presence of a purine at $+1$ prevents substantial metal induced cleavage at $+1$ and that the Hoogsteen surface of purine plays an important role. The observed cleavage at $+1$ for the U_{+1} and C_{+1} derivatives could be due to the electronegative environment generated by the oxygen at position 2 of U or C in the shallow groove (see also below). In all other cases tested there is either an exocyclic amine or no chemical group in the shallow groove at this position in the substrate.

RNase P RNA has leadzyme properties

The presence of Pb^{2+} induces hydrolysis of the phosphate backbone at the RNase P cleavage site (and in its vicinity) in the precursor substrate while combinations of metal ions that do not promote cleavage by themselves (or do so poorly) results in increased RNase P RNA mediated cleavage (9). Pb^{2+} was not included in our previous studies since we argued that addition of Pb^{2+} results in cleavage of RNase P RNA as well as other RNAs in particular at $pH > 7$ (2,3,9). Nonetheless, we decided to investigate whether RNase P RNA can mediate cleavage at the correct position in the presence of Pb^{2+} .

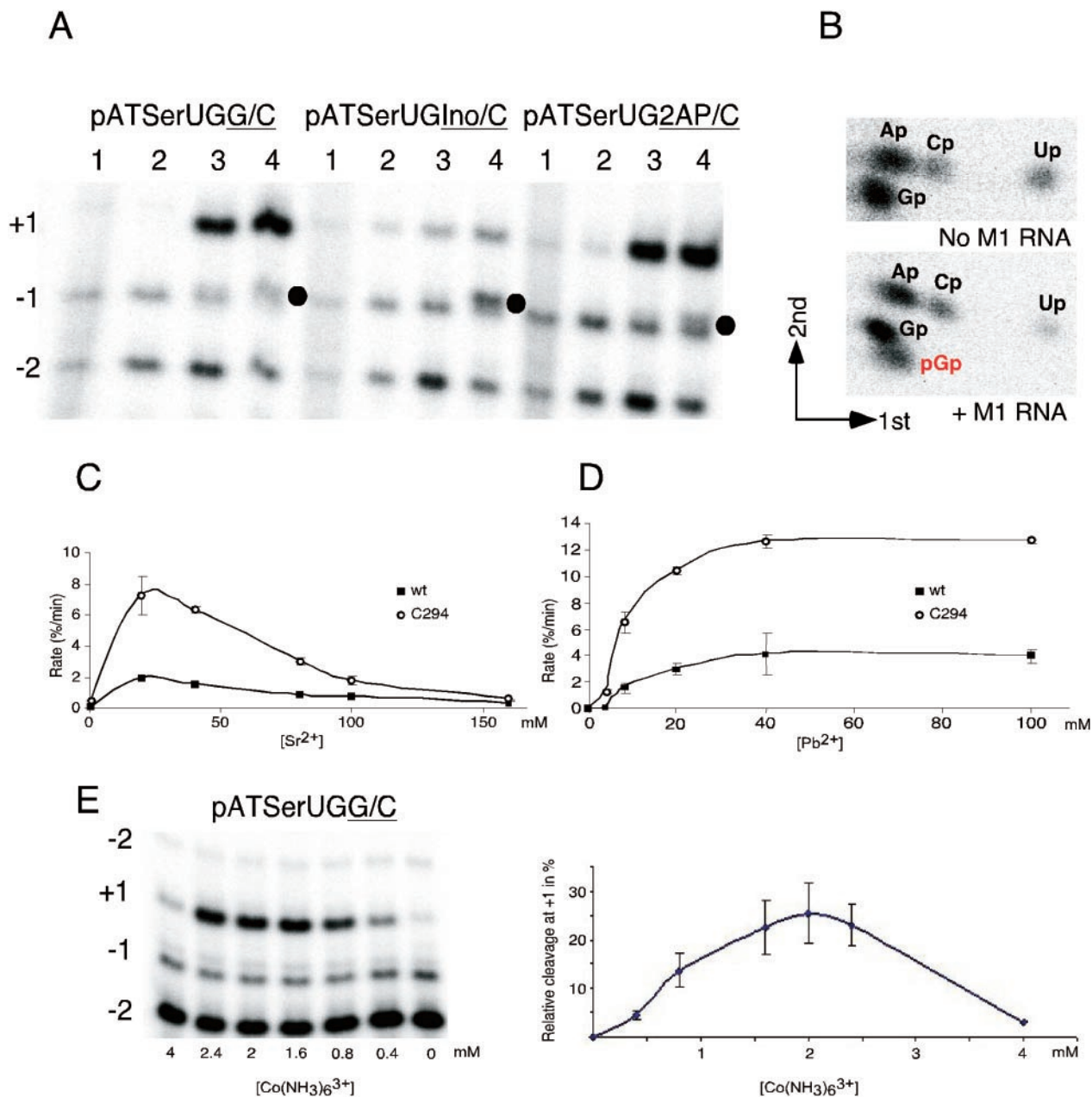


Figure 3. (A) M1 RNA mediated cleavage of pATSerUGG/C, pATSerUGIno/C and pATSerUG2AP/U under single turnover conditions at pH 7.2 in the presence of 8 mM Pb²⁺ and 40 mM Sr²⁺ as indicated. Two different M1 RNA variants (≈ 3.2 μ M final concentration; final concentration of substrate 0.04 μ M), wild-type M1 RNA (the number 3 lanes) and M1_{C294} RNA (the number 4 lanes) were used. Two controls, cleavage of the substrates in the absence of M1 RNA and \pm tRNA were included (no tRNA added the number 1 lanes while in the number 2 lanes tRNA was added). The filled circles indicate M1 RNA mediated cleavage at -1. The cleavage reaction was terminated after 5 min (for details see text). The different fragments as a result of cleavage at -2, -1 and +1 are shown. (B) Two-dimensional thin-layer chromatography according to Nishimura (30) demonstrating the presence of 5' pGp (in red) as a result of M1 RNA mediated cleavage in the presence of Pb²⁺. Internally labeled [α -³²P]GTP pATSerUGG/C was incubated in the presence of Pb²⁺/Sr²⁺ and in the absence and in the presence of M1 RNA as indicated. The large cleavage product was isolated and subjected to RNase digestion as outlined in Materials and Methods. For further experimental details see text. (C) and (D) M1 RNA mediated cleavage expressed as rate of cleavage in % per min as a function of increasing concentration of Sr²⁺ (C) and Pb²⁺ (D) using wild-type M1 RNA and M1_{C294} RNA as indicated. When Sr²⁺ was increased the Pb²⁺ concentration was kept constant at 8 mM likewise when we varied the Pb²⁺ concentration the Sr²⁺ concentration was kept constant at 40 mM. The experiments were performed at pH 6.1 at 37°C and the concentrations of M1 RNA and substrate were 3.2 μ M and ≤ 40 nM, respectively. (E) M1 RNA mediated cleavage expressed as relative cleavage at +1 in % as a function of increasing concentration of Co(NH₃)₆³⁺ as indicated using wild-type M1 RNA. The Pb²⁺ concentration was 8 mM. The experiment was performed in Buffer B at pH 7.2 at 37°C and the concentrations of M1 RNA and substrate were 3.2 μ M and ≤ 40 nM, respectively.

Conceivably this would give us a tool to identify factors important for generating 5'P and 3'OH as cleavage products on the one hand, and 5'OH and 2';3' cyclic phosphate on the other, where the former cleavage products are hallmarks for

RNase P RNA promoted cleavage and the latter the signature for Me²⁺- (here Pb²⁺-) induced cleavage [Figure 1D; (2, 6)]. Addition of Pb²⁺ will result in cleavage of both M1 RNA and the substrate pATSer at specific as well as unspecific positions

(3,7). Thus, cleavage conditions had to be short to minimize RNA degradation by Pb^{2+} but long enough to be able to detect RNase P RNA mediated cleavage. To achieve this objective we used *E.coli* RNase P RNA, M1 RNA and pATSerUGG/C as substrate. The pATSerUGG/C substrate (referred to as 'wild type') has in our previous studies been demonstrated to be as efficiently cleaved as precursor tRNAs (5,11,12). We preincubated M1 RNA for 10 min at 37°C in the presence of various concentrations of Sr^{2+} to allow M1 RNA to fold into an active conformation. No difference in the conformation of M1 RNA due to replacement of Mg^{2+} with Sr^{2+} was observed using Pb^{2+} -induced cleavage to monitor conformational changes (21). Note also that Sr^{2+} alone does not promote M1 RNA mediated cleavage under these conditions [(9); data not shown]. The substrate was added (amount added to ensure single turnover conditions) and after an additional incubation for 5 min Pb^{2+} was added to a final concentration of 8 mM (or as indicated). The cleavage reaction was terminated after 5 min and the cleavage products were separated by gel-electrophoresis (see Materials and Methods). Surprisingly, we observed M1 RNA mediated cleavage at the correct position +1 under these conditions with an optimum cleavage rate at 8 mM Pb^{2+} and 40 mM Sr^{2+} (Figure 3A). That cleavage was indeed mediated by M1 RNA was confirmed by employing thin-layer chromatography to demonstrate the presence of pGp at the 5' end of the cleavage product (Figure 3B). We also tried the combination Pb^{2+} and $Co(NH_3)_6^{3+}$. Here we had to reduce the concentration of $Co(NH_3)_6^{3+}$ significantly to detect M1 RNA mediated cleavage since higher concentrations of $Co(NH_3)_6^{3+}$ resulted in inhibition (Figure 3E). The lower concentration of $Co(NH_3)_6^{3+}$ is rationalized by that $Co(NH_3)_6^{3+}$ binds to RNA with higher affinity compared to e.g. Sr^{2+} (21). Consequently, at higher concentration of $Co(NH_3)_6^{3+}$, $Co(NH_3)_6^{3+}$ most likely displace Pb^{2+} and thereby cleavage is inhibited (see also below). Taken together, these findings are in keeping with our study where we observed cleavage when we mixed Sr^{2+} or $Co(NH_3)_6^{3+}$ with Zn^{2+} (9). In this context we note that Taira and coworkers (4) have studied the hammerhead cleavage reaction in the presence of different metal ions. Their data suggest that La^{3+} can act both as an activator and inhibitor of cleavage [see also the work by Komiyama *et al.* (31)].

A possibility is that the observed activities are due to contamination of Mg^{2+} or Ca^{2+} . However, in the presence of 40 mM Sr^{2+} and 0.1 mM Mg^{2+} we did not observe M1 RNA mediated cleavage (data not shown). Also, cleavage in the presence of 15 mM Ca^{2+} and 25 mM Sr^{2+} reduce the efficiency of cleavage >100-fold compared to cleavage in the presence of 15 mM Mg^{2+} and 25 mM Sr^{2+} (9). Hence, since addition of 0.1 mM Mg^{2+} did not result in cleavage we do not expect that addition of Ca^{2+} would. These data argue against contamination of Mg^{2+} and Ca^{2+} .

Replacing pATSerUGG/C with a conventional tRNA precursor, pSu3, also resulted in cleavage at the correct position at +1 in the presence of Pb^{2+} and Sr^{2+} (data not shown). Finally, Pb^{2+} induced cleavage of ^{32}P -labeled M1 RNA showed very little degradation of M1 RNA after 5 min in the presence of Pb^{2+} (data not shown).

We conclude that M1 RNA mediated cleavage can be promoted in the presence of Pb^{2+} and Sr^{2+} (or $Co(NH_3)_6^{3+}$), where Sr^{2+} and $Co(NH_3)_6^{3+}$ do not promote cleavage when present

alone. Thus, M1 RNA has leadzyme properties. This finding is in keeping with a model that Pb^{2+} is involved in generating the nucleophile i.e. corresponding to Me_A in Figure 1C. Given this, the Me_B binding site in the M1 RNA substrate (RS-) complex would most likely be occupied by Sr^{2+} (or $Co(NH_3)_6^{3+}$) since a Pb^{2+} at this position would result in hydrolysis of the M1 RNA phosphate backbone in its vicinity [for further discussion see Ref (9)].

The leadzyme-like activity is not a unique feature of M1 RNA

To investigate whether leadzyme-like activity could be observed for other RNase P RNAs we replaced M1 RNA with RNase P RNA derived from *Mycoplasma hyopneumoniae*, Hyo P RNA (32) and *Yersinia pestis*, Yer P RNA (Figure 4; B. M. F. Pettersson and L. A. Kirsebom, unpublished data). The former is a type B RNase P RNA and the latter is closely related to M1 RNA i.e. type A. While Hyo P RNA did not mediate cleavage under these conditions, partly due to poor substrate binding (data not shown), Yer P RNA

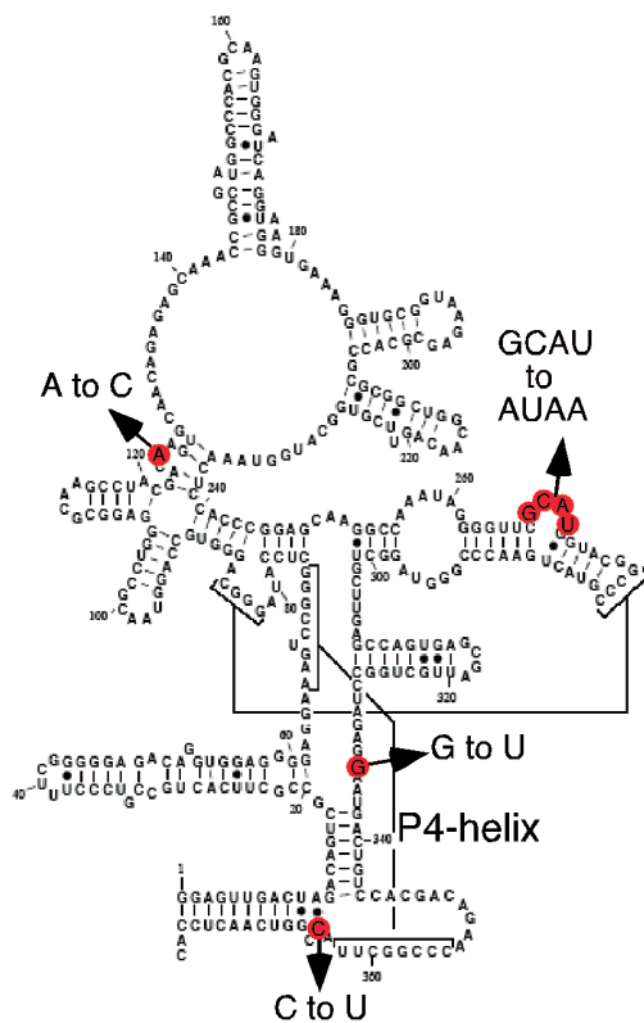


Figure 4. The predicted secondary structure of *Yersinia pestis* RNase P RNA according to Brown (48). The positions/region of the different changes that were introduced are highlighted in red.

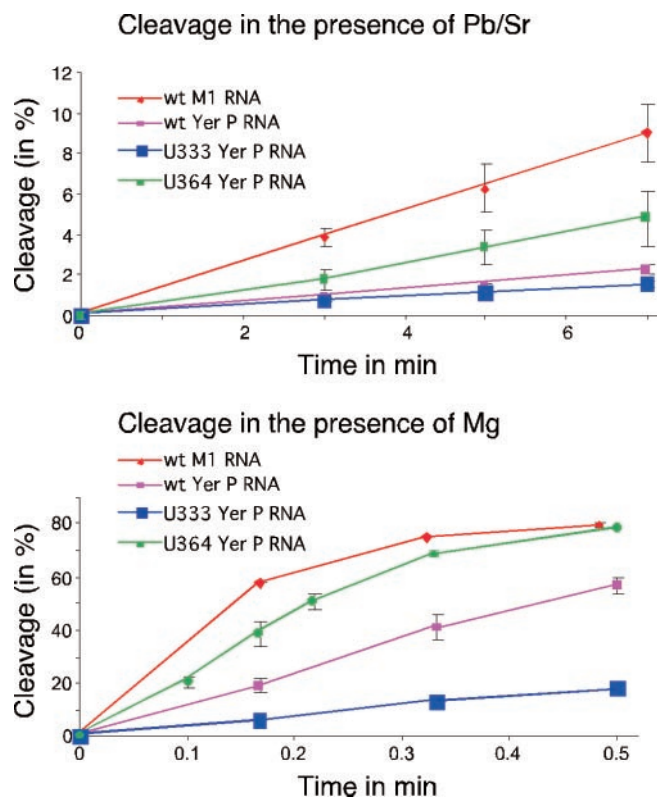


Figure 5. Cleavage rates under single turnover conditions at pH 6.1 as expressed in percentage of cleavage as a function of time for the different RNase P RNA variants as indicated where Yer P represents RNase P RNA derived from *Yersinia pestis*. The upper panel shows cleavage in the presence of 8 mM Pb^{2+} and 40 mM Sr^{2+} and the lower panel in the presence of 40 mM Mg^{2+} . The concentrations of M1 RNA and substrate were 3.2 μ M and \leq 40 nM, respectively.

promoted cleavage of pATSerUGG/C at the correct position +1 in the presence of Pb^{2+}/Sr^{2+} although less efficiently compared to M1 RNA (Figure 5). Both Hyo P RNA and Yer P RNA cleaved pATSerUGG/C efficiently and correctly in the Mg^{2+} alone cleavage reaction i.e. 40 mM Mg^{2+} (Figure 5; data not shown for Hyo P RNA). Moreover, the $appK_d$ value for Yer P RNA indicated no apparent difference in substrate binding compared to M1 RNA (see below; Table 1). Thus, the leadzyme-like activity is not a unique feature of M1 RNA.

Determinations of the apparent binding constant, $appK_d$ and the rate constant k_{obs}

Next we determined the apparent binding constant, $appK_d$, at two different Sr^{2+} concentrations 40 and 160 mM at pH 6.0. This was followed by determinations of the rate constant k_{obs} under single saturating conditions at pH 6.1. At this pH the rate of cleavage is linearly dependent on $[OH^-]$ in the presence of 8 mM Pb^{2+} and 40 (or 160 mM) mM Sr^{2+} suggesting that chemistry is rate-limiting at this pH [data not shown; (5)]. The linear dependence on $[OH^-]$ is also consistent with that a hydroxyl ion is acting as the nucleophile [e.g. see Ref (17)]. To be able to evaluate Pb^{2+} as a promoter of RNase P RNA mediated cleavage we also determined k_{obs} in the presence of 8 mM Mg^{2+} and 40 mM Sr^{2+} at pH 6.1. The results are shown in Table 1.

Comparing $appK_d$ for wild-type M1 RNA determined in the presence of Ca^{2+} or Sr^{2+} indicated that pATSerUGG/C binds with 10- and \approx 2.5-fold reduced affinity at 40 or 160 mM Me^{2+} , respectively, in the presence of Sr^{2+} . The rate of cleavage at position +1 in the presence of Pb^{2+} was only reduced 6-fold compared to cleavage in the presence of Mg^{2+} under the same conditions indicating that Pb^{2+} promoted cleavage with reasonable rates. Moreover, increasing the Sr^{2+} concentration resulted in better binding (20-fold) of the substrate but an almost 10-fold reduction in the rate of cleavage. Based on the model that Pb^{2+} generates the nucleophile this suggested that Sr^{2+} presumably had replaced Pb^{2+} in the vicinity of the cleavage site i.e. Me_A (Figure 1).

Structural architecture of the cleavage site affects positioning of Pb^{2+}

The observation that Pb^{2+} is positioned close to the cleavage site and likely involved in generating the nucleophile gives us a handle to identify residues and chemical groups in M1 RNA as well as in the substrate influencing RNase P RNA mediated cleavage promoted by Pb^{2+} (and most likely Mg^{2+}). To address which chemical groups at the cleavage site are important for efficient cleavage in the presence of Pb^{2+} , we cleaved pATSerUGG/C (Figure 3A). This suggested that the exocyclic amine at position 2 in guanosine plays an important role possibly influencing positioning of Pb^{2+} . The importance of the exocyclic amine at this position is consistent with previous findings [Kikovska *et al.* unpublished data; (33–35)]. Note also that we observed M1 RNA mediated cleavage at –1 in particular using M1_{C294} RNA (see below). We also tried to cleave the substrate where G_{+1}/C_{+72} had been replaced with U_{+1}/A_{+72} in the presence of Pb^{2+}/Sr^{2+} but no cleavage was observed under these conditions most likely due to this substrate being a poor substrate for M1 RNA mainly due to its influence on metal ion binding in its vicinity [data not shown; (12)]. These data suggest that the structural environment at the cleavage site and in particular the exocyclic amine positioned in the shallow groove influenced Pb^{2+}/Sr^{2+} promoted cleavage. We argue that these results are due to a change in the positioning of the Pb^{2+} involved in activating the nucleophile.

Structural architecture of ‘the +73/294 interaction’ affects positioning of Pb^{2+}

We recently provided experimental evidence that there is cross talk between ‘the +73/294 interaction’ and the cleavage site. During this process metal ions are suggested to play important roles [see Figure 1B; (5)]. Hence, we asked whether a change in the structural architecture of the +73/294 interaction influences the rate of cleavage in the Pb^{2+} -promoted reaction. Based on our model that Pb^{2+} is located close to the cleavage site (see above) we reasoned that this would give information about whether the positioning of this Pb^{2+} ion(s) is affected or not. Thus, we used a mutant M1 RNA where U294 had been replaced with a C, M1_{C294} RNA, resulting in a G_{+73}/C_{294} interaction instead of a G_{+73}/U_{294} in the RS-complex

Table 1. Summary of the kinetic constants k_{obs} and $k_{\text{obs}}/K^{\text{sto}}$, and the apparent binding constants, $\text{app}K_{\text{d}}$ for various RNase P RNAs and pATSerUGG/C at Me^{2+} concentrations as indicated

M1 RNA Metal ion conditions	k_{obs} (min^{-1})	$k_{\text{obs}}/K^{\text{sto}}$ ($\text{min}^{-1} \times \mu\text{M}$)	$\text{app}K_{\text{d}}$ (μM)
wt M1 RNA; $[\text{Mg}^{2+}] = 40 \text{ mM}^{\text{a}}$	1.2 ± 0.2	3.8 ± 0.6	$0.11 \pm 0.056^{\text{b}}$
M1 _{C294} RNA; $[\text{Mg}^{2+}] = 40 \text{ mM}$	0.8 ± 0.23	7.6 ± 2.8	$0.13 \pm 0.031^{\text{b}}$
wt M1 RNA; $[\text{Mg}^{2+}] = 160 \text{ mM}$	$8.8 \pm 0.35^{\text{c}}$	$14 \pm 2^{\text{c}}$	$0.014 \pm 0.002^{\text{d}}$
wt M1 RNA; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Mg}^{2+}] = 8 \text{ mM}$	0.038 ± 0.02	0.074 ± 0.03	$1.1 \pm 0.16^{\text{e}}$
M1 _{C294} RNA; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Mg}^{2+}] = 8 \text{ mM}$	0.10 ± 0.04	0.78 ± 0.6	$0.17 \pm 0.01^{\text{e}}$
wt M1 RNA; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	0.0059 ± 0.0033	0.017 ± 0.01	$1.1 \pm 0.16^{\text{e}}$
wt M1 RNA; $[\text{Sr}^{2+}] = 160 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	0.0009 ± 0.0006	0.0082 ± 0.0034	$0.053 \pm 0.0039^{\text{e}}$
M1 _{C294} RNA; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	0.024 ± 0.001	0.20 ± 0.098	$0.17 \pm 0.02^{\text{e}}$
M1 _{C294} RNA; $[\text{Sr}^{2+}] = 160 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	0.0016 ± 0.00015	0.064 ± 0.017	$0.018 \pm 0.004^{\text{f}}$
wt Yer P RNA; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	nd	nd	$1.7 \pm 0.22^{\text{e}}$
Yer P RNA _{U364} ; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	nd	nd	$1.2 \pm 0.33^{\text{e}}$
Yer P RNA _{U333} ; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	nd	nd	$0.95 \pm 0.16^{\text{e}}$
Yer P RNA _{C125} ; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	nd	nd	$2.8 \pm 1.3^{\text{e}}$
Yer P RNA _{AUAA} ; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	nd	nd	$2.7 \pm 1.2^{\text{e}}$
Yer P RNA _{U333U364} ; $[\text{Sr}^{2+}] = 40 \text{ mM}$; $[\text{Pb}^{2+}] = 8 \text{ mM}$	nd	nd	$1.2 \pm 0.58^{\text{e}}$

$k_{\text{obs}}/K^{\text{sto}} = k_{\text{cat}}/K_{\text{m}}$. The experiments were performed as described in Materials and Methods. Each value is an average of three independent experiments. In relevant cases, no Pb^{2+} was added when $\text{app}K_{\text{d}}$ was determined. nd = not determined.

^aTaken from Kikowska *et al.* (12).

^b $[\text{Ca}^{2+}] = 40 \text{ mM}$.

^cTaken from Brännvall and Kirsebom (49).

^d $[\text{Ca}^{2+}] = 160 \text{ mM}$.

^e $[\text{Sr}^{2+}] = 40 \text{ mM}$.

^f $[\text{Sr}^{2+}] = 160 \text{ mM}$.

(Figure 1B). The ground state binding ($\text{app}K_{\text{d}}$ at 40 mM Ca^{2+}) of this mutant is very similar compared to the wild-type (Table 1). Moreover, as shown in Table 1 in the Mg^{2+} alone reaction the rate of cleavage of pATSerUGG/C was only modestly reduced in keeping with our previous data (5). By contrast in the presence of Sr^{2+} , $\text{app}K_{\text{d}}$ was determined to be 6.5- to 2.5-fold lower relative to wild-type at 40 and 160 mM Sr^{2+} , respectively, indicating an improved ground state binding for this mutant in the presence of Sr^{2+} . Cleavage under single turnover conditions as a function of $[\text{Sr}^{2+}]$ revealed that the C294 mutant cleaved the substrate more efficiently compared to the wild-type. This was observed to be the case irrespective of whether Mg^{2+} or Pb^{2+} promoted cleavage was studied (Figure 4; data only shown for the case when 8 mM Pb^{2+} was used). This was further corroborated when we determined k_{obs} for M1_{C294} RNA under the conditions indicated in Table 1. Here we observed a 2.6-fold increase at $40 \text{ mM Sr}^{2+}/8 \text{ mM Mg}^{2+}$ and a 4.1-fold increase at $40 \text{ mM Sr}^{2+}/8 \text{ mM Pb}^{2+}$. At $160 \text{ mM Sr}^{2+}/8 \text{ mM Pb}^{2+}$, we observed only a 1.8-fold increase relative to wild-type again indicating (see above) that higher concentration of Sr^{2+} inhibits the rate of cleavage under these conditions. These findings suggested that changes of the structural architecture of the +73/294 interaction influence metal ion binding most likely in its vicinity (Me_A in Figure 1C; see also below). We argue that as a consequence, this affects the rate of cleavage possibly due to a repositioning of the metal ion involved in generating the nucleophile. This further indicates the role of metal ions bound at different sites in RNase P RNA mediated cleavage [see also Ref (9)].

Identification of a residue in RNase P RNA affecting positioning of Pb^{2+}

The efficiency of the $\text{Pb}^{2+}/\text{Sr}^{2+}$ promoted cleavage for Yer P RNA was lower compared to that of M1 RNA. The secondary

structures of these two RNase P RNAs are very similar and differences were identified in four regions: C125A, U333G, U364C (residue in M1 RNA underlined) and AUAA in M1 RNA (see Figure 4) while Yer P RNA carries GCAU at this site. This gave us a way to identify other residues/regions in RNase P RNA potentially involved in positioning Pb^{2+} in the vicinity of the cleavage site. Thus, we generated four Yer P RNA variants carrying C125, U333, U364 and AUAA₂₆₉ (M1 RNA numbering; Figure 4) to investigate whether the Yer P RNA activity could be rescued. As shown in Figure 5, only the C to U replacement at 364 resulted in an increased cleavage activity while the other changes did not. This was observed both in the $\text{Pb}^{2+}/\text{Sr}^{2+}$ and Mg^{2+} promoted reactions. Neither of these changes affected ground state binding (Table 1). These findings raise the possibility that the residue at 364 in Yer P RNA influenced positioning of the Pb^{2+} in the vicinity of the cleavage site.

DISCUSSION

Lead(II) ion is an efficient inducer of hydrolysis of the phosphate backbone of RNA generating $5'\text{OH}$ and $2';3'\text{P}$ as cleavage products (2,3). Here we showed that RNase P RNA is able to cleave its substrate at the canonical site in the presence of the Pb^{2+} and Sr^{2+} or $\text{Co}(\text{NH}_3)_6^{3+}$.

The $\text{p}K_{\text{a}}$ value for Sr^{2+} is significantly higher compared to Mg^{2+} and Ca^{2+} that do promote RNase P RNA mediated cleavage (2,7). In a previous study Sr^{2+} was demonstrated to promote cleavage at elevated pH, pH 9 (14). Thus, one could argue that Pb^{2+} binding in the RS-complex 'activates' Sr^{2+} that subsequently results in generation of the nucleophile. Here we observed RNase P RNA mediated cleavage in the presence of $\text{Pb}^{2+}/\text{Sr}^{2+}$ at a significantly lower pH (6.1 and 7.2). When present alone, Sr^{2+} (or $\text{Co}(\text{NH}_3)_6^{3+}$) has not been demonstrated to promote cleavage by RNase P RNA under these

conditions, i.e. at $\text{pH} \leq 7.5$ [(9) and this report]. Also, an increase in the Sr^{2+} concentration resulted in inhibition of cleavage. Moreover, mixing Pb^{2+} with $\text{Co}(\text{NH}_3)_6^{3+}$ resulted in RNase P RNA mediated cleavage and increasing concentration of $\text{Co}(\text{NH}_3)_6^{3+}$ also inhibited cleavage. Together this argues against 'activation' of Sr^{2+} . Rather the role of Sr^{2+} (or $\text{Co}(\text{NH}_3)_6^{3+}$) is suggested to promote RNA folding and facilitate/stabilize the interaction with the substrate. Note that available data suggest that RNase P RNA is not correctly folded as well as that it is efficiently cleaved in the presence of only Pb^{2+} (21). Our data also argues against the presence of contamination of e.g. Mg^{2+} in our solutions. We conclude that our data are consistent with a model where Pb^{2+} most likely is responsible for generating the nucleophile. To conclusively demonstrate that this is indeed the case will require detailed structural analysis of the RNase P RNA substrate complex.

Compared to Mg^{2+} that is the preferred metal(II) ion in promotion of RNase P RNA mediated cleavage, Pb^{2+} shows different physico-chemical properties but their preferred coordination numbers are similar: Pb^{2+} 6 to 8 and Mg^{2+} 6 (36). Moreover, although the softer Pb^{2+} ion is larger than Mg^{2+} , Pb^{2+} and Mg^{2+} bind to overlapping sites (2). This, together with that Pb^{2+} can act as an efficient general base ($\text{p}K_a$ 7.8) as well as the similarity in coordination numbers might be the only reasons to why Pb^{2+} can promote RNase P RNA mediated cleavage. Whether other criteria also has to be fulfilled remains to be elucidated. But, unlike lead(II)-induced hydrolysis or that by the *in vitro* evolved leadzyme which generate 5'OH and 2':3'P as cleavage products (37), Pb^{2+} -promoted cleavage by RNase P RNA resulted in 5'P and 3'OH. Therefore, the mechanism of cleavage has to be different and, in the case of RNase P RNA the nucleophilic attack has to come from the opposite side relative to the positioning of the 2'OH at the cleavage site (Figure 1C). Available data suggest that H_2O is the nucleophile in RNase P RNA mediated cleavage (13,17,24). In keeping with this we demonstrated that cleavage by RNase P RNA in the presence of $\text{Pb}^{2+}/\text{Sr}^{2+}$ is linearly dependent on $[\text{OH}^-]$ (see above). Thus, most likely a H_2O ligand is also the nucleophile in RNase P RNA cleavage in the presence of Pb^{2+} i.e. Pb^{2+} acts as a general base. To our knowledge this is the first time that Pb^{2+} has been suggested to activate H_2O to act as a nucleophile in RNA mediated cleavage of RNA. To conclude, our present finding that Pb^{2+} likely act as a catalytic metal ion in M1 RNA mediated cleavage gives us a handle to identify factors/chemical groups that promote M1 RNA mediated cleavage and suppress cleavage of the phosphate backbone in the 5' leader due to activation of neighboring 2'OH groups and thereby increase our understanding of cleavage of RNA by RNA.

Close and distant residues affect positioning of Pb^{2+} in the RS-complex

Based on the discussion above Pb^{2+} promotes cleavage at and in the vicinity of the RNase P cleavage site both in the absence and presence of RNase P RNA (Figure 3), where efficient cleavage at the canonical site (with correct cleavage products and G at +1) was only observed in its presence. Hence, it is conceivable that Pb^{2+} positioned in the vicinity of the cleavage site is re-positioned as a result of formation of the RS-complex arguing for the possibility that the metal ion responsible for

generating the nucleophile is associated with the substrate [Figure 1C; (5,15,16)]. The importance of the structural architecture of the +1/+72 for metal ion binding in the vicinity of the cleavage site as well as the finding that the presence of the exocyclic amine at position 2 of the guanosine at +1 is important for M1 RNA mediated cleavage in the presence of Pb^{2+} is in keeping with this model [(12); this report; unpublished data]. However, we cannot exclude the possibility that the metal(II) ion(s) involved in generating the nucleophile is recruited from the solution or is associated with RNase P RNA e.g. Me^{2+} bound in the P4 helix (8).

Irrespective of whether Me_B (Figure 1C) is associated with the substrate, RNase P RNA or is recruited from the solution, our data indicate that the structural architecture of the +73/294 interaction influenced the rate of cleavage promoted in the presence of Pb^{2+} as well as metal(II) binding in its vicinity. This is consistent with our previous findings where we showed that the structural architecture of the +73/294 interaction, that is part of the RCCA-RNase P RNA interaction (25), affects the charge distribution at the cleavage site, ground state binding, rate of cleavage and metal ion binding in its vicinity [(5,9,11,26,38); this report]. Thus, our present findings provide further evidence for cross talk between the +73/294 interaction and the site of cleavage and experimental evidence for the involvement of metal ion(s) in this process.

Our data also indicate that U364 in M1 RNA is likely to influence positioning of Pb^{2+} in the vicinity of the cleavage site. The significance of this finding is not clear yet but it might indicate that either that this region is positioned in the vicinity of the cleavage site or that this change results in distance effects e.g. by affecting positioning of the metal(II) ions positioned in the P4 helix [(8); see above]. However, based on the recent RNase P RNA crystal structures the former alternative is less likely (39,40). Clearly we need to investigate this in further detail but nonetheless this finding opens for new possibilities and approaches using hybrid RNase P RNA molecules in our efforts to understand the complex orchestration in RNase P RNA mediated cleavage. In this context we note that the classical double mutant M1 RNA ts709 carries a G to A change at 365. However, no phenotype has been linked to this change [(41–43); L. A. Kirsebom and S. Altman, unpublished data]. Thus, our findings raise the possibility that this mutation indeed influences M1 RNA mediated cleavage by affecting positioning of the metal ion(s) involved in activating the nucleophile.

Structural evidence for positioning a metal(II) ion at the RNase P cleavage site

Our present and previous data clearly indicate that a divalent metal ion(s) is positioned close to the RNase P cleavage site (5,13–24). The high-resolution structure of the RNase P cleavage site is not yet available. However, we noticed that the structures of the ends of the leadzyme are almost identical to the sequences at and in the vicinity of the pATSerCGG/C and pATSerCGU/A cleavage sites with the exception that in the case of the ${}^{\text{G}}_{+1}/\text{C}_{+72}$ variant the C_{-1} is single stranded [Figure 6; (44)]. But in another high-resolution structure of SRP RNA in which the structure at one of the ends is also identical to the sequence of the pATSerCGG/C cleavage site the C_{-1} is base paired to a G as in pATSerCGG/C [Figure 6;

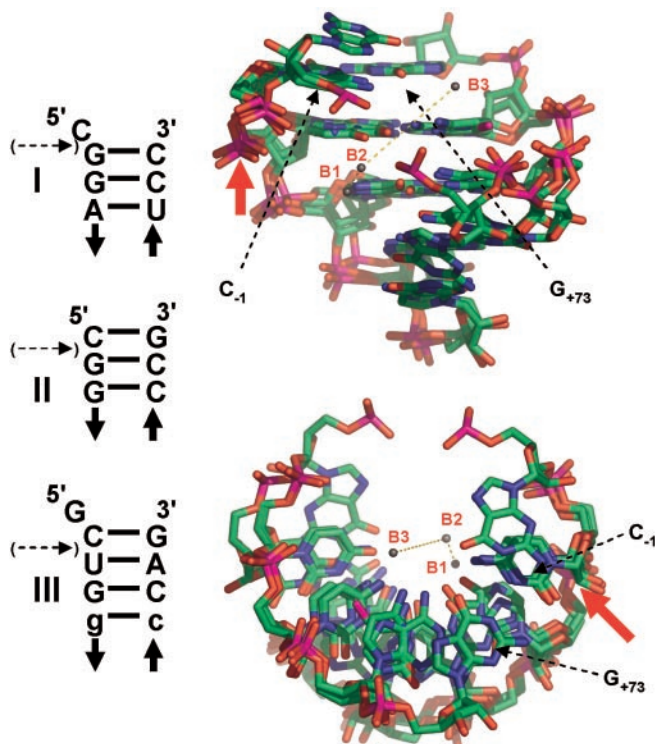


Figure 6. The three dimensional structures derived from the RNA leadzyme (43) and SRP RNA (44) superimposed where the upper representation is viewed from the side and the lower from the top. Two structures, I and III, were taken from the RNA leadzyme (to the left in the figure) whereas structure II was derived from the SRP RNA. The hexahydrated Mg^{2+} ions are designed B1, B2 and B3 where B1 corresponds to the Mg^{2+} located in the SRP RNA (II), while B2 and B3 are the Mg^{2+} ions located in the vicinity of 'G₊₁C₊₇₂' (structure I) and 'U₊₁A₊₇₂' (III) variants in the RNA leadzyme, respectively. The dashed arrows in parenthesis in the left half of the figure indicate the sites that correspond to the canonical RNase P cleavage site. The red arrows mark the phosphorous atom that corresponds to the one to be attacked in RNase P RNA mediated cleavage. The residues that would correspond to C₋₁ and G₊₇₃ in our pATSer substrate derivatives are indicated in the three dimensional structure representations.

(45)]. These structural studies suggested that a hexahydrated Mg^{2+} is bound in the deep groove in the vicinity of the base pairs that would correspond to G₊₁/C₊₇₂ and U₊₁/A₊₇₂, respectively, in our substrates. Superimposing the three Mg^{2+} binding sites revealed no apparent structural differences. But, the positioning of Mg^{2+} is suggested to be shifted >7 Å due to the G₊₁/C₊₇₂ to U₊₁/A₊₇₂ replacement, while formation of the 'C₋₁/G₊₇₃' base pair only gives a shift of 2.2 Å. In our substrates this would correspond to the Mg^{2+} near the cleavage site. This supports our functional data suggesting that U/A at +1/+72 affects Pb^{2+} as well as Mg^{2+} binding most likely in the vicinity of the cleavage site [(12); this study]. Hence, we suggest that there is a functionally important Mg^{2+} , most likely in the hexahydrated state, positioned in the deep groove at the cleavage site. Based on this model it is apparent that hexahydrated Mg^{2+} is positioned close to the cleavage site (≈ 7 –8 Å) and to the phosphorous centers of +1, –1 and –2 (Figure 6). Assuming that this is the metal ion that is involved in generating the nucleophile (see above) a repositioning has to occur as a result of the interaction with RNase P RNA to ensure cleavage at +1 i.e. positioning of the nucleophile for an in line attack on the +1 phosphorous center. During this

conformational rearrangement, the data suggest that the '73/294 interaction', residue 364 and the exocyclic amine of G₊₁ in the substrate play important roles (see above). At the same time, attack on the phosphorous center at –1 and –2 should be avoided. In the case of cleavage in the presence of Pb^{2+} this is not observed due to that Pb^{2+} is very efficient to activate the neighboring 2'OH to act as a nucleophile while Mg^{2+} is significantly less efficient (2). This rationalizes why cleavage at other positions within the 5' leader under Pb^{2+} /Sr²⁺ conditions even in the presence of M1 RNA was observed.

Concluding remarks

RNase P RNA is the first natural ribozyme demonstrated to show leadzyme-like activity. Hence, our findings raise the question whether other ribozymes can mediate catalysis using Pb^{2+} . In this context we note that Mg^{2+} and Mn^{2+} promote correct splicing while Mn^{2+} also induce cleavage at an additional site in the Group I intron. However, Pb^{2+} does not promote splicing but induce cleavage at the additional site due to that Pb^{2+} do not promote correct folding (46,47). In view of our present findings it would be of interest to test whether Pb^{2+} in combination with other divalent metal ions e.g. Sr²⁺ can promote Group I intron splicing. To the best of our knowledge this has not been tested.

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