Published online October 11, 2004

5418–5429 *Nucleic Acids Research, 2004, Vol. 32, No. 18*
doi:10.1093/nar/gkh883

Cross talk between the +73/294 interaction and the cleavage site in RNase P RNA mediated cleavage

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Received June 28, 2004; Revised September 9, 2004; Accepted September 21, 2004

ABSTRACT

To monitor functionally important metal ions and possible cross talk in RNase P RNA mediated cleavage we studied cleavage of substrates, where the 2′OH at the RNase P cleavage site (at −1) and/or at position +73 had been replaced with a 2′ amino group (or 2′H). Our data showed that the presence of 2′ modifications at these positions affected cleavage site recognition, ground state binding of substrate and/or rate of cleavage. Cleavage of 2′ amino substituted substrates at different pH showed that substitution of Mg^{2+} by Mn^{2+} (or Ca^{2+}), identity of residues at and near the cleavage site, and addition of C5 protein influenced the frequency of miscleavage at −1 (cleavage at the correct site is referred to as +1). From this we infer that these findings point at effects mediated by protonation/deprotonation of the 2′ amino group, i.e. an altered charge distribution, at the site of cleavage. Moreover, our data suggested that the structural architecture of the interaction between the 3′ end of the substrate and RNase P RNA influence the charge distribution at the cleavage site as well as the rate of cleavage under conditions where the chemistry is suggested to be rate limiting. Thus, these data provide evidence for cross talk between the +73/294 interaction and the cleavage site in RNase P RNA mediated cleavage. We discuss the role metal ions might play in this cross talk and the likelihood that at least one functionally important metal ion is positioned in the vicinity of, and use the 2′OH at the cleavage site as an inner or outer sphere ligand.

INTRODUCTION

Catalytic RNAs or ribozymes catalyze a large number of different reactions including cleavage of other RNA molecules, RNA polymerization and peptide bond formation. Ribozymes can be divided into classes based on size and reaction products. Large ribozymes such as RNase P RNA, Group I and Group II introns catalyze reactions that generate 3′ hydroxyls and 5′ phosphates as cleavage products. The hairpin, hammerhead and hepatitis delta virus RNA are referred to as small ribozymes and yield 2′, 3′-cyclic phosphates and 5′ hydroxyls upon cleavage of other RNA molecules [(1) and references therein].

RNase P RNA is a naturally occurring *trans*-acting ribozyme and is the catalytic subunit of RNase P (2), an endoribonuclease responsible for generating the 5′ end of mature tRNA molecules (3). In bacteria, RNase P consists of two subunits, RNase P RNA and the C5 protein, in a 1:1 ratio (4,5). The protein moiety of RNase P is essential for activity *in vivo* whereas *in vitro*, RNase P RNA cleaves various substrates correctly and efficiently in the absence of C5 protein (2). As with other large ribozymes, RNase P RNA requires the presence of divalent metal ions for activity. Considering both correctness/accuracy and efficiency of cleavage, Mg^{2+} is the preferred divalent metal ion [e.g. see (6) and references therein]. The roles of Mg^{2+} in RNase P RNA mediated cleavage are to promote correct folding, facilitate substrate binding and participate in the chemistry of cleavage [(3,7,8) and references therein].

To understand RNA catalysis in general, an important task is to identify Mg^{2+} ions that have specific functions and/or metal-ion-binding sites that are unique for a specific functional trait. Phosphorothioate modifications of the non-bridging oxygens at the RNase P cleavage site and analysis of the resulting substrates in the presence of hard (Mg^{2+}) and soft (Mn^{2+} and Cd^{2+}) metal ions suggest that Mg^{2+} coordinates to the *R*p-oxygen via inner sphere interaction (9–11). Substitution of the 2′OH at the cleavage site (at −1) results in significant reduction in the rate of cleavage under single turnover conditions, and available data have been interpreted to indicate that the absence of the 2′ hydroxyl influences metal ion binding in the vicinity of the cleavage site (12–16). Recently we suggested that Mg^{2+} is positioned in the vicinity of and stabilizes the '73/294-interaction', where the '73/294-interaction' is part of the 'RCCA-RNase P RNA interaction' [interacting residues underlined (15,17)]. We argued that this Mg^{2+} cross talk with the Mg^{2+} ion(s) involved in generating the nucleophile (6,18,19). To investigate the role of divalent metal ions in RNase P RNA mediated cleavage we decided to revisit the importance of the 2′OH at the −1 position and study cleavage of various model substrates carrying 2′ amino modifications at −1. The idea was to use the metal ion switch approach that has successfully been used to reveal functionally important metal ions in the Group I ribozyme system (20–23). This approach is based on the observation that the 2′ amino group is a better ligand for Mn^{2+} compared to Mg^{2+}. Thus, monitoring changes in RNase P RNA cleavage site recognition, substrate binding and/or activity due to the

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presence of a 2′ amino group at −1 in the substrate in combination with metal ion substitution is therefore a rational approach to study the role of the 2′OH for metal ion binding in cleavage promoted by RNase P RNA. We will refer to the 2′ amino group as 2′N throughout this study.

Here we have used this approach and *Escherichia coli* RNase P RNA (M1 RNA) to investigate the role of the 2′OH at the cleavage site. Based on our findings we suggest that the 2′OH is involved in coordination (inner or outer sphere) of a functionally important metal ion. In addition, we used the 2′N at the cleavage site as a probe to monitor the possibility of cross talk in RNase P RNA mediated cleavage. Here, our data suggested that the structural architecture of the +73/294 interaction influences the charge distribution at the cleavage site (i.e. ionization state of the 2′N when present at the cleavage site) as well as the rate of cleavage under conditions where the chemistry is suggested to be rate limiting. These findings provide evidence for cross talk between the +73/294 interaction and the cleavage site in the reaction catalyzed by RNase P RNA. These data will be discussed in the perspective of the role metal ions in cleavage of RNA by RNase P RNA and for RNA based activities in general.

MATERIALS AND METHODS

Preparation of substrates and M1 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 (18,19,24). The M1 RNA variants were generated as run-off transcripts using T7 DNA-dependent RNA polymerase (24–26).

Binding assay conditions

Spin columns were used to determine apparent equilibrium dissociation constants (appK_d) for different model pATSer substrate derivatives in: 50 mM Mes at pH 6.0 at 37°C (or 50 mM Hepes at pH 7.2), 0.8 M NH_4OAc, 0.05% (w/v) NP-40, 0.1% (w/v) SDS and 40 mM $CaCl_2$ as described elsewhere (10,27) except that the time of preincubation was 20 min and time after mixing substrate with M1 RNA was 20 min. The substrate concentration was <10 nM and M1 RNA concentration was varied from 0.01 to 22.1 μM (concentration range dependent on substrate). The appK_d values were determined by non-linear regression analysis using Origin 7.0 software (Originlab) and the equation $f_c = f_t \times [\text{M1 RNA}]_{free}/(K_d + [\text{M1 RNA}]_{free})$, where f_c is the fraction of pATSer in complex with M1 RNA and f_t is the maximum fraction of pATSer able to bind M1 RNA, i.e. endpoint.

Cleavage assay conditions

M1 RNA activity was monitored under single turnover conditions in buffer A: 50 mM Tris–HCl (pH 7.2), 5% (w/v) PEG 6000, 100 mM NH_4Cl and 40 mM $MgCl_2$, $MnCl_2$ or $CaCl_2$ (6) or in buffer B: 50 mM Bis-Tris Propane (pH 5.5, 6.1, 6.5, 6.9, 7.2, 8.1 and 8.6), 5% (w/v) PEG 6000, 100 mM NH_4Cl and 40 mM $MgCl_2$, $MnCl_2$ or $CaCl_2$ (with respect to cleavage in the presence of 160 mM $MgCl_2$ see also legend to Figure 2C). The pH was adjusted with HCl to 5.5, 6.1, 6.5, 6.9, 7.2, 8.1 or 8.6. The given pH values were measured at 37°C with all components added, except M1 RNA and substrate. The concentrations of M1 RNA and substrates were ≥0.24 μM and ≤0.05 μM, respectively. All reactions were performed at 37°C and the reaction products were separated on denaturing 20–22% (w/v) polyacrylamide gels and cleavage was quantified on a Phosphorimager (Molecular Dynamics 400S) as described elsewhere [(19) and references therein].

Cleavage by reconstituted RNase P was performed in the presence of excess of C5 protein as described elsewhere (28). The concentration of M1 RNA was 0.0024–0.0032 μM and substrate was ≤0.05 μM. In these experiments the following buffers (pH was adjusted with HCl) were used: 50 mM Tris–HCl, pH 7.3, 7.6, 7.9, 8.2; 50 mM MES buffer pH 5.8, 6.1, 6.4, 6.7, 7.0; 50 mM Bis-Tris Propane, pH 6.6, 7.1, 8.2, 9.1. The given pH values were determined as described above and the reactions were performed in the presence of 11 or 20 mM $MgCl_2$ as indicated (see legend to Figure 2D).

Determination of the kinetic constant k_{obs} under single turn over conditions

The kinetic constant k_{obs} was determined at pH 6.1 and 7.2 as described elsewhere (26,29) in buffer B in the presence of 40 mM Mg^{2+} (see above). The final concentration of substrate was 40 nM while for M1 RNA the concentration was varied between 0.040 μM and 6.1 μM. For the calculations we used the 5′ cleavage fragments and the time of cleavage for each substrate was adjusted to ensure that we were in the linear part of the curve of kinetics. The k_{obs} values were obtained by linear regression from Eadie–Hofstee plots.

To investigate linear dependence between cleavage rate and pH the conditions were adjusted to ensure single turnover and cleavage rates were determined at different pH and plotted as a function of pH.

Determination of the site of cleavage

The frequency of cleavage at different positions was quantified from the relative amounts of 5′ cleavage products generated from cleavage at the different positions essentially as described elsewhere (30). These numbers were subsequently used to calculate the percentage of miscleavage at −1.

RESULTS

A 2′N at the RNase P RNA cleavage site influences cleavage site recognition

To probe for divalent metal ion(s) positioned at the cleavage site, the 2′OH immediately upstream of the cleavage site, i.e. at the −1 position, was substituted with a 2′N (Figure 1). These modified substrates are derivatives of the model substrate pATSer, which is a well-characterized RNase P substrate that is not rate-limited by product release [(6,18,19,24, 26,30); data not shown]. The residue preceding the 3′ CCA-motif in the model substrates corresponds to position +73 in tRNA precursors and will therefore be referred to as position +73. As a model system, we used M1 RNA in the absence of the C5 protein unless stated otherwise. First, we compared cleavage by M1 RNA of pATSerUA and pATSerU_{am}A, where the latter carried 2′N at −1. Note that replacement of

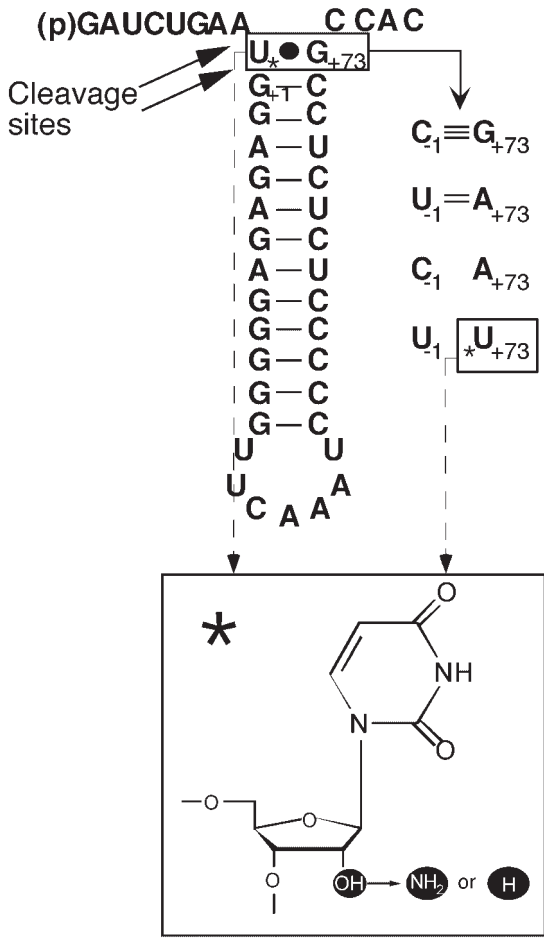

Figure 1. The predicted secondary structure of the model substrate pATSerUG is depicted. The substitutions that resulted in the pATSer variants are indicated and the stars (*) indicate where the 2′N (or 2′H) modifications were introduced. The residue immediately preceding the 3′ CCA motif is referred to as residue +73 since it corresponds to residue +73 in precursor tRNA. The arrows indicate cleavage sites and the inset shows where the 2′N (or 2′H) was inserted.

the 2′OH at −1 with 2′N does not result in any apparent change in the overall structure as revealed by Pb^{2+} cleavage analysis (M. Brännvall and E. Kikovska, unpublished data). The experiments described below were conducted under single turnover conditions except for cleavage in the presence of C5 protein (see Materials and Methods).

As shown in Figure 2A, in the Mg^{2+} promoted reaction the all-ribo substrate was cleaved only at the correct position +1 while cleavage of pATSerU_{am}A at both −1 and +1 was observed. Thus, replacement of the 2′OH with a 2′N at −1 influenced cleavage site recognition under our standard assay conditions i.e. buffer A at pH 7.2. The correct cleavage site is between residues −1 and +1 whereas miscleavage occurs between −1 and −2. Hence, we will refer to cleavage at these positions as cleavage at +1 and at −1, respectively (Figure 1).

Our previous data suggest that residues −1 and +73 (Figure 1) are important for cleavage efficiency and site recognition (6,18,19,30,31). We therefore decided to investigate the influence of these residues in the context of cleavage of substrates with 2′N at position −1. As shown in Figure 2B (data not shown for pATSerC_{am}A), irrespective of identity of the residue at −1 and/or +73 (except pATSerC_{am}G, which was only cleaved at −1 under the same assay conditions at pH 7.2) cleavage at both position −1 and +1 in the Mg^{2+} alone reaction was observed. These findings are in contrast to the cleavage pattern of the corresponding all-ribo substrates (except pATSerCG), which were cleaved only at the correct position +1. The pATSerCG was cleaved with a low frequency at −1 as previously reported [(30); data not shown]. Thus, the observed miscleavage at −1 due to the presence of 2′N does not appear to depend on the identity of the residue at −1 and/or +73. Note that miscleavage of pATSerU_{am}U was observed in particular at low pH (see below).

Changing the metal ion, i.e. replacing Mg^{2+} with Mn^{2+}, resulted in a reduced frequency of −1 miscleavage and increased cleavage at +1 compared with the Mg^{2+} alone reaction (Figure 2A shows the data only for the cleavage of pATSerU_{am}A; see also below). In contrast, substitution of Mg^{2+} with Ca^{2+} resulted in a significant shift of the cleavage site such that cleavage mainly occurred at the −1 position (Figure 2A). Switching the metal ion gave no miscleavage of the all-ribo substrate, pATSerUA, in keeping with our previous data (19). This indicated that switching to the softer metal ion Mn^{2+} suppressed miscleavage of the substrate carrying an amino group at the 2′ position. We emphasize that we previously have identified Mn^{2+} as an inducer of miscleavage using several different all-ribo pATSer derivatives [e.g. see (30)].

The influence of protonation of the 2′N at the RNase P RNA cleavage site on cleavage site recognition

The pK_a value of the 2′N of dinucleotides has been determined by NMR spectroscopy to be 6.0–6.2 (32,33). In cleavage by the Group I ribozyme, the data suggest that the 2′OH at the cleavage site is involved in stabilizing the oxyanion leaving group. This was based on the finding that the rate of cleavage of a substrate carrying a 2′N was stimulated below pH 6.5 relative to cleavage of a substrate with a 2′OH at the cleavage site and that a protonated amine is a good H-donor (23). To investigate whether the frequency of cleavage at +1 versus −1 changed due to protonation of the 2′N at −1 we studied cleavage of pATSerU_{am}G at different pH values. Among unmodified pATSer substrate derivatives, pATSerUG is the most efficiently cleaved substrate in the M1 RNA alone reaction (19). Moreover, the pK_a values for U and G are >9 while for C and A the corresponding values are close to 4 (34). Thus, in the experiments where we studied cleavage as a function of pH, we increased the likelihood that we monitored changes at the 2′N at −1 by using pATSerU_{am}G and pATSerUG as substrates. We also included pATSerdUG (Figure 1) to investigate whether the presence of a 2′H at −1 changed the miscleavage pattern in the pH range used here.

As shown in Figure 3, a decrease in pH resulted in an increased frequency of cleavage at −1 relative to +1. It is also apparent that cleavage at −1 diminished with increasing pH (Figure 2B). Control experiments in which we studied cleavage of the all-ribo substrate, pATSerUG, showed no shift of the cleavage site at 40 mM Mg^{2+} as a function of pH (Figure 2A). The cleavage pattern of pATSerU_{am}G at different pH was essentially unchanged when the Mg^{2+} concentration was increased to 160 mM (Figure 2, see also

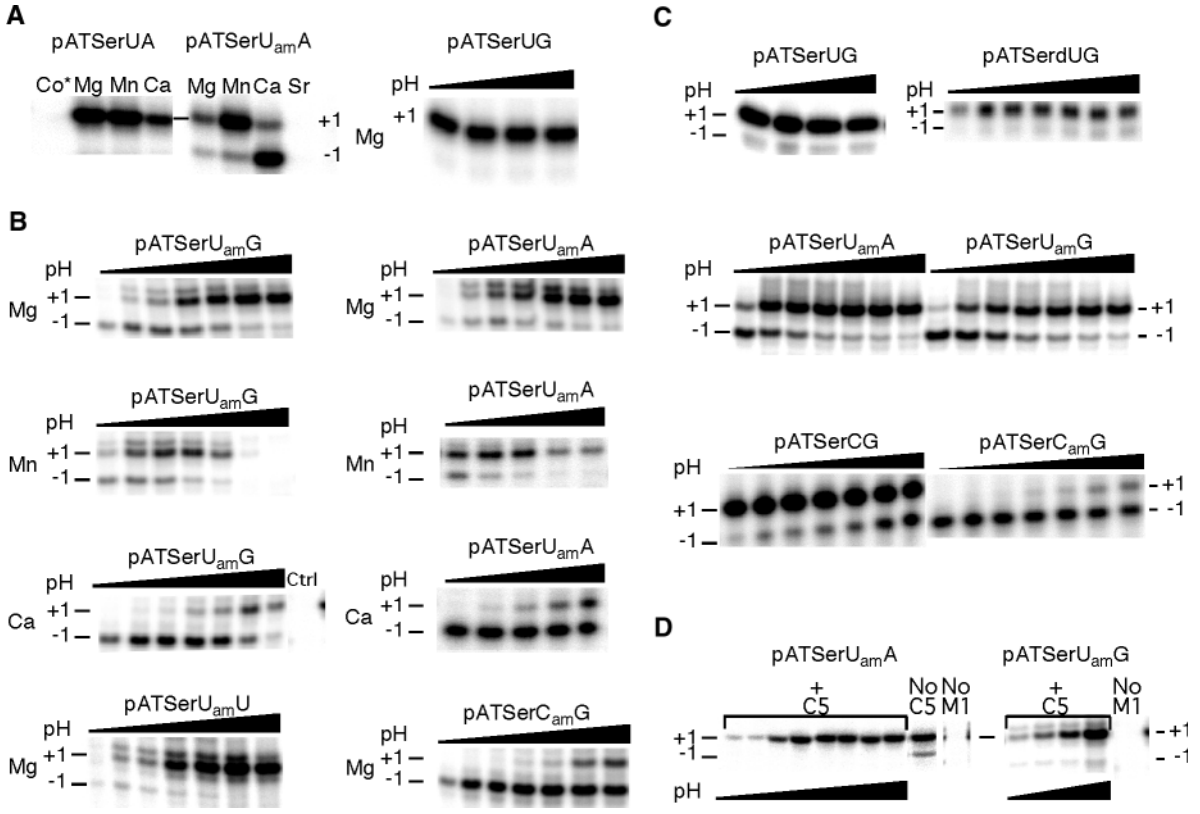

Figure 2. Cleavage site recognition of different pATSer model substrates under different conditions as indicated. [γ-^{32}P]ATP 5′ end-labeled substrates were cleaved and only the 5′ cleavage fragments are shown. The cleavage sites are indicated as +1 and −1. (**A**) Cleavage of pATSerUA and pATSerU$_{am}$A in the presence of different divalent metal ions as indicated under our standard assay conditions at pH 7.2 (for pATSerUG, cleavage was performed at pH 5.5, 6.5, 7.2 and 8.6). The concentrations of M1 RNA and substrates were 0.24 and ≤0.05 μM, respectively. As controls, substrate and M1 RNA were incubated for 180 min in the presence of $Co(NH_3)_6^{3+}$ and Sr^{2+} [neither of these alone can promote cleavage (6)]. Time of incubation for cleavage of pATSerUA was 8 min in the presence of Mg^{2+} and Mn^{2+} and 180 min in the presence of Ca^{2+}; pATSerU$_{am}$A was 180 min irrespective of metal ion and; pATSerUG was 40 min (note in the presence of 40 mM Mg^{2+}). (**B**) Cleavage of pATSerU$_{am}$G, pATSerU$_{am}$A, pATSerU$_{am}$U and pATSerC$_{am}$G in the presence of different divalent metal ions and different pH as indicated (increasing pH from left to right). The experiments were performed in 50 mM Bis-Tris Propane buffer at pH 5.5, 6.1, 6.5, 6.9, 7.2, 8.1 and 8.6 in the presence of 40 mM divalent metal ion Mg^{2+}, Mn^{2+} or Ca^{2+}. Cleavage of pATSerU$_{am}$A in the presence of Mn^{2+} or Ca^{2+} was performed at pH 5.6, 6.4, 6.8, 7.1 and 7.4, respectively. The concentrations of M1 RNA and substrates were 0.16 and ≤0.05 μM, respectively (in the case of cleavage of pATSerU$_{am}$A, 0.19 μM and 0.08 μM, respectively). Time of incubation in the presence of: Mg^{2+} = 260 min; Mn^{2+} = 285 min; Ca^{2+} = 262 min. For pATSerU$_{am}$A, the incubation time in the presence of Mn^{2+} and Ca^{2+} was 180 min. Ctrl = control in the presence of Ca^{2+} with no M1 RNA added. (**C**) Cleavage of pATSerUG, pATSerdUG, pATSerU$_{am}$A, pATSerU$_{am}$G, pATSerCG and pATSerC$_{am}$G in the presence of 160 mM Mg^{2+} at different pH as indicated (increasing pH from left to right). Same buffer conditions (50 mM Bis-Tris Propane, 5% (w/v) PEG 6000, 100 mM NH_4Cl and 160 mM $MgCl_2$) and pH as described in Figure 2B were used for cleavage of these substrates except pATSerUG, which was cleaved at pH 5.0, 6.1, 7.3 and 8.5 with the same buffer conditions. The concentrations of M1 RNA and substrates were 0.19 and 0.08 μM, respectively (in cleavage of pATSerUG, 0.04 μM substrate and 1.31 μM M1 RNA, while for cleavage of pATSerdUG, pATSerCG and pATSerC$_{am}$G 0.02 μM substrate and 1.02 μM M1 RNA were used). Time of incubation for cleavage of: pATSerUG, 15 min; pATSerdUG, 150 min; pATSerU$_{am}$A and pATSerU$_{am}$G, 265 min; pATSerCG, 50 min; pATSerC$_{am}$G, 150 min. In the experiments using pATSerU$_{am}$A and pATSerU$_{am}$G, the reactions were terminated by the addition of 99% ethanol. The precipitates were resolved in loading buffer as described elsewhere (23). (**D**) Cleavage of pATSerU$_{am}$A and pATSerU$_{am}$G in the presence of the C5 protein at different pH as indicated (increasing pH from left to right). Cleavage of pATSerU$_{am}$A was performed in the presence of 50 mM MES and 50 mM Tris–HCl buffers (see Materials and Methods) at 20 mM Mg^{2+} while cleavage of pATSerU$_{am}$G was performed in 50 mM Bis-Tris Propane buffer at pH 6.6, 7.1, 8.2 and 9.1 and 10 mM Mg^{2+}. The concentrations of M1 RNA and substrates were 0.0024 and ≤0.05 μM, respectively, irrespective of substrate. The C5 protein was added in excess as previously described (28). Time of incubation for cleavage of pATSerU$_{am}$A, and pATSerU$_{am}$G was 210 and 227 min, respectively. The controls were: no C5 protein, 0.24 μM M1 RNA incubated with ≤0.05 μM pATSerU$_{am}$A for 210 min; no M1 RNA, substrate incubated at 40 mM Mg^{2+} in the absence of M1 RNA and C5 protein for 210 and 227 min, respectively.

Table 1). However, an increase in Mg^{2+} concentration to 160 mM resulted in a low frequency of miscleavage at −1 using pATSerUG that did not change under single turnover conditions in the pH range 5.0–8.6 (Figures 2C and 3). Moreover, as shown in Figure 2C and Figure 3 pATSerdUG (with 2′H at −1) was cleaved both at +1 and −1 at 160 mM Mg^{2+}. However, note that the frequency of cleavage at −1 increased rather than decreased, as was observed for the 2′N substituted substrate, with increasing pH. Taken together, these findings provided support for the idea that we were monitoring protonation of the 2′N at −1 by changing pH and therefore also the presence/absence of a positive charge at the cleavage site. Furthermore, this suggested to us that the 2′N at −1 could be used as a tool to probe for factors influencing the charge distribution at the cleavage site.

Plotting relative frequency of cleavage at −1 as a function of increasing pH gave a pH-value of 6.6 that corresponds to the pH where we observed equal frequency of cleavage at +1 and −1. We will refer to this pH value as pH^{50} (see Table 1). Cleavage of the other substrates with substitutions at −1

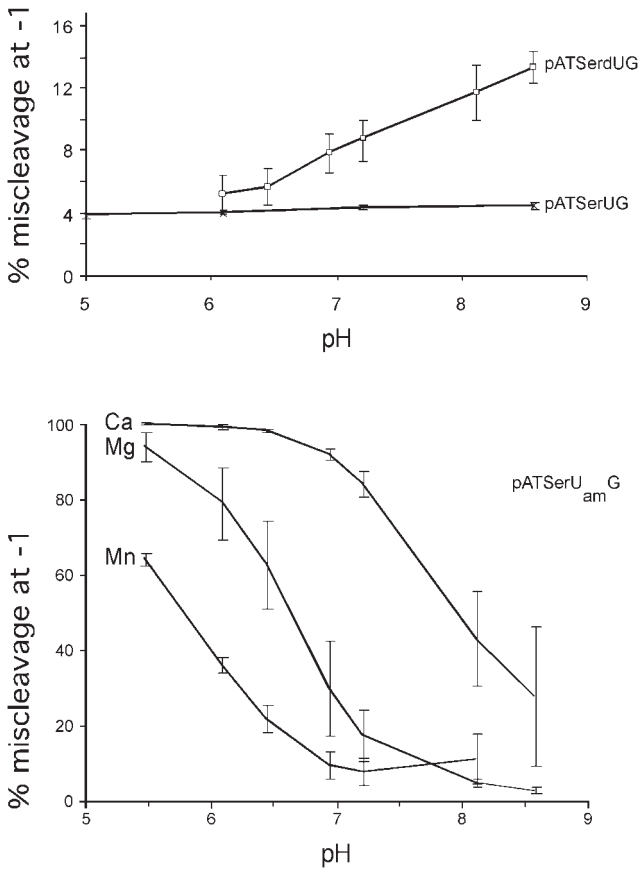

Figure 3. Frequency of cleavage at −1 of pATSerUG, pATSerdUG and pATSerU_{am}G as a function of pH in the presence of different divalent metal ions as indicated. The curves are averages of several independent experiments and the bars indicate experimental errors. The experiments were performed at 37°C as outlined in Materials and Methods. Data shown for pATSerUG and pATSerdUG were taken from experiments performed at 160 mM Mg^{2+}.

Table 1. Summary of pH^{50} values[a] for cleavage of different substrates in the presence of various divalent metal ions as indicated

Substrate	M1 RNA	pH^{50}_{Mg}	pH^{50}_{Mn}	pH^{50}_{Ca}
−1/+'73'				
U_{am}•G	Wild type	6.6 ± 0.18	5.8 ± 0.03	8.1 ± 0.41
U_{am}–A	Wild type	6.4 ± 0.3	<5.6[b]	7.2 ± 0.1
C_{am} A	Wild type	6.5 ± 0.4	nd	7.4 ± 0.2
C_{am}–G	Wild type	>8.6[b]	nd	nd
U_{am} U	Wild type	<6[b]	nd	nd
U_{am} U	A294	6.9 ± 0.2	nd	8.1 ± 0.1
U_{am} U	G294	6.5 ± 0.05	nd	7.7 ± 0.1
U_{am}•G (160 mM)	Wild type	6.5 ± 0.2	nd	nd
U_{am}–A (160 mM)	Wild type	5.6 ± 0.1	nd	nd

nd, not determined.
[a]Each pH^{50} value refers to the pH at which equal frequency of cleavage at −1 and +1 was observed; for details see text. The given values are averages of several independent experiments performed at 37°C as outlined in Materials and Methods.
[b]Given numbers are approximate since pH^{50} values could not be accurately determined.

and/or +73 as a function of pH revealed similar results for cleavage of pATSerU_{am}G, pATSerU_{am}A and pATSerC_{am}A, i.e. pH^{50} values were approximately the same (Table 1). This is in contrast to the study by Hartmann and coworkers (35) who observed a difference in pH^{50} value as a result of substituting the residue U at −1 with a C. The reason for this discrepancy could be differences in reaction conditions and/or substrate, i.e. a hairpin model versus a tRNA precursor substrate. The pH^{50} value for cleavage of pATSerU_{am}U was estimated to be <6 while for pATSerC_{am}G we only observed a modest cleavage at +1 at higher pH and we were unable to determine a pH^{50} value for this substrate (Figure 2B and Table 1). However, note that the all-ribo substrate pATSerCG was cleaved with an increased frequency at −1 with increasing pH (Figure 2C). Therefore it is possible that the observed high pH^{50} value in cleavage of pATSerC_{am}G is due to an increased miscleavage at higher pH and/or that the C_{-1} residue in pATSerC_{am}G being base paired with G_{+73} [Figure 1, see also (30)]. A shift of the cleavage site with increasing pH is in keeping with earlier observations (36).

Given that we were monitoring protonation of the 2′N at −1, we conclude that the presence of a protonated amino group at the correct cleavage site influences cleavage by RNase P RNA negatively. Moreover the identity of residue −1 does not appear to affect protonation/deprotonation of the 2′N at −1 with the exception of when C_{-1} is engaged in base pairing with G_{+73} in the substrate. For those substrates with C and/or A at −1 and +73, respectively, we cannot conclusively exclude that protonation/deprotonation of C and/or A affected the result (see above).

Metal ion substitution or addition of the RNase P protein influences cleavage of the 2′ amino substituted substrate by changing pH^{50}

Cleavage in the presence of the softer metal ion Mn^{2+} suppressed (while Ca^{2+} accentuated) miscleavage of the substrate carrying an amino group at the 2′ position (see above). Hence, we decided to study the cleavage pattern of pATSerU_{am}G as a function of pH in the presence of Mn^{2+} and Ca^{2+}. With Mn^{2+}, we noted to our surprise that equal frequency of cleavage at +1 and −1 was observed at a lower pH relative to cleavage in the Mg^{2+} alone reaction. We estimated pH^{50} to be 5.8 for Mn^{2+}. In contrast, a higher estimated pH^{50} value of 8.1 was observed when cleavage was performed in the presence of Ca^{2+} (Figures 2B and 3; Table 1). Approximately similar shifts of the pH^{50} values in cleavage of the other 2′ amino substituted substrates (Figure 1) were observed when cleavage was studied with increasing pH in the presence of Mn^{2+} or Ca^{2+} (Figure 2B, Table 1, and data not shown). In the control experiments, cleavage of the all-ribo substrates at different pH in the presence of Mn^{2+} or Ca^{2+} showed no change of the cleavage site (data not shown). The differences in the observed pH^{50} values in the presence of Mn^{2+}, Mg^{2+} and Ca^{2+} correlate with the difference in pK_a for these metal ions 10.6, 11.4 and 12.7, respectively (37). Given that the estimated pH^{50} values reflect pK_a for protonation of the 2′N at −1, our data suggest that metal ion substitution can perturb the pK_a value for a chemical group, in our case 2′N. These data also indicated that the observed suppression (see above) of miscleavage by Mn^{2+} is due to a decreased pH^{50} value.

The RNase P protein, C5, has been suggested to interact with residues in the 5′ leader of the substrate (38). This, together with our findings, prompted us to investigate cleavage of the 2′ amino substituted substrates in the presence of C5

Table 2. K_D values for different pATSer derivatives and M1 RNA variants in the presence of Ca^{2+} at pH 6.0 and 7.2 as indicated

Substrate	M1 RNA	pH	K_D (μM)
pATSerUG	Wild type	6.0	0.091 ± 0.04
pATSerU_{am}G	Wild type	6.0 (7.2)	1.5 ± 0.2 (0.85 ± 0.2)
pATSerdUG	Wild type	6.0	2.4 ± 0.84
pATSerUG	C294	6.0	0.12 ± 0.015
pATSerU_{am}G	C294	6.0	1.5 ± 0.2
pATSerUU	Wild type	6.0	0.12 ± 0.005
pATSerUU$_{am}$	Wild type	6.0	0.31 ± 0.05
pATSerUU	G294	6.0	0.67 ± 0.084
pATSerUU$_{am}$	G294	6.0	0.13 ± 0.01

nd, not determined.
The experiments were performed as described in Materials and Methods. Each value is an average of several independent experiments.

Table 3. Cleavage efficiencies expressed as k_{obs} in the presence of Mg^{2+} at pH 6.1 and 7.2 as indicated

Substrate	M1 RNA		pH 6.1 k_{obs} (min^{-1})	pH 7.2 k_{obs} (min^{-1})
pATSerUG	wt	−1	nc	nc
		+1	1.2 ± 0.2	3.2 ± 0.2
pATSerU_{am}G	wt	−1	0.0012 ± 0.0001	0.0021 ± 0.00012
		+1	0.001 ± 0.00004	0.024 ± 0.0031
pATSerdUG	wt	−1	nd	nd
		+1	0.0007 ± 0.00014	0.011 ± 0.0022
pATSerUU	wt	−1	nc	nc
		+1	0.41 ± 0.07	nd
pATSerUU$_{am}$	wt	−1	nc	nc
		+1	0.18 ± 0.06	nd
pATSerUU	G294	−1	nc	nc
		+1	0.29 ± 0.05	nd
pATSerUU$_{am}$	G294	−1	nc	nc
		+1	0.82 ± 0.18	nd

nd, not determined; nc, no cleavage detected; wt, wild type (U294); −1 and +1 refer to the cleavage sites.
The experiments were performed as described in Materials and Methods. Each value is an average of several independent experiments.

protein at different pH. Surprisingly, as shown in Figure 2D (cleavage of pATSerU_{am}A and pATSerU_{am}G), we only observed cleavage of these substrates at the correct position +1 irrespective of pH. M1 RNA cleaved the all-ribo substrate both in the presence and in the absence of C5 protein only at +1 (data not shown). Moreover, the other 2′ amino substituted substrates (pATSerC_{am}A, pATSerU_{am}U and pATSerC_{am}G) were also cleaved mainly at the correct position +1 in the presence of the RNase P protein C5 (data not shown). Taken together, replacement of the 2′OH with a 2′N revealed a difference in the cleavage pattern comparing cleavage by M1 RNA and the reconstituted holoenzyme, irrespective of the identity of residues at −1 and/or +73 under the conditions tested.

A 2′N (or 2′H) at −1 affects ground state binding and the rate of cleavage

To investigate whether replacement of the 2′OH with 2′N (or 2′H) at −1 influenced the interaction with M1 RNA, we determined the apparent binding constant, appK_d, for pATSerUG, pATSerU_{am}G and pATSerATdUG at pH 6.0 using a spin-column assay (see Materials and Methods). From the data presented in Table 2 it is evident that M1 RNA bound the all-ribo substrate significantly (17-fold) better compared to the 2′N substituted substrate. Moreover, a raise in pH to 7.2, where the 2′N is mainly in the deprotonated state, only resulted in a slight improvement in binding of pATSerU_{am}G. For the 2′H substituted substrate we also detected a significant change in appK_d of the same order of magnitude as for the substrate with 2′N. We conclude that the presence of the 2′N (or 2′H) at the cleavage site affected ground state binding of the hairpin model substrate emphasizing the importance of a 2′OH at this position in keeping with previous data (see Introduction).

To study the influence of the 2′N (or 2′H) on the rate of cleavage at the cleavage site we determined the rate constant, k_{obs}, under saturating single turnover conditions in the presence of Mg^{2+} at pH 6.1 and 7.2. Cleavage of pATSerUG at different pH showed linear dependence of k_{obs} on $[OH^-]$ between pH 5.5 and 6.5 indicating that the chemistry is rate limiting in this pH range. In contrast, the rate of cleavage showed linear dependence on $[OH^-]$ up to pH >8.1 in cleavage of the 2′N and 2′H substituted substrates, pATSerU_{am}G and pATSerdUG, respectively (data not shown). These findings are in keeping with previous reports (9,14,16,30,35). As shown in Table 3, the 2′ amino as well as the 2′H modification resulted in a dramatic drop (1200-fold) in k_{obs} at pH 6.1. Based on the discussion above, a significant fraction of the 2′N is most likely in the protonated state at pH 6.1. We therefore determined k_{obs} at pH 7.2 where the majority of the 2′N is in the deprotonated state. Here the difference in k_{obs} was 133-fold for cleavage of the all-ribo and 2′N modified substrates while only a 2-fold difference was observed for cleavage of the 2′N and 2′H substituted substrates. The chemistry is not rate limited for cleavage of pATSerUG at pH 7.2 and therefore this difference might be larger that 133- and 290-fold, respectively. These findings are in keeping with kinetic measurements (using the 2′N substituted substrate) performed under multiple turnover conditions where a decrease in k_{cat} was observed with decreasing pH (M. Brännvall and E. Kikovska, unpublished data). Taken together, the presence of 2′N (or 2′H) at the cleavage site influenced the rate of cleavage significantly, in addition to the effect on ground state binding, indicating the importance of the 2′OH at −1 for the chemistry of cleavage of hairpin model substrates.

Cross talk between the 73/294-interaction and the cleavage site

We recently suggested that the structural architecture of the +73/294 interaction in M1 RNA substrate (RS-) complexes affects the binding of a functionally important metal ion in its vicinity. Moreover, we argued that this metal ion is involved in cross talk with metal ion(s) positioned in the vicinity of the cleavage site (see Introduction). Hence, based on this and together with our findings discussed above, we decided to use the 2′N at the cleavage site as a probe to investigate whether the structural architecture of the +73/294 interaction influences protonation/deprotonation of the 2′N (i.e. charge distribution) and thereby obtain information about possible cross talk between the +73/294 interaction and the cleavage site. The M1 RNA variants we used carried substitutions at position 294 in M1 RNA (U to C or G). For $M1_{C294}$ RNA this

will give a G_{+73}/C_{294} interaction (for wild-type G_{+73}/U_{294}) in cleavage of pATSerUG derivatives while in the case of cleavage of pATSerUU derivatives with $M1_{G294}$ RNA we will have a U_{+73}/G_{294} interaction (see Figure 4).

First, we studied cleavage of pATSerU_{am}G by $M1_{C294}$ RNA that harbored a U to C substitution at 294. This M1 RNA variant was determined to be 2- to 3-fold reduction in the rate of cleavage of pATSerUG and pATSerU_{am}G under single turnover conditions relative to wild type (data not shown). Also, $M1_{C294}$ RNA binds the substrate with similar appK_d as wild type (Table 2). Moreover, cleavage of pATSerUG using this M1 RNA variant showed linear dependence of the rate of cleavage on [OH^-] between pH 5.5 and 6.5 suggesting that the chemistry is rate limiting at pH < 6.5 (data not shown). As shown in Figure 5, cleavage by $M1_{C294}$ RNA resulted in significantly higher frequency of miscleavage at −1 at pH 6.1 compared to wild type indicating a higher pH^{50} value for $M1_{C294}$ RNA relative to wild type.

Next, we compared the cleavage patterns of pATSerU_{am}U variants using wild-type M1 RNA and $M1_{G294}$ RNA. As demonstrated above, wild type cleaved pATSerU_{am}U with an estimated pH^{50} value <6 (Table 1), while the pH^{50} value for cleavage of the same substrate with the G294 variant was increased (Table 1 and Figure 5). Furthermore, previous data suggest that the 2′OH at +73 is involved in metal ion binding (19). Therefore, we also used substrates where the 2′OH at position +73 had been replaced with 2′N, pATSerUU_{am} and pATSer$U_{am}U_{am}$, where the latter carried a 2′N modification both at −1 and at +73 (Figure 1). These substrates were cleaved at pH 6.1. At this pH we observed increased miscleavage at −1 for pATSer$U_{am}U_{am}$ using wild-type M1 RNA compared to cleavage of pATSerU_{am}U (2′N at −1 and 2′OH at +73). In contrast, for $M1_{G294}$ RNA a decrease in miscleavage using pATSer$U_{am}U_{am}$ was detected compared to cleavage using pATSerU_{am}U. No miscleavage of pATSerUU_{am} harboring a 2′N only at +73 was observed irrespective of M1 RNA, wild type or G294 (data not shown). These findings suggested that changes in the structural architecture of the +73/294 interaction (including the 2′OH at +73) influence protonation/deprotonation of the 2′N at the cleavage site.

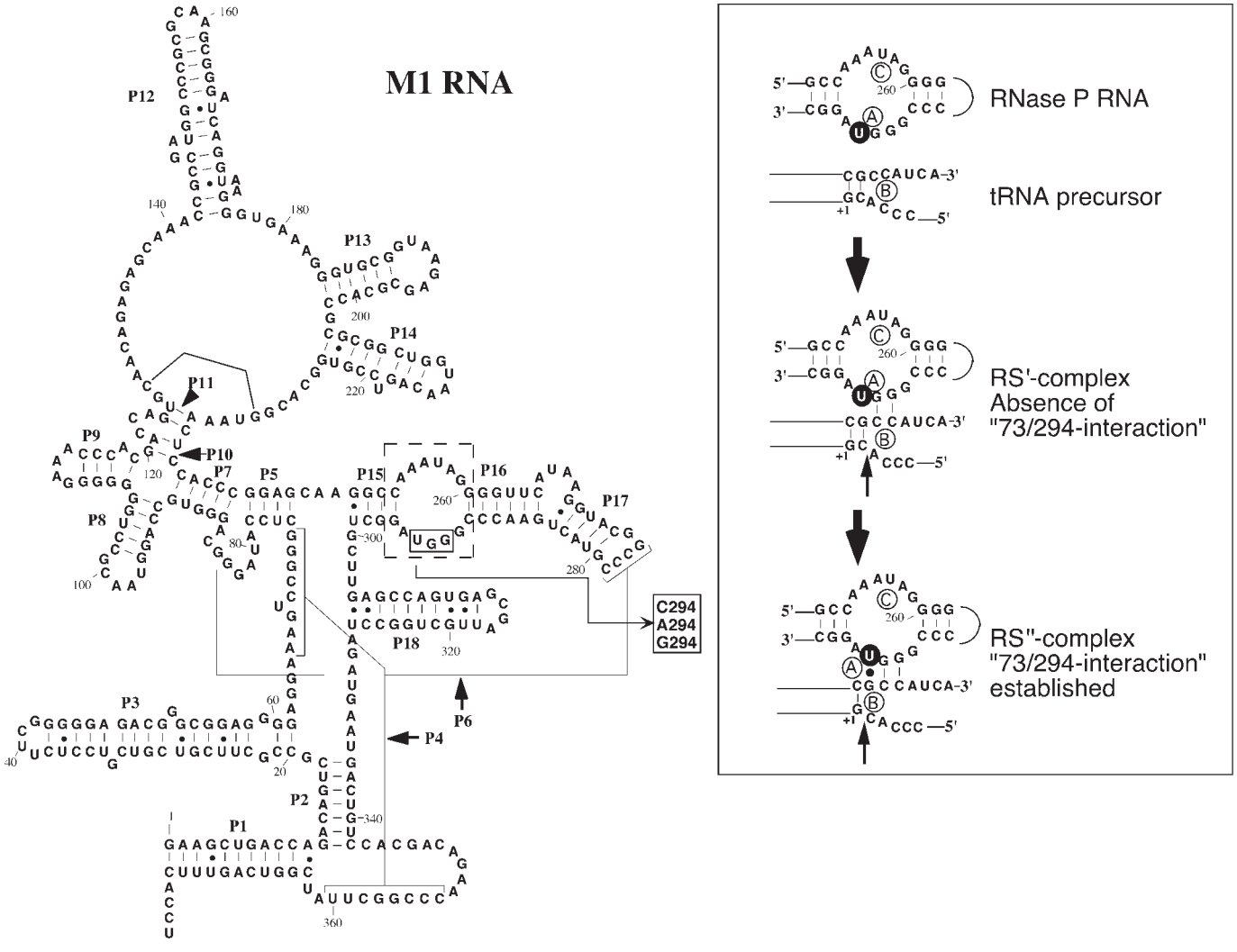

Figure 4. The predicted secondary structure of *E.coli* RNase P RNA (M1 RNA) according to Haas and Brown (63). The broken-line box represents the P15-loop while the boxed GGU-motif represents the residues that base pair with the 3′ RCCA motif of the substrate – the RCCA-RNase P RNA interaction – as illustrated in the inset. The M1 RNA variants C294, A294 and G294 used in this study are indicated. The inset illustrates a model of the RCCA-RNase P RNA interaction. Here A,B and C (encircled) represent divalent metal ions—for details see text and (19). The U at position 294 is highlighted in black.

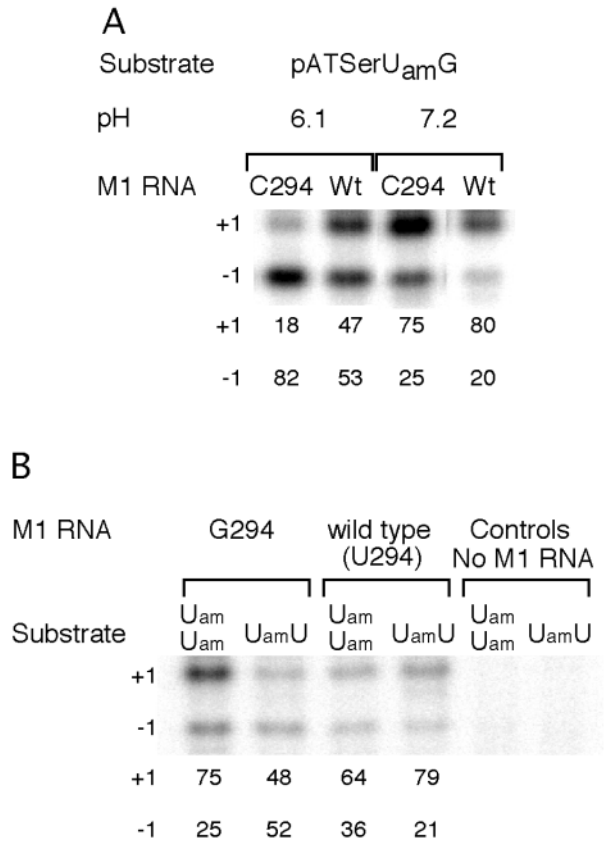

Figure 5. Cleavage as a function of the structural architecture of the +73/294 interaction. The experiments were conducted in buffer B in the presence of 40 mM at indicated pH under single turnover conditions (see Materials and Methods). Given numbers refer to the frequency of cleavage at +1 and −1. The controls represent incubation of indicated substrates in buffer B and 40 mM Mg^{2+} in the absence of M1 RNA. (**A**) Cleavage of $pATSerU_{am}G$ with wild type and $M1_{C294}$ RNA. (**B**) Cleavage of $pATSerU_{am}U$ and $pATSerU_{am}U_{am}$ with wild type and $M1_{G294}$ RNA.

Finally, we asked whether a change in the structural architecture of the +73/294 interaction (including the 2′OH at +73) had an impact on the rate of cleavage for substrates with 2′OH at the cleavage site. Hence, we determined the rate constants (k_{obs}) for cleavage of pATSerUU and $pATSerUU_{am}$ for wild type and $M1_{G294}$ RNA under saturating single turnover conditions at pH 6.1. Cleavage of pATSerUU as well as $pATSerUU_{am}$, with wild type or $M1_{G294}$ RNA, showed a linear dependence of the rate of cleavage on [OH^-] up to pH 6.5 suggesting that the chemistry is rate limiting at pH 6.1 in keeping with the discussion above (data not shown). As shown in Table 3, substitution of the 2′OH with 2′N at +73 gave a modest (2-fold) decrease in k_{obs} for wild type while for the G294 variant an almost 3-fold increase was observed. This experiment was followed by determination of appK_d values for the same combinations (Table 2). Here we observed a slightly weaker binding (∼2.5-fold) for wild type due to the presence of the 2′N at +73 whereas the opposite was the case for $M1_{G294}$ RNA, i.e. ∼5-fold stronger binding. Thus, weaker binding correlated with decreased k_{obs}, in particular when comparing cleavage of pATSerUU and $pATSerUU_{am}$ with wild type or G294, respectively. We conclude that the structural architecture of the +73/294 interaction influences catalysis and ground state binding. Taken together, these data provide evidences for cross talk between the +73/294 interaction and the cleavage site.

DISCUSSION

Involvement of the 2′OH at the cleavage site in metal ion binding

The main consequence of replacing the 2′OH at −1 is a significant reduction in the rate of cleavage [this report; (12–15,16,35,39)]. But replacement of the 2′OH with 2′N at the cleavage site for RNase P RNA also resulted in cleavage both at the correct position, +1, and at −1. However, the frequency of miscleavage at −1 changed with decreasing pH. In contrast cleavage of a 2′H modified substrate resulted in a low and reproducible frequency of miscleavage at −1 that increased with increasing pH, while cleavage of the corresponding all-ribo substrate at −1 did not change within this pH range (this report). The 2′H cannot be protonated and it is very unlikely that the 2′OH is protonated within this pH range. The pK_a value for a 2′N in the context of dinucleotides is 6.0–6.2 as determined by NMR spectroscopy (32,33). This is close to the pH^{50} values (∼6.5) for the cleavage, in the presence of Mg^{2+} of some of the substrates used in this report (Table 1). Moreover, the pH^{50} value shifted as a result of replacing Mg^{2+} with Mn^{2+} or Ca^{2+} such that the shift correlated with the difference in pK_a for these metal ions. No apparent change in the conformation of M1 RNA was observed substituting Mg^{2+} by Mn^{2+} or Ca^{2+} (40). We also note that addition of Hg^{2+} to DNA can result in deprotonation of the N3 of thymidine such that the equilibrium of binding is pH-dependent (41). Taken together, we conclude that the reduction of cleavage at the correct position with lowering pH is most likely due to protonation of the 2′N at −1 and that the presence of a protonated amine, i.e. a positive charge, at the correct cleavage site influences cleavage by M1 RNA negatively. Moreover, together with previous data [see above and also (42)], we suggest that the 2′OH at the cleavage site is involved in metal ion coordination either as inner or outer sphere ligand. An alternative reason to the observed shift of cleavage site for the 2′N substituted substrates with increased pH would be differences in the rate-limiting steps for cleavage at +1 and −1. For example, one possibility is that the rate-limiting step for cleavage at +1 and −1 is the chemistry and binding, respectively. This would favor cleavage at +1 with increasing pH. However, K_m for cleavage of pATSer derivatives is approximately the same irrespective of the cleavage site [(30); M. Brännvall and E. Kikovska, unpublished data] and cleavage of pATSerdUG at −1 increased with increasing pH (this report). We therefore consider this possibility less likely and favor the conclusion that protonation of the 2′N at −1 is the reason for the observed shift of the cleavage site with decreasing pH.

Our conclusion is in keeping with the recent work by Hartmann and coworkers in which they used full-size precursor tRNA variants with 2′ modifications at −1 (35). However, this study complements their study emphasizing the importance of metal ion identity and the use of another type of substrate. Note that RNase P interacts and cleaves a large number of substrates with different structures, e.g. precursors to tRNA and 4.5S RNA, where the latter has a hairpin-like structure, similar to the model substrate used in the present

study [reviewed in (3)]. Based on this, it is important to study cleavage under various conditions using different types of substrates in order to elucidate the cleavage mechanism and importance of various chemical groups in RNase P RNA mediated cleavage. Moreover, our data and the data of Persson *et al*. (35) are in contrast to the Group I ribozyme system where the presence of a protonated 2′N at the cleavage site makes a positive contribution to the cleavage reaction (23).

Possible role of the metal ion coordinated at the cleavage site

For the hammerhead ribozyme it has been suggested that a metal ion bound in the vicinity of the 2′OH at the cleavage site functions either as a general base or as a Lewis acid, thereby activating the nucleophile (43,44). The cleavage products in the hammerhead cleavage reaction as well as in the metal ion induced cleavage of RNA are 5′ hydroxyls and 2′,3′-cyclic phosphates (37) while cleavage by RNase P RNA generates a 5′ phosphate and a 3′ OH (2). Therefore, a metal ion acting as a general base, i.e. interacting with the 2′OH at −1 at the RNase P RNA cleavage site via an outer sphere interaction could result in a nucleophilic attack on the phosphate and 'incorrect' cleavage products, i.e. a 5′ OH and a 2′,3′-cyclic phosphate. In fact, metal ions cleave precursor tRNA and model substrates at and close to the cleavage site in the absence of M1 RNA (12,40,45). Moreover, the (*pro*)-Rp oxygen is a ligand for Mg^{2+} at the cleavage site (9–11). This would make the phosphorous more electrophilic and vulnerable for a nucleophilic attack. The strategy for RNase P RNA must therefore be to prevent the 2′OH at −1 (when coordinated to a divalent metal ion as we suggest here) from acting as a nucleophile and favor an attack on the phosphorous from the other side of the phosphate to ensure 'correct' cleavage products, i.e. a 5′ phosphate and a 3′ OH. This strategy is also relevant to the other two large ribozymes, Group I and Group II, since both these ribozymes give the same cleavage products as RNase P RNA. For the Group I ribozyme it has been conclusively demonstrated that the proton of the 2′ OH is shared between the 2′ and the 3′ oxygen in the transition state (23). In addition, the 2′ OH is engaged in hydrogen bonding with the 2′ OH of residue A207 [e.g. see (46)]. For the Group II ribozyme it has been suggested that a metal ion is directly coordinated to both the 3′ oxygen and the 2′OH in the transition state (47). To conclude, it is therefore conceivable that a metal ion is coordinated in a similar way in the cleavage reaction mediated by RNase P RNA as in the Group II ribozyme system (Figure 6). Possibly the positioning of this metal ion prevents the 2′OH from acting as a nucleophile in addition to its plausible involvement in stabilization of the (3′) oxyanion in the transition state. Alternatively, the Mg^{2+} could be coordinated via inner sphere interactions both to the 2′OH and the (*pro*)-Rp oxygen. This type of Mg^{2+} binding was hypothesized to exist in the Group I system but was rejected (23). In this situation, we envision that there is an increased probability that the 2′OH will act as a nucleophile and attack the phosphorous center (see above), arguing against this alternative. Irrespective of the alternative there is also the possibility that N7 and/or O6 of the +1 G residue is involved in Mg^{2+} binding. At present we do not exclude other alternatives (35).

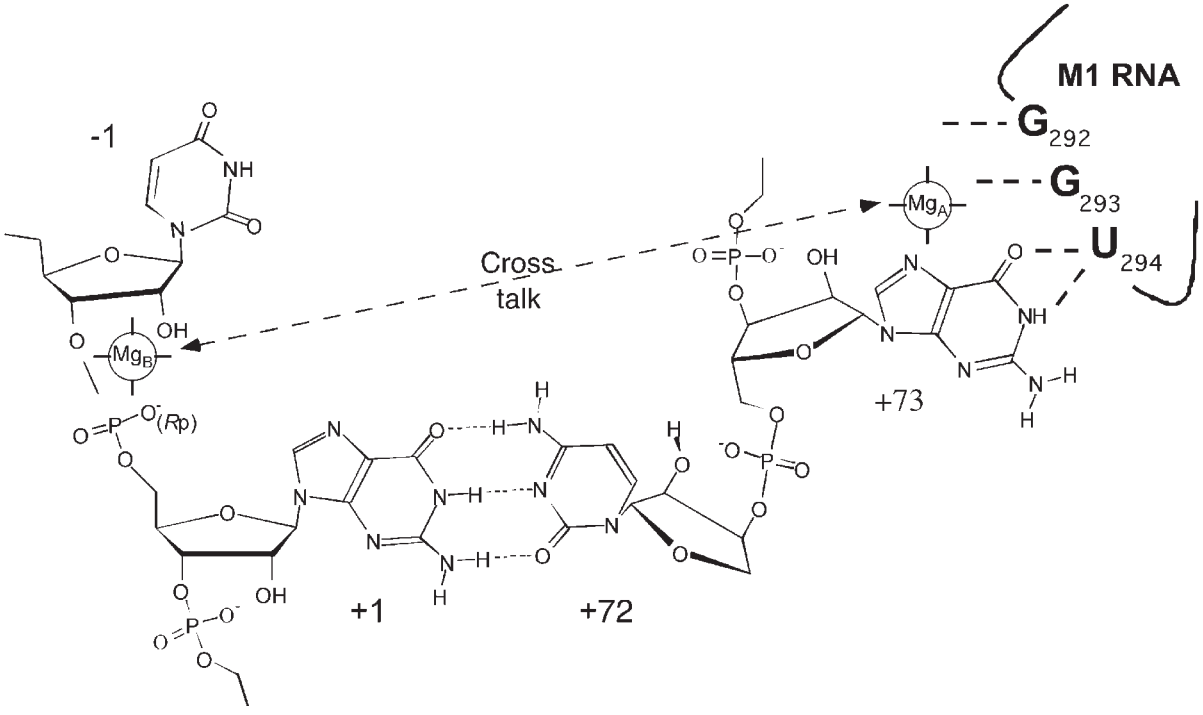

Figure 6. Model of the cleavage site where the upper part illustrates the −1, +1, +72 and +73 in the substrate as indicated. The RCCA-RNase P RNA interaction between M1 RNA and the 3′ end of the substrate is shown in dashed lines (see Figure 4). The dashed line with double arrow heads indicate cross talk between Mg^{2+}, positioned in the vicinity of the interaction between residues +73 in the substrate and U294 in M1 RNA (metal ion A in Figure 4), and the cleavage site including $Mg_B{}^{2+}$ (metal ion B in Figure 4). As outlined in the text, at present we cannot distinguish whether the 2′OH is engaged in an inner or outer sphere interaction with $Mg_B{}^{2+}$.

It has been suggested that the 2′OH at −1 mediate an interaction with RNase P RNA and that this interaction plays a role for positioning the scissile bond in the active site (16). Recent data suggest that the residue at the −1 position interacts with A248 in M1 RNA via Watson–Crick base pairing (48). As discussed in these studies the 2′OH at −1 plays an essential role and probably act in concert with the interaction between residues −1 and 248. Apart from ensuring cleavage at the correct site, the function of these interactions might be to prevent the 2′OH to act as a nucleophile. Thus, it is not excluded that replacing the 2′OH at −1 with a 2′N (or 2′H) would affect the −1/248 interaction and/or the proposed interaction between the 2′OH and M1 RNA. Given that Mg^{2+} is positioned in the vicinity of the 2′OH at −1 makes it therefore plausible that Mg^{2+} plays an active role in establishing these interactions.

Cross talk in M1 RNA mediated cleavage

We have suggested that to ensure correct and efficient cleavage the +73/294 interaction operates in concert with displacement of residue −1 [(6,17,19,30) and references therein]. The +73/294 interaction is part of the 'RNase P RNA-RCCA' interaction (Figure 4). Disruption of the 'RNase P RNA-RCCA' interaction, i.e. the base pairing between C_{+75} and G_{292} in M1 RNA, resulted in miscleavage at −1 of a substrate with a 2′ deoxy substitution at −1 while cleavage still occurred mainly at the correct site when the corresponding all-ribo substrate was cleaved (35). Moreover, base substitution of the −1 residue affects both the rate of cleavage and cleavage site recognition [(17,19,30,48,49) and references therein]. These data are in keeping with the fact that at least one determinant for cleavage site recognition has been disrupted/changed (50). Here, we demonstrated that changes in the structural architecture of the +73/294 interaction affected the charge distribution at the cleavage site (ionization state of the 2′N at −1), ground state binding and the rate of cleavage under conditions where the chemistry is suggested to be rate limiting (pH 6.1). Thus, the present findings extend and increase our understanding of the function of the 'RNase P RNA–RCCA' interaction by providing evidence for cross talk between the +73/294 interaction and the cleavage site. Moreover, the pH^{50} value in cleavage of $pATSerU_{am}A$ by wild-type M1 RNA decreased when the Mg^{2+} concentration was increased to 160 mM while for $pATSerU_{am}G$ it remained essentially unchanged (Table 1 and Figure 2C). This is in keeping with the fact that the structural architecture of the +73/294 interaction, including the 2′OH at +73, in the M1 RNA substrate complex influence metal in coordination in its vicinity (19). It is conceivable that this cross talk influences the charge distribution and/or Mg^{2+} coordination at the cleavage site such that substrates with different +73 base identities (resulting in a different structural architecture of the +73/294 interaction) are cleaved with different efficiencies (19). Furthermore, it is also plausible that the interaction between M1 RNA and the −1 residue (including the 2′OH) actually depend on the formation of the +73/294 interaction (see also above). This would be consistent with the proposal that cleavage at −1 does not depend on the +73/294 interaction (30). We envision that Mg^{2+} ions (or other metal ions when present) might play important/essential roles in mediating this cross talk where Mg_A is proposed to stabilize the +73/294 interaction [Figure 6 and (6)]. In this context, we note that in the revised mechanism for alkaline phosphatase, Mg^{2+} is not directly coordinating the serine nucleophile but there is a correlation with Mg^{2+} binding and the conformation of this serine that affects deprotonation of the serine nucleophile (51). This suggests that Mg^{2+} can indeed influence catalysis without being directly bound to the active site. Moreover, the data from Harris and coworkers suggested the existence of a polynuclear binding site in the vicinity of the P4 helix (52). We do not exclude the possibility that these metal ions are positioned close to Mg_A and/or Mg_B, however, whether this is the case or not remains to be seen.

Influence of the RNase P protein C5

Surprisingly, addition of the RNase P protein C5 resulted in cleavage of the 2′N substrates almost exclusively at the correct site +1 irrespective of pH. This could indicate that the presence of the C5 protein influences the chemical environment either by allowing the presence of a positive charge (protonated amino group) at the 2′ of the −1 residue or following the discussion above by perturbing pK_a for the 2′N. Cross-linking experiments suggests that the C5 protein is located in close proximity to substrate residues −3, −4 and −5 in the 5′ leader (38). Therefore it is plausible that interaction between these residues and the C5 protein affects the environment at the cleavage site perhaps by mediating cross talk along the 5′ leader of the RNA. Recent data actually suggest cross talk from the first to the sixth nucleotide residue in an RNA hexamer that results in changes in the local electronic environment along the RNA chain (53). Also, based on the structure of the 50S ribosomal subunit it has been suggested that a buried phosphate (between A2450 and A2451) might result in a perturbed pK_a of A2451, which is positioned in the peptidyl transferase center (54). Moreover, data suggest that the C5 protein enhances Mg^{2+} affinity of the pre-tRNA RNase P RNA complex indirectly in the *Bacillus subtilis* system (55). Therefore Mg^{2+} might be involved in mediating cross talk along the 5′ leader. Another possibility is that the positioning of the C5 protein near residues −3 to −5 in the 5′ leader results in a more rigid complex than when the C5 protein is absent, and this rigidity prevents cleavage at alternative sites in the 5′ leader, i.e. at −1 [for a recent three-dimensional model of the RNase P holoenzyme in complex with its substrate (56)]. In this context, we note that the presence of a 2′H at −1 in a similar hairpin model substrate (as used here) resulted in a significant reduction in cleavage in the RNA alone reaction and that addition of the C5 protein suppressed this reduction in cleavage efficiency (12). Obviously, further experiments are required to explain our findings. But, nevertheless our data stresses the importance of the C5 protein in the cleavage site recognition process [e.g. see (57,58)].

Metal ions and regulation of RNA based activities

Available structural and biochemical data suggest that pK_a values for specific chemical groups can be perturbed and that this perturbation is of biological importance. For example in the peptidyl transferase center on the 50S ribosomal subunit, the pK_a for N1 (or N3) of residue A2451 (*E.coli* numbering) has been suggested to be 7.6, which is ∼4 pH units higher than

the pK_a for N1 (or N3) of the nucleotide base (34,54,59). For protein-based activities it is clear that binding of different metal ions to, for example, the active site affects activity e.g. specificity and processivity [reviewed in (60)]. Also, it is known that addition of Ca^{2+} inhibits charging of $tRNA^{Ala}$ by mammalian and *E.coli* alanyl-tRNA-synthetase [(61), L. A. Kirsebom, unpublished data] and RNase P RNA mediated cleavage [e.g. see (6)]. Recently we raised the possibility that the intracellular concentrations of the bulk biological ions Mg^{2+} and Ca^{2+} influence the activity of biocatalysts that depend on RNA for activity (6). From structural studies it is evident that different metal ions such as Mg^{2+}, Mn^{2+} and Ca^{2+} can bind to RNA if not to the same at least to overlapping sites [e.g. see (37,62)]. Therefore based on our present findings it is conceivable that binding of different metal ions e.g. Mg^{2+}, Ca^{2+} or Mn^{2+}, to RNA results in different degrees of perturbation of pK_a values for specific chemical groups. As a consequence this could give changes in activity such that the activity is either up- or downregulated and/or result in stabilization/destabilization of interaction with other factors.

ACKNOWLEDGEMENTS

In particular, we thank Dr R. K. Hartmann for fruitful discussions, collaboration and exchange of data. Drs J. Chattopadhyaya, S. Dasgupta and A. Virtanen are acknowledged for fruitful discussions and critical reading of the manuscript. We acknowledge constructive criticism by the anonymous reviewers. This work was supported by grants from the Swedish Natural Research Council and the Foundation for Strategic Research Nucleic Acid Strategic Network (to L.A.K.).

REFERENCES

1. Doudna,J.A. and Cech,T.R. (2002) The chemical repertoire of natural ribozymes. *Nature*, **418**, 222–228.
2. Guerrier-Takada,C., Gardiner,K., Marsh,T., Pace,N. and Altman,S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell*, **35**, 849–857.
3. Altman,S. and Kirsebom,L.A. (1999) Ribonuclease P. In Gesteland,R.F., Cech,T.R. and Atkins,J.F. (eds.), *The RNA World, 2nd edn*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 351–380.
4. Vioque,A., Arnez,J. and Altman,S. (1988) Protein-RNA interactions in the RNase P holoenzyme from *Escherichia coli*. *J. Mol. Biol*., **202**, 835–848.
5. Fang,X.W., Yang,X.J, Littrell,K., Niranjanakumari,S., Thiyagarajan,P., Fierke,C.A., Sosnick,T.R. and Pan,T. (2001) The *Bacillus subtilis* RNase P holoenzyme contains tow RNase P RNA and two RNase P protein subunits. *RNA*, **7**, 233–241.
6. Brännvall,M. and Kirsebom,L.A. (2001) Metal ion cooperativity in ribozyme cleavage of RNA. *Proc. Natl Acad. Sci. USA*, **98**, 12943–12947.
7. Frank,D.N. and Pace,N.R. (1998) Ribonuclease P: unity and diversity in a tRNA processing ribozyme. *Annu. Rev. Biochem*., **67**, 152–180.
8. Kirsebom,L.A. (1999) The structure and function of the ribozyme RNase P RNA is dictated by magnesium(II) ions. In Barciszewski,J. and Clark,B.F.C. (eds.), *RNA Biochemistry and Biotechnology*. NATO ASI Series, Kluwer Academic Publishers, pp. 89–109.
9. Warnecke,J.M., Fürste,J.P., Hardt,W.-D., Erdmann,V.A. and Hartmann,R.K. (1996) 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. *Proc. Natl Acad. Sci. USA*, **93**, 8924–8928.
10. Warnecke,J.M., Held,R., Busch,S. and Hartmann,R.K. (1999) Role of metal ions in the hydrolysis reaction catalyzed by RNase P RNA from *Bacillus subtilis*. *J. Mol. Biol*., **290**, 433–445.
11. Chen,Y., Li,X. and Gegenheimer,P. (1997) Ribonuclease P catalysis requires Mg^{2+} coordinated to the pro-Rp oxygen of the scissile bond. *Biochemistry*, **37**, 2425–2438.
12. Perreault,J-P. and Altman,S. (1992) Important 2′-hydroxyl groups in model substrates for M1 RNA, the catalytic RNA subunit of RNase P from *Escherichia coli*. *J. Mol. Biol*., **226**, 399–409.
13. Perreault,J-P. and Altman,S. (1993) Pathway of activation by magnesium ions of substrates for the catalytic subunit of RNase P RNA from *Escherichia coli*. *J. Mol. Biol*., **230**, 750–756.
14. Smith,D. and Pace,N.R. (1993) Multiple magnesium ions in the ribonuclease P reaction mechanism. *Biochemistry*, **32**, 5273–5281.
15. Kufel,J. and Kirsebom,L.A. (1996) Different cleavage sites are aligned differently in the active site of M1 RNA, the catalytic subunit of *Escherichia coli* RNase P. *Proc. Natl Acad. Sci. USA*, **93**, 6085–6090.
16. Loria,A. and Pan,T. (1998) Recognition of the 5′ leader and the acceptor stem of a pre-tRNA substrate by the ribozyme from *Bacillus subtilis* RNase P. *Biochemistry*, **37**, 10126–10133.
17. Kirsebom,L.A. and Svärd,S.G. (1994) Base pairing between *Escherichia coli* RNase P RNA and its substrate. *EMBO J*., **13**, 4870–4876.
18. Brännvall,M., Pettersson,B.M.F. and Kirsebom,L.A. (2002) The residue immediately upstream of the RNase P cleavage site is a positive determinant. *Biochimie*, **84**, 693–703.
19. Brännvall,M., Pettersson,B.M.F. and Kirsebom,L.A. (2003) Importance of the +73/294 interaction in *Escherichia coli* RNase P RNA substrate complexes for cleavage and metal ion coordination. *J. Mol. Biol*., **325**, 697–709.
20. Sjögren,A.-S., Pettersson,E., Sjöberg,B.-M. and Strömberg,R. (1997) Metal ion interaction with cosubstrate in self-splicing of group I introns. *Nucleic Acids Res*., **25**, 648–653.
21. Shan,S., Narlikar,G.J. and Herschlag,D. (1999) Protonated 2′-aminoguanosine as a probe of the electrostatic environment of the active site of the Tetrahymena group I ribozyme. *Biochemistry*, **38**, 10976–10988.
22. Shan,S. and Herschlag,D. (1999) Probing the role of a metal ion in RNA catalysis: kinetic and thermodynamic characterization of a metal ion interaction with the 2′-moiety of the guanosine nucleophile in the Tetrahymena group I ribozyme. *Biochemistry*, **38**, 10958–10976.
23. Yoshida,A., Shan,S., Herschlag,D. and Piccirilli,J.A. (2000) The role of the cleavage site 2′-hydroxyl in the Tetrahymena group I ribozyme reaction. *Chem. Biol*., **7**, 85–96.
24. Kufel,J. and Kirsebom,L.A. (1998) The P15-loop of *Escherichia coli* RNase P RNA is an autonomous divalent metal ion binding domain. *RNA*, **4**, 777–788.
25. Milligan,J.F., Groebe,D.R., Whiterell,G.W. and Uhlenbeck,O.C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and DNA templates. *Nucleic Acids Res*., **15**, 8783–8798.
26. Brännvall,M., Mattsson,J.G., Svärd,S.G. and Kirsebom,L.A. (1998) RNase P RNA structure and cleavage reflect the primary structure of tRNA genes. *J. Mol. Biol*., **283**, 771–783.
27. Beebe,J.A. and Fierke,C.A. (1994) A kinetic mechanism for cleavage of precursor $tRNA^{Asp}$ catalyzed by the RNA component of *Bacillus subtilis* ribonuclease P. *Biochemistry*, **33**, 10294–10304.
28. Svärd,S.G., Kagardt,U. and Kirsebom,L.A. (1996) Phylogenetic comparative mutational analysis of the base-pairing between RNase P RNA and its substrate. *RNA*, **2**, 463–472.
29. Tallsjö,A. and Kirsebom,L.A. (1993) Product release is a rate-limiting step during cleavage by the catalytic RNA subunit of *Escherichia coli* RNase P. *Nucleic Acids Res*., **21**, 51–57.
30. Brännvall,M. and Kirsebom,L.A. (1999) Manganese ions induce miscleavage in the *Escherichia coli* RNase P RNA-catalyzed reaction. *J. Mol. Biol*., **292**, 53–63.
31. Mans,R.M.W., Guerrier-Takada,C., Altman,S. and Pleij,C.W.A. (1990) Interaction of RNase P from *Escherichia coli* with pseudoknotted structures in viral RNAs. *Nucleic Acids Res*., **18**, 3479–3487.
32. Miller,P.S., Bhan,P. and Kan,L-S. (1993) Synthesis and interactions of oligodeoxyribonucleotides containing 2′-amino-2′-deoxyuridine. *Nucleosides Nucleotides*, **12**, 785–792.
33. Aurup,H., Tuschl,T., Benseler,F., Ludwig,J. and Eckstein,F. (1994) Oligonucleotide duplexes containing 2′-amino-2′-deoxycytidines: thermal stability and chemical reactivity. *Nucleic Acids Res*., **22**, 20–24.
34. Saenger,W. (1984) *Principles of Nucleic Acid Structure*. Springer-Verlag, NY.

35. Persson,T., Cuzic,S., Siedler,S. and Hartmann,R.K. (2003) Catalysis by RNase P RNA: unique features and unprecedented active site plasticity. *J. Biol. Chem.*, **278**, 43394–43401.
36. Kufel,J. and Kirsebom,L.A. (1994). Cleavage site selection by M1 RNA, the catalytic subunit of *Escherichia coli* RNase P is influenced by pH. *J. Mol. Biol.*, **244**, 511–521.
37. Feig,A. and Uhlenbeck,O.C. (1999) The role of metal ions in RNA biochemistry. In Gesteland,R.F., Cech,T.R. and Atkins,J.F. (eds) *The RNA World, 2nd Edn*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 287–319.
38. Niranjanakumari,S., Stams,T., Crary,S.M., Christianson,D.W. and Fierke,C.A. (1998) Protein component of the ribozyme ribonuclease P alters substrate recognition by directly contacting precursor tRNA. *Proc. Natl Acad. Sci. USA*, **95**, 15212–15217.
39. Forster,A.C. and Altman,S. (1990) External guide sequences for an RNA enzyme. *Science*, **249**, 783–786.
40. Brännvall,M., Mikkelsen,N.E. and Kirsebom,L.A. (2001). Monitoring the structure of *Escherichia coli* RNase P RNA in the presence of various metal ion. *Nucleic Acids Res.*, **29**, 1426–1432.
41. Kosturko,L.D., Folzer,C. and Stewart,R.F. (1974) The crystal and molecular structure of a 2:1 complex of 1-methylthymine-mercury(II). *Biochemistry*, **19**, 3949–3951.
42. Guerrier-Takada,C., Haydock,K., Allen,L. and Altman,S. (1986) Metal ion requirements and other aspects of the reaction catalyzed by M1 RNA, the RNA subunit of ribonuclease P from *Escherichia coli*. *Biochemistry*, **25**, 1509–1515.
43. Dahm,S.C., Derrick,W.B. and Uhlenbeck,O.C. (1993) Evidence for the role of solvated metal ion hydroxide in the hammerhead cleavage mechanism. *Biochemistry*, **32**, 13040–13045.
44. Pontius,B.W., Lott,W.B. and von Hippel,P.H. (1997) Observations on catalysis by hammerhead ribozymes are consistent with a two-metal-ion mechanism. *Proc. Natl Acad. Sci. USA*, **94**, 2290–2294.
45. Kazakov,S. and Altman,S. (1991) Site-specific cleavage by metal ion cofactors and inhibitors of M1 RNA, the catalytic subunit of RNase P from *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **88**, 9193–9197.
46. Szewczak,A.A., Kosek,A.B., Piccirilli,J.A. and Strobel,S.A. (2002) Identification of an active site ligand for a group I ribozyme catalytic metal ion. *Biochemistry*, **41**, 2516–2525.
47. Gordon,P.E., Sontheimer,E.J. and Piccirilli,J.A. (2000) Kinetic characterization of the second step of group II intron splicing: Role of metal ions and the cleavage site 2′-OH in catalysis. *Biochemistry*, **39**, 12939–12952.
48. Zahler,N.H., Christian,E.L. and Harris,M.E. (2003) Recognition of the 5′ leader of pre-tRNA substrates by the active site of ribonuclease P. *RNA*, **9**, 734–745.
49. Crary,S.M., Niranjanakumari,S. and Fierke,C.A. (1998) The protein component of *Bacillus subtilis* ribonuclease P increases catalytic efficiency by enhancing interactions with the 5′ leader sequence of pre-tRNA^{Asp}. *Biochemistry*, **37**, 9409–9416.
50. Svärd,S.G. and Kirsebom,L.A. (1992) Several regions of a tRNA precursor determine *Escherichia coli* RNase P cleavage site. *J. Mol. Biol.*, **227**, 1019–1031.
51. Stec,B., Holtz,K.M. and Kantrowitz,E.R. (2000) A revised mechanism for the alkaline phosphatase reaction involving three metal ions. *J. Mol. Biol.*, **299**, 1303–1311.
52. Christian,E.L., Kaye,N.M. and Harris,M.E. (2002) Evidence for a polynuclear metal ion binding site in the catalytic domain of ribonuclease P RNA. *EMBO J*, **21**, 2253–2262.
53. Acharya,P., Acharya,S., Cheruku,N.V., Amirkhanov,A., Földesi,A. and Chattopadhyaya,J. (2003) Cross-modulation of the pK_a of nucleobases in a single-stranded hexameric-RNA due to tandem electrostatic nearest-neighbor interactions. *J. Am. Chem. Soc.*, **125**, 9948–9961
54. Nissen,P., Hansen,J., Ban,N., Moore,P.B. and Steitz,T.A. (2000) The structural basis of ribosome activity in peptide bond synthesis. *Science*, **289**, 920–930.
55. Kurz,J.C. and Fierke,C.A. (2002) The affinity of magnesium binding sites in the *Bacillus subtilis* RNase P•Pre-tRNA complex is enhanced by the protein subunit. *Biochemistry*, **41**, 9545–9558.
56. Tsai,H-Y., Masquida,B., Biswas,R., Westhof,E. and Gopalan,V. (2003) Molecular modeling of the three-dimensional structure of the bacterial RNase P holoenzyme. *J. Mol. Biol.*, **325**, 661–675.
57. Krupp,G., Kahle,D. and Vogt,T. (1991) Sequence changes in both flanking sequences of a pre-tRNA influence the cleavage specificity of RNase P. *J. Mol. Biol.*, **217**, 637–648.
58. Svärd,S.G. and Kirsebom,L.A. (1993) Determinants of *Escherichia coli* RNase P cleavage site selection: a detailed *in vitro* and *in vivo* analysis. *Nucleic Acids Res.*, **21**, 427–434.
59. Muth,G.W., Ortoleva-Donnelly,L. and Strobel,S.A. (2000) A single adenosine with a neutral pK_a in the ribosomal peptidyl transferase center. *Science*, **289**, 947–950.
60. Cowan,J.A. (1998) Metal activation of enzymes in nucleic acid biochemistry. *Chem. Rev.*, **98**, 1067–1087.
61. Roy,A.K. (1971) Inhibition of the alanine tRNA aminoacylation by Ca^{2+}. *Biochem. Biophys. Acta*, **246**, 349–352.
62. Jack,A., Ladner,J.E., Rhodes,D., Brown,R.S. and Klug,A. (1977) A crystallographic study of metal-ion binding to yeast phenylalanine transfer tRNA. *J. Mol. Biol.*, **111**, 315–328.
63. Haas,E.S. and Brown,J.W. (1998) Evolutionary variation in bacterial RNase P RNAs. *Nucleic Acids Res.*, **26**, 4093–4099.