Substrate discrimination in RNase P RNA-mediated cleavage: importance of the structural environment of the RNase P cleavage site

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ABSTRACT

Like the translational elongation factor EF-Tu, RNase P interacts with a large number of substrates where RNase P with its RNA subunit generates tRNAs with matured 5' termini by cleaving RNA precursors immediately 5' of the residue at +1, i.e. at the position that corresponds to the first residue in tRNA. Most tRNAs carry a G_{+1}C_{-72} base pair at the end of the aminoacyl acceptor-stem whereas in tRNA^{Gin} G_{+1}C_{-72} is replaced with U_{+1}A_{-72}. Here, we investigated RNase P RNA-mediated cleavage as a function of having G_{+1}C_{-72} versus U_{+1}A_{-72} in various substrate backgrounds, two full-size tRNA precursors (pre-tRNA^{Gin} and pre-tRNA^{Tyr Su3}) and a model RNA hairpin substrate (pATSer). Our data showed that replacement of G_{+1}C_{-72} with U_{+1}A_{-72} influenced ground state binding, cleavage efficiency under multiple and single turnover conditions in a substrate-dependent manner. Interestingly, we observed differences both in ground state binding and rate of cleavage comparing two full-size tRNA precursors, pre-tRNA^{Gin} and pre-tRNA^{Tyr Su3}. These findings provide evidence for substrate discrimination in RNase P RNA-mediated cleavage both at the level of binding, as previously observed for EF-Tu, as well as at the catalytic step. In our experiments where we used model substrate derivatives further indicated the importance of the +1/+72 base pair in substrate discrimination by RNase P RNA. Finally, we provide evidence that the structural architecture influences Mg^{2+} binding, most likely in its vicinity.

INTRODUCTION

The majority of tRNAs in bacteria and eukaryotic cells carry a guanosine at the 5' termini (1). One of the exceptions is tRNA^{Gin} that harbors a U at this position that pairs with an A at position 72, and throughout this study we have inferred that this is the case. The U_{-1}A_{-72} base pair in tRNA^{Gin} is an important determinant in the charging of tRNA^{Gin} [2] and references therein]. In contrast to the various specific aminoacyl tRNA synthetases, elongation factor EF-Tu binds to all elongator aminoacyl-tRNAs. However, based on determination of the equilibrium dissociation constants differences in binding between EF-Tu and different correctly aminoacylated tRNAs have been detected (3–6). Like EF-Tu, the endoribonuclease RNase P interacts with a large number of substrates where tRNA precursor transcripts constitute the majority. The role of RNase P is to generate tRNA molecules with matured 5' termini and as such RNase P is essential and found in all different kingdoms of life (7). Bacterial RNase P consists of the catalytic RNA moiety and a basic protein in a 1:1 ratio (8–10).

Available data suggests that it is the tRNA structure of the precursor that is recognized by RNase P and that the aminoacyl acceptor stem is a major determinant (7). More specifically, base substitutions of the residues at and in the vicinity of the cleavage site affect both cleavage efficiency and cleavage site recognition (7,11–16) indicating their importance for cleavage. The majority of these studies have been conducted using one specific substrate, i.e. different full-size tRNA precursor substrates [7]; see also the more recent Ref (17). However, to date very few studies have analyzed in detail the consequences of base and/or base pair replacement at different positions in different substrates. Consequently, the importance of the identity of, for example, the +1/+72 base pair in different substrate contexts with respect to cleavage rates, substrate binding and cleavage site recognition remain an open question.

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This together with the findings in the EF-Tu system (see above) and the fact that while most tRNAs carry $G_{+1}$, $C_{+2}$ tRNA$^{Gln}$ harbors a $U_{+1}$ $A_{+2}$ base pair encouraged us to investigate RNAse P RNA-mediated cleavage as a function of having $G_{+1}$, $C_{+2}$ versus $U_{+1}$ $A_{+2}$ in various substrate backgrounds, two full-size tRNA precursors (pre-tRNA$^{Gln}$ and pre-tRNA$^{Tyr}$ $Su_3$) and a model RNA hairpin substrate (pATSer). Considering that available data also suggest that the RNAse P protein, the C5 protein, does not interact with the residues at positions $+1$ and $+2$ (18 and references therein), we decided to perform our experiments in the absence of the C5 protein. As model system, we used Escherichia coli RNAse P RNA, M1 RNA.

Our data showed that replacement of $G_{+1}$ $C_{+2}$ with $U_{+1}$ $A_{+2}$ influenced ground state binding, cleavage efficiency under multiple and single turnover (under conditions where chemistry is rate limiting) in a substrate-dependent manner. Interestingly, comparison of M1 RNA-mediated cleavage of ‘wild type’ precursors to tRNA$^{Gln}$ and tRNA$^{Tyr}$ $Su_3$ revealed differences both in ground state binding and rate under single turnover conditions. Moreover, based on our studies using various model RNA hairpin substrate derivatives, we consider it likely that the $G_{+1}$ $C_{+2}$ to $U_{+1}$ $A_{+2}$ influence metal ion binding in the vicinity of the cleavage site and that it is the presence of U at $+1$ which is the main reason to the observed effects. These findings provide evidence for substrate discrimination in RNAse P RNA-mediated cleavage, and indicate the importance of the $+1$/$+2$ base pair in this process. These data will be discussed in relation to the findings in the EF-Tu system.

**MATERIALS AND METHODS**

**Preparation of substrates and M1 RNA**

The various pATSer derivatives were purchased from Dharmaco, USA, purified, labeled at the 5’ end and gel purified according to standard procedures as described elsewhere (12,13,19). The pGln and pSu3 variants and M1 RNA were generated as run-off transcripts using T7 DNA-dependent RNA polymerase (19–21).

**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$_4$Cl] (22) or in buffer B [50 mM Bis-Tris Propane (pH 6.1 or as indicated), 5% (w/v) PEG 6000, 100 mM NH$_4$Cl] in the presence of MgCl$_2$ and/or MnCl$_2$ as indicated. The pH was adjusted with HCl and 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 $\geq 0.24$ and $\leq 0.05$ μM, respectively. All reactions were performed at 37°C and the reaction products were separated on denaturing 20–22% (v/v) polyacrylamide gels and cleavage was quantified on a Phosphorimager (Molecular Dynamics 400S) as described elsewhere [(13 and references therein).

**Determination of the site of cleavage**

The frequency of cleavage at different positions was quantified from the relative amounts of 5’ cleavage products as a result of cleavage at the different positions as described elsewhere (23). In the case of the pGln derivatives, the cleavage sites were also determined by primer extension analysis using an oligonucleotide complementary to positions A31 through C48 in tRNA$^{Gln}$ (see Figure 1).

**Binding assay conditions**

Spin columns were used to determine apparent equilibrium dissociation constants (appKd) in Buffer C: 50 mM MES at pH 6.0 at 37°C, 0.8 M NH$_4$OAc, 0.05% (w/v) Nonidet P40, 0.1% (w/v) SDS in the presence of various concentrations of CaCl$_2$ as described elsewhere (24,25) except that preincubation was 20 min and the 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 13.7 μM. appKd values were determined by non-linear regression analysis using Origin 7.0 software (Originlab) and the equation $f_c = f_c \times [M1 RNA]_{free}/([K_{d} + [M1 RNA]_{free})$ where $f_c$ = fraction of precursor substrate in complex with M1 RNA and $f_i$ = maximum fraction of substrate able to bind M1 RNA, i.e. endpoint.

**Determination of the kinetic constants under multiple turnover conditions**

The kinetic constants $k_{cat}$ and $K_m$ were determined under multiple turnover conditions in buffer A at 160 mM MgCl$_2$ and pH 7.2 as described elsewhere (12,13).

**Determination of the kinetic constants, $k_{obs}/K^{sto}$ and/or $k_{obs}$ under single turnover conditions**

The kinetic constants $k_{cat}/K^{sto}$ ($k_{cat}/K_m$) and/or $k_{obs}$ (the rate constant of cleavage) were determined following two protocols.

Protocol A was used to determine $k_{obs}/K^{sto}$ and $k_{obs}$ for cleavage of the various pATSer derivatives using Buffer B (pH 6.1) in the presence of 40 mM MgCl$_2$ as described elsewhere (20,22,26). In the case of cleavage of the different tRNA$^{Gln}$ (pGlnG/C and pGlnU/A) and tRNA$^{Tyr}$ $Su_3$ (pSu3G/C and pSu3U/A) precursors, we used Buffer C (without Nonidet P40 and SDS) in the presence of 40 mM Mg(OAc)$_2$. The final concentration of substrate was 40 nM while for M1 RNA the concentration was varied between 0.040 and 5.16 μM. The $k_{obs}$ values were obtained by linear regression from Eadie–Hofstee plots.

Protocol B was used to determine the pseudo-first order rate constant of cleavage, $k_{obs}$, at higher Mg$^{2+}$ concentrations, 160 and 400 mM according to Warnecke et al. (24). The $k_{obs}$ was calculated from single turnover experiments with 40 nM substrate and 4.1 μM M1 RNA by non-linear regression analysis using Origin 7.0 software (Originlab) and the equation $f_{cleaved} = f_{endpoint} \times \left(1 - e^{-k_{obs}t}\right)$ where $f_{cleaved}$ = fraction of cleaved substrate, $t$ = time, $f_{endpoint}$ = maximum cleavable fraction of substrate.

Irrespective of protocol (A or B), the 5’ cleavage fragments were used in our calculations to determine the frequency of cleavage at each time point (and/or M1 RNA concentration).
RESULTS

To investigate the impact on cleavage site recognition, rate of cleavage ($k_{\text{obs}}$) and ground state binding (app $K_d$) in M1 RNA-mediated cleavage as a result of substituting the G$_{+1}$C$_{+72}$ with U$_{+1}$A$_{+72}$ (or vice versa in the case of tRNAGln), we decided to use two full-size tRNA precursors, a tRNAGln precursor (pGln) and the tRNA TyrSu3 precursor (pSu3), and one model RNA hairpin substrate, pATSer (Figure 1). Model RNA hairpin substrates like pATSer have been used to obtain information about RNase P RNA substrate interaction, cleavage site recognition, chemical groups important for cleavage and role of metal ions (12,13,22,23,27–30). We will first describe the result obtained using full-size tRNA precursors, and this will be followed by our studies using various pATSer derivatives.

Replacement of U$_{+1}$A$_{+72}$ to G$_{+1}$C$_{+72}$ in a tRNA$^{\text{Gln}}$ precursor affected both ground state binding and rate of cleavage

The tRNA$^{\text{Gln}}$ precursor carries U$_{+1}$A$_{+72}$ while the majority of tRNAs harbor G$_{+1}$C$_{+72}$ (see above). Thus, to understand whether this difference in the identity of the +1/+72 base pair influenced M1 RNA-mediated cleavage we replaced U$_{+1}$A$_{+72}$ with G$_{+1}$C$_{+72}$ in a tRNA$^{\text{Gln}}$ precursor generating pGlnG/C (Figure 1; underlined residues correspond to the +1/+72 residues throughout this study). The corresponding ‘wild type’ is referred to as pGlnU/A where the underlined residues indicate G$_{+1}$C$_{+72}$ and U$_{+1}$A$_{+72}$, respectively. In addition, we substituted G$_{+1}$C$_{+72}$ with U$_{+1}$A$_{+72}$ in the tRNA$^{\text{TyrSu3}}$ (pSu3) precursor that normally carries a GC-base pair at +1/+72. These two variants are referred to as pSu3G/C (‘wild type’) and pSu3U/A (Figure 1). These different tRNA precursor variants were analyzed with respect to ground state binding (app $K_d$) and rate of cleavage ($k_{\text{obs}}$) under saturating single turn-over conditions at pH 6.0. At this pH, available data suggest that the chemistry of cleavage is rate-limiting (22,31–34; see also below). Note that RNase T1 structural probing revealed no apparent overall structural changes due to substituting the +1/+72 base pair in these tRNA precursors (data not shown).

As shown in Table 1 substituting U$_{+1}$A$_{+72}$ with G$_{+1}$C$_{+72}$ in pGln resulted in a significant reduction in app$K_d$, while only a modest change was observed comparing app$K_d$ for pSu3G/C and pSu3U/A. Moreover, the U$_{+1}$A$_{+72}$ to G$_{+1}$C$_{+72}$ substitution in pGln resulted in a 5-fold increase in $k_{\text{obs}}$ while $k_{\text{obs}}/K_{\text{sto}}$ (= $k_{\text{cat}}/K_m$) was changed almost 50-fold (Table 2) in keeping with the observed difference in app$K_d$ (65-fold). Likewise, the rate of cleavage ($k_{\text{obs}}$) was reduced replacing G$_{+1}$C$_{+72}$ with U$_{+1}$A$_{+72}$ in the pSu3 context (Table 2). But, in this case we
observed a dramatic reduction, >150-fold. Finally, while the pSu3 derivatives were cleaved only at the correct position +1 (data not shown) the pGln derivatives were cleaved at several positions (at −1, +1 and +2), irrespective of U+1A72 or G+1C72 and +/− C5 protein (Figure 2A, data not shown for cleavage of pGlnG/C or in the presence of C5). Taken together, these findings suggested that replacement of U+1A72 with G+1C72 in pGln influenced both ground state binding and rate of cleavage under conditions where chemistry is rate limiting. By contrast, in the case of pSu3 we only observed a dramatic effect on the rate of cleavage. Thus, the observed effects as a result of substituting the +1/+72 base pair appears to depend on tRNA/tRNA precursor identity. Moreover, comparison of the rate of cleavage and ground state binding for ’wild type’ pGln/U/A and pSu3G/C, suggested substrate discrimination in M1 RNA-mediated cleavage both at the level of binding and at the catalytic step.

Table 1. Apparent $K_d$ values for the different substrate variants at different Ca$^{2+}$ concentrations as indicated.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>app$K_d$ (µM)</th>
<th>40 mM</th>
<th>160 mM</th>
<th>400 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pATSerUGG/C</td>
<td>0.11 ± 0.056</td>
<td>0.020 ± 0.0093</td>
<td>0.026 ± 0.018</td>
<td></td>
</tr>
<tr>
<td>pATSerUGU/A</td>
<td>1.3 ± 0.787</td>
<td>0.13 ± 0.023</td>
<td>0.030 ± 0.024</td>
<td></td>
</tr>
<tr>
<td>pATSerUGU/DAP</td>
<td>4.8 ± 1.2</td>
<td>0.73 ± 0.49</td>
<td>0.94 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>pATSerCGG/C</td>
<td>11 ± 7</td>
<td>0.24 ± 0.06</td>
<td>0.057 ± 0.027</td>
<td></td>
</tr>
<tr>
<td>pATSerUGU/DAP</td>
<td>n.d.</td>
<td>3.2 ± 0.38</td>
<td>1.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>pGln/U/A</td>
<td>0.98 ± 0.18</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>pGln/G/C</td>
<td>0.015 ± 0.005</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>pSu3G/C</td>
<td>0.079 ± 0.044</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>pSu3U/A</td>
<td>0.14 ± 0.043</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

The experiments were performed as described in Materials and Methods. Each value is an average of several independent experiments and n.d = not determined. Underlined residues correspond to the identity of the +1/+72 base pair.

Table 2. Summary of cleavage rate constants in cleavage of different substrate variants at pH 6.1 at various Mg$^{2+}$ concentrations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>40 mM</th>
<th>160 mM</th>
<th>400 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{obs}$</td>
<td>$k_{obs}$/k$^{*}$</td>
<td>$k_{obs}$</td>
</tr>
<tr>
<td>pATSerUGG/C</td>
<td>n.d.</td>
<td>3.8 ± 0.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>pATSerUGU/A</td>
<td>1.2 ± 0.2$^*$</td>
<td>n.d.</td>
<td>1.4 ± 0.18</td>
</tr>
<tr>
<td>pATSerUGU/DAP</td>
<td>n.d.</td>
<td>0.11 ± 0.05</td>
<td>n.d.</td>
</tr>
<tr>
<td>pATSerCGG/C</td>
<td>n.d.</td>
<td>0.015 ± 0.004</td>
<td>n.d.</td>
</tr>
<tr>
<td>pATSerCGU/A</td>
<td>0.0005 ± 0.0005</td>
<td>n.d.</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>pATSerUGU/DAP</td>
<td>n.d.</td>
<td>0.055 ± 0.0061</td>
<td>n.d.</td>
</tr>
<tr>
<td>pATSerCGG/C</td>
<td>n.d.</td>
<td>0.19 ± 0.026</td>
<td>n.d.</td>
</tr>
<tr>
<td>pATSerCGU/A</td>
<td>n.d.</td>
<td>0.013 ± 0.001a</td>
<td>n.d.</td>
</tr>
<tr>
<td>pATSerUGU/DAP</td>
<td>n.d.</td>
<td>0.015 ± 0.001a</td>
<td>n.d.</td>
</tr>
<tr>
<td>pGln/G/C</td>
<td>2.8 ± 0.50</td>
<td>9.4 ± 1.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>pSu3G/C</td>
<td>2.5 ± 0.77</td>
<td>4.3 ± 1.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>pSu3U/A</td>
<td>0.12 ± 0.0035</td>
<td>0.053 ± 0.027</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*$^*$Based on two independent determinations. Underlined residues correspond to the identity of the +1/+72 base pair. The finding that the rates of cleavage for pATSerUGG/C, pATSerUGU/A and pATSerUGU/DAP were linear dependent on [OH−] up to at least pH 6.5 suggested that the rate of cleavage at pH 6.1 is rate limiting (data not shown).
(DAP) at position +72, where U and DAP has the potential to form a base pair with three H-bonds. In this context, we also used two substrates where U_{1C} cannot interact with the residue at +72 by conventional base-pairing: pATSerUG variants with U_{1C}C_{72} [but see Ref (35)] and 3mU_{1A}C_{72} (where 3mU = 3-methyl-U). The residue at −1 plays an important role in cleavage by RNase P RNA (12–14,23). Therefore, we also studied pATSer-(+1/+72) variants with either C or U at −1 where position −1 corresponds to the position immediately 5′ of the canonical cleavage site at +1 (Figure 1).

These substrates were analyzed with respect to cleavage site recognition, cleavage rates (efficiency) under multiple as well as single turnover conditions, ground state binding (appK_{d} = apparent K_{d}) and metal ion binding.

### Cleavage site recognition

At pH 6.1 in the presence of 160 mM Mg^{2+}, the pATSerCGG/C substrate is cleaved at the correct site (+1) as well as with a low frequency at position −1 in keeping with our previous observations [Figure 2B; Refs (12,22)]. Replacement of G_{1C}C_{72} with U_{1A}A_{72} or U_{1A}DAP_{72} resulted in mis-cleavage both at the −1 and +2 positions. However, the total frequency of mis-cleavage (i.e. both at −1 and +2) of the U_{1A}DAP_{72} variant was approximately the same compared to that for the G_{1C}C_{72} variant.

Replacing C_{72} with U resulted in a similar mis-cleavage pattern for the U_{1A}A_{72} and U_{1A}DAP_{72} variants, i.e. mis-cleavage at −1 and at +2. But, here a significant increase in mis-cleavage was observed for the U_{1A}DAP_{72} variant mainly due to an increased frequency of cleavage at +2 (Figure 2C). Just replacing G_{1A} with U in pATSerUGG/C or U_{1A} in pATSerUGU/A with a 3-methyl U also resulted in mis-cleavage both at −1 and at +2 where pATSerUGU/C was cleaved preferentially at +2 (Figure 2C).

We conclude that the presence of U at +1 influence cleavage site recognition however, our data suggested that this depends on substrate context (see above). Moreover, in keeping with previous data, cleavage site recognition is also affected by the identity of the −1 residue (12–14,22).

### Replacement of G_{+1}C_{+72} influenced the efficiency of cleavage under multiple turnover conditions

We previously reported the kinetic constants for cleavage of several pATSerCGG/C substrates under multiple turnover conditions at pH 7.2 (12,13,23,27). Hence, we first determined cleavage efficiency for the various +1/+72 derivatives by calculating k_{cat}/K_{m} values based on determination of k_{cat} and K_{m} under multiple turnover conditions at pH 7.2 and 160 mM Mg^{2+} (see Materials and Methods). As shown in Figure 3, reductions ranging between 16- and ~150-fold in k_{cat}/K_{m} (for cleavage at +1) for pATSerCGU/DAP and pATSerCGU/A, respectively, were observed compared to cleavage of the corresponding G_{1}C_{72} variant. This was mainly due to an increased K_{m} (∼10-fold) for the former, while for pATSerCGU/A k_{cat} was down 5-fold and K_{m} was increased.
30-fold. In keeping with our previous data (13), pATSer-UGG/C was cleaved with a higher efficiency compared to cleavage of pATSerCG/C indicating the importance of the identity of residue −1 (Figure 3). These data indicated that replacement of G72 with U/A or U/A resulted in changes in the rate of cleavage under the context of the model substrate, pATSer.

Replacement of G72/C72 influenced the rate of cleavage under conditions where chemistry is rate limiting also in the context of a model hairpin substrate

At pH 7.2, chemistry is not rate limiting, therefore these data do not provide information about the importance of the G72/C72 to U/A change with respect to the chemistry of cleavage. We therefore determined the rate constant kcat for cleavage under single turnover conditions at pH 6.1 where the rate of cleavage is rate limiting in the context of the model RNA hairpin substrate. Our data also indicated that increasing Mg2+ concentration indicated different Mg2+ requirements due to replacing G72/C72 with U/A (Figure 4A). We therefore determined kobs at different Mg2+ concentrations. The results are summarized in Table 2.

In general, replacement of G72/C72 to any of the other combinations tested resulted in a reduction in the rate of cleavage at the correct (+1) position irrespective of U or C at −1 with the exception of when kobs values for cleavage of the U/A derivatives were compared (see below). The extreme case was the 2500-fold decrease in rate of cleavage of pATSerUGU/DAP at 40 mM Mg2+ while kcat was reduced >150-fold for pATSerUGA/A at the same [Mg2+]. By increasing the Mg2+ concentration, a rescue in the rate of cleavage for the U/A variants was observed and at 400 mM Mg2+, the rates of cleavage for the U/A and U/A/DAP variants were only down 2.8- and 16-fold, respectively, compared to cleavage of the corresponding G72/C72 variant, pATSerUGG/C.

The C−1 derivatives pATSerCG/C and pATSerCGUA were cleaved with lower rates relative to the corresponding U−1 derivatives irrespective of [Mg2+] indicating the importance of the −1 residue (and/or +73, see below). Comparing kobs for cleavage of the U/A/DAP72 variants suggested an increased rate of cleavage with a C at position −1. Moreover, in contrast to what we observed for the U−1 variants, no rescue in the rate of cleavage was observed for the C−1 derivatives as a result of increasing [Mg2+] under the conditions tested. It is conceivable that this difference is influenced by the fact that C−1 is base-paired to G73 in the substrate (see Figure 5).

In conclusion, replacing G72/C72 and/or the −1 residue resulted in changes in the rate of cleavage under conditions where the rate of cleavage is rate limiting in the context of the model RNA hairpin substrate. Our data also indicated that substituting G72/C72 to U/A or U/DAP72 influenced Mg2+ binding (see also below), conceivably the Mg2+ ion(s) bound in the vicinity of the cleavage site. It also appears that the influence of Mg2+ was dependent on the identity of the −1 residue. In the case of U/DAP72, we have to consider that the introduction of DAP at this position also affects the
electrostatics. Therefore, this might be part of the reason for our observations using the U1DAP72 variants.

The $k_{obs}$ values at 40 mM Mg$^{2+}$ were determined from Eadie-Hofstee plots ($V_i = \frac{f(V[M1RNA])}{K}$). The intercept with the x-axis gives estimates of $k_{obs}/K^{cat} (= k_{cat}/K_m)$, which corresponds to the association rate constants $k_{-1}$ (assuming that $k_{-1} < k_{r1}$, where $k_{-1}$ represents the dissociation constant and $k_{r1}$ the rate constant for cleavage, see scheme 1 in Figure 1), for the pATSerUG(+1/U72) derivatives tested under these conditions. Based on this, our data suggested that replacing G4+C72 with U1A72 and U41DAP72 resulted in a 35- and 250-fold decrease in $k_{+1}$, respectively ($k_{obs}/K^{cat}$; Table 2).

Replacing G4+C72 and the −1 residue in the model substrate affected ground state binding

Next, we investigated whether substitution of G4+C72 with U1A72 (or U41DAP72) and/or the −1 residue influenced ground state binding. As shown in Table 1, substituting G4+C72 with U1A72 or U41DAP72 resulted in increased appKd values irrespective of Mg$^{2+}$-concentration and U or C at the −1 position. But, comparison of appKd values for pATSerUGGC/C and pATSerUGUA showed a decreasing difference in appKd with increasing Mg$^{2+}$. This was not observed in the case of the U1DAP72 variant. Moreover, comparing appKd values for the U1DAP72 derivatives with the respective ‘wild type’ values, i.e. appKd for pATSerUGGC/C and pATSerCGGC/C, respectively, showed a larger difference in the case where the substrate carried a U at −1 (∼40-fold versus 1.7-fold difference at 160 mM Ca$^{2+}$). The appKd values were also found to be generally higher for the substrates carrying a C−1 compared to substrates with U−1. The reason to these observations might be that the C−1 and G4+C72 interact via base-pairing in the absence of M1 RNA (see also above).

As a consequence, this would make these residues less accessible to interact with M1 RNA upon M1 RNA–substrate (RS) complex formation, e.g. establishing the +73/294 interaction [Figure 5; (13)]. In addition, U−1 would probably result in a more favorable interaction with A248 in M1 RNA than C−1 [(14); see also (12)]. To conclude, in addition to the influence on the rate of cleavage and possibly Mg$^{2+}$ binding, replacing G4+C72 with U41A72 (or U41DAP72) and the residue at −1 influenced ground state binding. This is similar to what was observed comparing binding of the pGln variants, but different in relation to when the psu3 derivatives were used (see above).

Replacing G4+C72 influenced metal ion binding in its vicinity

The data presented above suggested that replacement of G4+C72 with U1A72 affects metal ion binding possibly in its vicinity (see Discussion). To study this in more detail, we took advantage of the fact that cleavage of pATSerCGGC/C results in substantial miscleavage at −1 in the presence of Mn$^{2+}$. The addition of Mg$^{2+}$ (without changing the total metal ion concentration, i.e. [Mg$^{2+}$]+[Mn$^{2+}$]) to the cleavage reaction suppresses miscleavage at −1 [Figure 4B; (12,13,23,27)]. We have used this approach elsewhere to obtain information about chemical groups important for Mg$^{2+}$ binding in the M1 RNA cleavage reaction (12). Here, we studied the frequency of miscleavage as a function of [Mg$^{2+}$]/([Mg$^{2+}$]+[Mn$^{2+}$]) for pATSerGC/G/C and pATSerCGUA. The data in Figure 4B showed that higher [Mg$^{2+}$] was required to suppress the Mn$^{2+}$-induced miscleavage as a result of replacement of G4+C72 with U1A72. These findings supported the suggestion that the G4+C72 to U1A72 change influences Mg$^{2+}$ binding in the M1 RNA substrate complex.

DISCUSSION

Substrate discrimination in RNase P RNA-mediated cleavage

RNase P has to recognize and cleave tRNA precursors irrespective of whether it has a G/C or U/A base pair at position +1/−2 (or any other combination, e.g. C4+A72 or G4+C72 as in the precursors to tRNA$^{\text{Glu}}$ and tRNA$^{\text{Pro}}$, respectively). Here, we demonstrated that replacement of G4+C72 with U1A72 in full-size tRNA precursor substrates and a model hairpin–loop substrate influenced cleavage by M1 RNA (RNase P RNA derived from E.coli) by affecting: ground state binding, rate of cleavage under conditions where chemistry is suggested to be rate limiting, cleavage site recognition and most likely Mg$^{2+}$ binding in the vicinity of the cleavage site. These findings are of particular relevance in view of the presence of U41A72 in the wild-type tRNA$^{\text{Glu}}$ precursor while most other tRNA precursors in bacterial cells carry G4+C72 (1). Indeed, the tRNA$^{\text{Glu}}$ precursor, ‘wild type’ pGlnU/A, used here was shown to bind to M1 RNA with a 10-fold lower binding affinity compared to the ‘wild type’ tRNA$^{\text{Glu}}$ precursor psu3G/C (this report). Hence, these findings provide evidence for substrate discrimination in RNase.
P RNA-mediated cleavage at the level of substrate binding. As RNase P, EF-Tu also interacts with a large number of different substrates, i.e. aminoacylated tRNAs, and a 10-fold difference in binding between EF-Tu and different correctly aminoacylated tRNA was reported [(6); see also (3–5)]. Thus, both RNase P RNA and EF-Tu discriminate at the level of substrate binding. It is conceivable that this is related to that RNase P RNA and EF-Tu bind the same tRNA regions: the aminoacyl acceptor stem, T-stem and T-loop (7,36,37). However, our kinetic data as determined under saturating single turnover conditions also suggested substrate discrimination at the catalytic step for RNase P RNA: 5-fold difference in $k_{\text{obs}}$ for pGlnU/A and pSu3G/C (Table 2).

Taken together, based on our studies where we replaced U$_{4}$A$_{72}$ with G$_{4}$C$_{72}$ in pGlnU/A (or vice versa in pSu3 and pATSer), we conclude that the +1/+72 plays an important role in substrate discrimination by RNase P RNA, and it will be of interest to investigate whether this is also the case for EF-Tu. Moreover, our findings provide evidence that the U$_{4}$A$_{72}$ base pair in tRNA$^{A}_{\text{he}}$ plays a role not only in the interaction with glutaminyl-tRNA synthetase (38), but also in tRNA processing. This extends and supports the suggestion that different substrates interact differently with RNase P RNA (20,30,39,40). Note that changing the structural architecture at the cleavage site did not affect the overall structure of the substrate however, with respect to local structural consequences (see below).

### The +1/+72 base pair and substrate context

Previous studies using P RNA from *Bacillus subtilis* indicated that substitution of G$_{4}$C$_{72}$ with U$_{4}$A$_{72}$ (A$_{4}$U$_{72}$ or C$_{4}$G$_{72}$) resulted only in modest effects on binding and chemistry of cleavage of a yeast tRNA$^{A}_{\text{he}}$ precursor in the context when the 2OH at −1 had been replaced with a 2H (15). But, in the presence of the *B. subtilis* RNase P protein, changing of G$_{4}$C$_{72}$ to U$_{4}$A$_{72}$ (A$_{4}$U$_{72}$ or C$_{4}$G$_{72}$) resulted in a significantly larger effect on both binding and rate of cleavage ([15]; see also below). Moreover, certain tRNA-like structures carrying a U/A base pair at the position corresponding to +1/+72 were cleaved at more than one position (41), similar to our previous findings that cleavage site recognition of this substrate. For example, the presence of the methyl at position 3 would influence the potential interaction between A248 and 3mU$_{4}$+. It is also likely, but not mutually exclusive, that Mg$^{2+}$ binding in the vicinity of the cleavage site is affected as a result of substituting G$_{4}$C$_{72}$ (see also below) and as a consequence this results in mis cleavage. Thus, our data are in keeping with the establishment of a functionally important interaction between the −1 residue in the 5′ leader and A248 [Figure 5; (14); see also (12)].

A U at +1 in pSu3 did not result in mis cleavage. This can be rationalized by our previous findings that cleavage site recognition of pSu3 is dictated by several determinants (11) and changing one in pSu3 is not enough to result in cleavage at other positions. Moreover, pGln that naturally carries U$_{4}$A$_{72}$ was mis cleaved at several positions irrespective of the identity of +1/+72, i.e. U$_{4}$A$_{72}$ or G$_{4}$C$_{72}$, or absence/presence of the RNase P protein C5. Thus, other factors must play a role in cleavage site recognition of this substrate. For example, the presence of the 6 nt long 3′ trailer and/or that the tRNA$^{G}_{\text{ln}}$ are not naturally transcribed as a monomeric precursor in *E.coli*, but as part of a multimeric tRNA gene transcript. Also, the length and structure of the 5′ leader might play a role. Indeed, earlier data suggested that the 43 nt long 5′ leader of pSu3 is involved in stabilizing the M1 RNA substrate interaction (45).

To conclude, these data indicate the importance of substrate context and structural architecture (in particular the +1/+72 base pair) of the cleavage site in RNase P RNA-mediated cleavage. Moreover, based on our studies, it is likely that the presence of U at +1 (and not A at +72) is the reason for the effects observed in the present study and in previous *in vitro* studies. Together this rationalizes earlier *in vivo* findings where it was suggested that RNase P processing of the dimeric Pro-Ser tRNA precursor was blocked due to replacement of G$_{4}$ with U$_{4}$ in tRNA$^{A}_{\text{Ser}}$ (42); the suppression efficiency for SupE (Sn2/gltn) encoding tRNA$^{G}_{\text{ln}}$ was significantly reduced in a mutant RNase P protein (rnpA49) background (43). We also note the importance of the identity of the +1/+72 base irrespective of type of RNase P RNA, i.e. type A as represented by M1 RNA and *B. subtilis* P RNA that belongs to type B [(44); see also (20)].

### Differences in cleavage and ground state binding of different substrates and residue −1

Recent data suggested that the residue at the −1 position immediately 5′ of the scissile bond interacts with residue A248 in M1 RNA [((11); see also (9)]. In *E.coli* as well as in other bacteria, a U at this position is the preferred residue (12–14,39). Substitution of G$_{4}$ with U$_{4}$ might therefore affect correct interaction with A248 and thereby influence the cleavage site recognition process resulting in mis cleavage as observed in the present study for the model substrate pATSer. This is corroborated by our findings using the U$_{4}$A$_{72}$ and 3mU$_{4}$A$_{72}$ variants. In the case of the former, the U at +1 would become accessible to interact with A248 (Figure 5) resulting in cleavage preferentially at +2 whereas the presence of the methyl at position 3 would influence the potential interaction between A248 and 3mU$_{4}$+. It is also likely, but not mutually exclusive, that Mg$^{2+}$ binding in the vicinity of the cleavage site is affected as a result of substituting G$_{4}$C$_{72}$ (see also below) and as a consequence this results in mis cleavage. Thus, our data are in keeping with the establishment of a functionally important interaction between the −1 residue in the 5′ leader and A248 [Figure 5; (14); see also (12)].

A U at +1 in pSu3 did not result in mis cleavage. This can be rationalized by our previous findings that cleavage site recognition of pSu3 is dictated by several determinants (11) and changing one in pSu3 is not enough to result in cleavage at other positions. Moreover, pGln that naturally carries U$_{4}$A$_{72}$ was mis cleaved at several positions irrespective of the identity of +1/+72, i.e. U$_{4}$A$_{72}$ or G$_{4}$C$_{72}$, or absence/presence of the RNase P protein C5. Thus, other factors must play a role in cleavage site recognition of this substrate. For example, the presence of the 6 nt long 3′ trailer and/or that the tRNA$^{G}_{\text{ln}}$ are not naturally transcribed as a monomeric precursor in *E.coli*, but as part of a multimeric tRNA gene transcript. Also, the length and structure of the 5′ leader might play a role. Indeed, earlier data suggested that the 43 nt long 5′ leader of pSu3 is involved in stabilizing the M1 RNA substrate interaction (45). This would be in keeping with the fact that no significant change in ground state binding was observed as a result of replacing G$_{4}$C$_{72}$ with U$_{4}$A$_{72}$ in pSu3.

We also observed that replacing U$_{4}$ with C$_{4}$ in the model pATSer substrate resulted in a significant increase in ground state binding. This is in keeping with Zahler et al. (14) who...
used a tRNA precursor substrate. As discussed in detail elsewhere, one likely reason for this is that C-1 in these substrates are engaged in base pairing with G73, and this results in G73 being less accessible for interaction with U294 in M1 RNA [Figure 5; (12–14,22,46)]. In addition, we observed a significant difference in the rate of cleavage ($k_{\text{obs}}$) as a result of introduction of C at −1 (Table 2). Harris and coworkers using a tRNA precursor substrate did not detect any difference in the rate of cleavage comparing cleavage with U-1 versus C-1 (14). But, Loria and Pan (15) did observe that changing the 5′ leader, where one carried U-1 and the other C-1, resulted in different rates of cleavage. Together, this again indicates differences in cleavage of different substrates and corroborates the importance of the −1 residue. It is conceivable that binding of Mg$^{2+}$ does play a role in this, given the difference in response to the Mg$^{2+}$ concentration where the C-1 variants required higher Mg$^{2+}$ concentration than the U-1 derivatives. Moreover, these differences again emphasize the importance to study different substrates [see also Discussion and ref. 22].

Structural architecture of the cleavage site and possible role of Mg$^{2+}$

Although the overall structure did not change, as a result of replacing G$^4_A$C$^2_T$2 with U$^1_A$T$^1$2 (or vice versa) and/or substitution of the residue at −1 (see above), the local structural topography has been changed by definition. From the above and previous data [Ref (22) and references therein; see also (47)], it is clear that different chemical groups at and in the vicinity of the cleavage site are involved in aligning the scissile bond to ensure efficient and correct cleavage. At present, we do not have access to a high-resolution structure of the RNase P cleavage site in the RS-complex. Nonetheless, a picture is emerging based on genetic and biochemical data from several laboratories, and our current understanding of RNase P RNA substrate interactions are modeled in Figure 5 (for references see legend in Figure 5). Moreover, based on our present results we can infer the following: Hexahydrated Mg$^{2+}$ ions bind to RNA and structural studies suggested binding in the deep groove of RNA [(48); see also for example (49)]. The structural studies further suggested that in one of the binding modes O6 and N7 of the guanosine of GpN in the deep groove of RNA [Figure 5; (12–14,22,46)]. In addition, we observed a significant difference in the rate of cleavage comparing cleavage with U-1 versus C-1 (14). But, Loria and Pan (15) did observe that changing the 5′ leader, where one carried U-1 and the other C-1, resulted in different rates of cleavage. Together, this again indicates differences in cleavage of different substrates and corroborates the importance of the −1 residue. It is conceivable that binding of Mg$^{2+}$ does play a role in this, given the difference in response to the Mg$^{2+}$ concentration where the C-1 variants required higher Mg$^{2+}$ concentration than the U-1 derivatives. Moreover, these differences again emphasize the importance to study different substrates [see also Discussion and ref. 22].

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