Interaction of the 3'-end of tRNA with ribonuclease P RNA

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ABSTRACT
Ribonuclease P, which contains a catalytic RNA subunit, cleaves 5' precursor-specific sequences from pre-tRNAs. It was previously shown that the RNase P RNA optimally cleaves substrates which contain the mature, 3'-terminal CCA of tRNA. In order to determine the contributions of those individual 3'-terminal nucleotides to the interaction, pre-tRNAs that have CCA, only CC or C or are without CCA at the 3'-end were synthesized by run-off transcription, tested as substrates for cleavage by RNase P RNA and used in photoaffinity crosslinking experiments to examine contact sites in the ribozyme. In order to generalize the results, analyses were carried out using three different bacterial RNase P RNAs, from Escherichia coli, Bacillus subtilis and Thermotoga maritima. At optimal (kcat/Km) ionic strength (1 M NH₄⁺/25 mM Mg²⁺), Km increases incrementally 3- to 10-fold upon stepwise removal of each nucleotide from the 3'-end. At high ionic strength (2 M NH₄⁺/50 mM Mg²⁺), which suppresses conformational effects, removal of the 3'-terminal A had little effect on Km, indicating that it is not a specific contact. Analysis of the deletion and substitution mutants indicated that the C residues act specially; their contribution to binding energy at high ionic strength (~1 kcal/mol) is consistent with a non-Watson-Crick interaction, possibly irregular triple-strand formation with some component of the RNase P RNA. In vitro, the Km of RNase P RNA for tRNA lacking CCA, or tRNA in which CCA is mutated to other sequences, is 10- to 20-fold higher than for the native tRNA (10, 12, 13, 15).

INTRODUCTION
RNase P cleaves precursor-specific sequences from the 5'-ends of pre-tRNAs (1, 2). The bacterial RNase P is composed of an ~400 nucleotide RNA and an ~120 amino acid protein (3, 4). At high ionic strength in vitro the RNA alone can cleave pre-tRNAs in the absence of the protein (5). The high ionic strength may assist in the folding of RNase P RNA or in the binding of the two RNAs, by the screening of ionic repulsion (6).

The main region of tRNA recognized by RNase P RNA is the coaxial helix formed by the acceptor stem and the common arm (7, 8). The only invariant sequences in this helix are CCA at the 3'-end of the tRNA and GUUCG in the common arm of tRNA. Since RNase P must act on all tRNAs, sequences that are common to all tRNAs are potential sites of specific interaction. The ubiquitous 3'-terminal CCA of tRNA has previously been shown both in vivo and in vitro to be important in the interaction with RNase P RNA (7, 9-15).

In order to evaluate the details of the interaction of CCA with RNase P RNA, we fabricated tRNAs that lack A, CA, or CCA, or contained alternative nucleotides. These mutant tRNAs were used in kinetic analyses to evaluate the energetic contribution of each nucleotide, and in crosslinking experiments to identify potential sites of interaction of the individual nucleotides in the CCA sequence with RNase P RNA. Experiments were carried out with RNase P RNAs from three different organisms in order to generalize the information. The RNase P RNA of Escherichia coli and Bacillus subtilis differ to some extent from one another in structure. The RNA from Thermotoga maritima is thermostable and phylogenetically diverse from the others. All contain a homologous core of sequence and structure responsible for catalysis.

MATERIALS AND METHODS
Construction of plasmids
The oligonucleotide CCGGACCCGCTTTCATCC-TGCA and its complement TGCCGTCGAAGGTAGG were used to create FokI, BbsI and BsrNI restriction sites at the 3'-end of the B.subtilis tRNAAsp gene. The vector plasmid
pDW152 (16) was digested with the restriction enzymes BspEl and PstI, removing 44 nucleotides at the 3'-end of the tRNA gene. After purification, the vector DNA was ligated to the annealed oligonucleotide fragments, which contained cohesive ends compatible with the vector DNA. In order to avoid transformants formed by intact vector DNA, the DNA purified from the ligation reaction mixture was digested with BamHI, which cleaves the parent plasmid DNA but not the plasmid containing the oligonucleotide fragments. The ligated DNA was used to transform *E. coli* DH5αF' and the constructs were confirmed by sequencing. A plasmid containing the desired sequences was named pDW152BF. The plasmid DNA digested with *BstN1, BstI* or *FokI* was used as the template for run-off transcription to yield tRNA with CCA at the 3'-end, or tRNA lacking A and CA, respectively. pT7-1LCCA (13) digested with *NciI* was used as a template to generate tRNA lacking CCA at the 3'-end. tRNA genes in which each C was mutagenized were obtained by cloning oligonucleotides in which A, G and T were substituted at the 3' terminus of CCA into pDW152 as indicated above.

Preparation of RNA by run-off transcription

Unlabeled RNase P RNAs were prepared by in vitro run-off transcription of plasmid DNA with T7 RNA polymerase (20 mM sodium phosphate, pH 7.7, 8 mM MgCl₂, 2 mM DTT, 5 mM spermidine, 1 mM each ribonucleotide triphosphate, 37°C, 2–3 h). The RNAs were purified by electrophoresis through 3.75% polyacrylamide, 0.25% bisacrylamide, 8 M urea in TBE (90 mM Tris–borate, pH 8.0, 9 mM EDTA), viewed by UV shadow, excised and eluted into 0.5 M ammonium acetate, 5 mM EDTA, and 0.1% SDS. Labeled tRNAs were prepared by in vitro transcription using T7 RNA polymerase (20 mM sodium phosphate, pH 7.7, 8 mM MgCl₂, 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.1 mM GTP, and [α-³²P]GTP (3,000 Ci/mmol; Amersham Corp.). The RNA concentrations were determined by the measurement of isotopic incorporation by UV spectrophotometry. The identities of the 3'-ends of tRNAs were confirmed by 3'-end-labeling with RNA ligase and [³²P]-3'-cytidine bisphosphate (pCp), followed by alkaline

### Table 1. Kinetic parameters of RNase P RNA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E. coli</th>
<th>B. subtilis</th>
<th>T. maritima</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optimal Salt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA</td>
<td>Km ± SD</td>
<td>kcat ± SD</td>
<td>ΔG</td>
</tr>
<tr>
<td>tRNAACA</td>
<td>12±9</td>
<td>0.2±0.1</td>
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<tr>
<td>tRNAACA</td>
<td>48±10</td>
<td>0.8±0.2</td>
<td>-10.4</td>
</tr>
<tr>
<td>tRNAACCA</td>
<td>180±40</td>
<td>1.0±0.1</td>
<td>-9.6</td>
</tr>
<tr>
<td>tRNAACCCA</td>
<td>350±40</td>
<td>2.1±1.0</td>
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<tr>
<td>tRNAACCCCA</td>
<td>380±50</td>
<td>4.6±0.5</td>
<td>-9.1</td>
</tr>
<tr>
<td>tRNAACCGC</td>
<td>510±200</td>
<td>3.0±0.9</td>
<td>-8.9</td>
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<td>tRNAACGGG</td>
<td>360±30</td>
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<tr>
<td>tRNAACCGGG</td>
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<td>3.4±2.9</td>
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<tr>
<td>tRNAACCGGG</td>
<td>370±100</td>
<td>2.2±1.0</td>
<td>-9.1</td>
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<tr>
<td>tRNAACCGGG</td>
<td>180±90</td>
<td>3.7±2.5</td>
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<tr>
<td>tRNAACCGGG</td>
<td>180±40</td>
<td>0.4±0.2</td>
<td>-9.6</td>
</tr>
<tr>
<td><strong>High Salt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA</td>
<td>Km ± SD</td>
<td>kcat ± SD</td>
<td>ΔG</td>
</tr>
<tr>
<td>tRNAACA</td>
<td>19±12</td>
<td>0.05±0.01</td>
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<tr>
<td>tRNAACA</td>
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<td>tRNAACCA</td>
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<td>tRNAACCA</td>
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<td>tRNAACCA</td>
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<td>-9.9</td>
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<td>tRNAACCC</td>
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<tr>
<td>tRNAACGG</td>
<td>60±10</td>
<td>0.9±0.2</td>
<td>-10.2</td>
</tr>
</tbody>
</table>

| Phenylazide attached | | | |
| High | tRNA | | |
| tRNAACA | 36 | 0.2 | -10.6 | 0.0 |
| tRNAACA | 27 | 0.2 | -10.7 | -0.1 |
| tRNAACCA | 25 | 0.7 | -10.8 | 0.1 |
| tRNAACCCCA | 61 | 1.9 | -10.2 | 0.4 |

### The values shown are the mean of 3–10 repetitions. ΔG (ΔG = ΔH/RT) was calculated from the mean of Kₘ and ΔΔG was obtained by the subtraction of ΔG for pre-tRNA from ΔG for experimental RNAs. tRNA with the CCA sequence is designated tRNA, and tRNAs lacking A, CA, and CCA are respectively indicated as tRNAΔA, tRNAΔCA and tRNAΔCCA. Mutagenized nucleotides in the 3'-terminal sequences of tRNA are underscored.
hydrolysis and chromatographic resolution of the released nucleotides (17).

Kinetic assays

Uniformly labeled ([α-32P]GTP) pre-tRNA<sub>Asp</sub> of <i>B. subtilis</i> was used as substrate for kinetic assays. Kinetic assays were carried out in 50 mM HEPES/NaOH buffer, pH 8.0, 0.05% Nonidet P-40 (NP-40), 0.1% SDS, 2 mM RNase P RNA and in salt concentrations as indicated. Assays were performed in the optimum (K<sub>eq</sub>/K<sub>n</sub>) salt condition for RNase P RNAs of <i>E. coli</i> and <i>B. subtilis</i> were carried out at 1.0 M NH4Cl and 25 mM MgCl<sub>2</sub> at 37°C. Assays of <i>E. coli</i> and <i>B. subtilis</i> RNase P RNA under 'high salt' conditions were carried out in 2.0 M NH4Cl and 50 mM MgCl<sub>2</sub> at 37°C. <i>T. maritima</i> RNase P RNA was assayed at 50°C in 3.0 M NH4Cl and 50 mM MgCl<sub>2</sub> (18). The RNase P RNAs and substrates were preincubated separately in reaction buffer at the reaction temperature for 5–10 min, and the reaction was started by the mixing of enzyme and substrate. The concentration of substrate ranged from 10 to 100 nM for the high salt condition and 100 nM to 1.0 μM for the optimum conditions. The reactions were stopped by the addition of 2 volumes of cold ethanol. The reaction products were resolved by electrophoresis in 8% denaturing polyacrylamide gels. The relative intensities of appropriate RNA bands were measured with a Phosphorimager (Molecular Dynamics).

Preparation of photoagent-attached tRNA at the 3'-end
tRNAs synthesized by transcription were oxidized in 100 mM sodium acetate, pH 5.4, 3 mM sodium periodate. The reaction was carried out in the dark at room temperature for 1 h. RNAs were collected by ethanol precipitation and dried in vacuo. Oxidized tRNAs were modified in 1 mM of either 1,6-hexyl diamine or ethylene diamine, 20 mM imidazole, pH 8.0, 5 mM NaCNBH<sub>3</sub> at 37°C for 1 h. NaBH<sub>4</sub> was added to 5 mM and allowed to react 5–15 min further. Modified tRNAs were precipitated twice in ethanol and then reacted in 10 mM NaCNBH<sub>3</sub> and 100 nM to 1.0 μM for the optimum conditions. The reactions were stopped by the addition of 2 volumes of cold ethanol. The reaction products were resolved by electrophoresis in 8% denaturing polyacrylamide gels. The relative intensities of appropriate RNA bands were measured with a Phosphorimager (Molecular Dynamics).

Formation of RNase P RNA–tRNA cross-links

Crosslinking reactions were performed in 2.0 M NH4Cl, 0.1% SDS, 0.05% NP-40 and with either 25 mM CaCl<sub>2</sub> in 16.5 mM PIPES/44 mM Tris, pH 6.0, or 25 mM MgCl<sub>2</sub> in 50 mM HEPES/NaOH, pH 8.0. Crosslinking reactions contained 10–100 nM photoagent-derivatized tRNA and a 6- to 40-fold molar excess of RNase P RNA. All the components were allowed to equilibrate for 5–10 min at room temperature in the dark. The mixtures were exposed to UV light (Model UVM-57 302 nm, UVP, San Gabriel, CA) at 5 cm. A polystyrene petri dish lid was used for screening UV light <300 nm. After irradiation, reaction mixtures were diluted 2- to 5-fold with distilled water, or with 0.3 M sodium acetate, and precipitated with ethanol. The crosslinked conjugates were separated by electrophoresis in a 4% denaturing polyacrylamide gel. Carrying out irradiations in liquid N<sub>2</sub> (−196°C) or dry ice (−78°C) (20, 21) did not improve the efficiencies of crosslinking (data not shown).

Primer extension for analysis of crosslink sites

Unlabeled, photoagent-derivatized tRNAs (≤1 μg) were crosslinked to the RNase P RNA (≤5 μg) as above and products were separated on a 4% denaturing polyacrylamide gel. Bands corresponding to crosslinked conjugates were visualized by staining with ethidium bromide, excised and eluted into 300–500 μl STE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 0.1% SDS) overnight. Conjugates were recovered by ethanol precipitation and resuspended in 5–8 μl TE buffer, pH 8.0.

Crosslinked species (1–3 μl) were heated with 1–5 ng 5'-end-labeled-primer in 50 mM Tris–HCl, pH 8.5, 25 mM KCl at 90°C for 2 min then slowly cooled to room temperature over 15–30 min. Primers for <i>E. coli</i> RNase P RNA were: TTACC-TGGCACCCTGGCC (60R), CTCTTACCSCACCATTTCA-CCTC (174RN), GGGTTGAGTTTACCGTG (225R), and AAGTGGACTAAATG (370R). For <i>B. subtilis</i> RNase P RNA: CTTGCCATGCGACGAGACT (80R), AAATTTGGGWMWCTCCGTCGA (235R), and GTGGTCTACGCGTCTG (382R) were used. For <i>T. maritima</i> RNase P RNA: RTAA-GCGGGRTTCTGTG (347R), CCGGCTGCAGGAATTC (388R), and 174RN were used. Primers were extended with reverse transcriptase in 50 mM Tris–HCl, pH 8.5, 50 mM KCl, 10 mM DTT, 10 mM MgCl<sub>2</sub>, and 100 μM of each nucleotide at 45–50°C for 30 min (22). The reaction was stopped by the addition of the same volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) and products were resolved in an 8% denaturing polyacrylamide gel. Sequencing reactions were done as above with the addition of 100 nM dideoxynucleotide to the appropriate reactions.
RESULTS
Kinetic analyses with mutant pre-tRNAs

As detailed in Materials and Methods, a transcription vector containing the gene for \textit{B. subtilis} pre-tRNA\textsuperscript{ASP} was modified by oligonucleotide mutagenesis to create a series of mutants in the 3'-terminal CCA of the gene. Uniformly labeled pre-tRNAs, produced by \textit{in vitro} transcription (Materials and Methods), contained the normal CCA (pre-tRNA), or lacked A (pre-tRNA\textsuperscript{AAP}), CA (pre-tRNA\textsuperscript{ACA}), or CCA (pre-tRNA\textsuperscript{ACCA}). All these pre-tRNAs contained a 33 nucleotide leader that is removed during processing. These substrates were used in kinetic assays with the three phylogenetically diverse RNase P RNAs at their respective optimal (maximum \(k_{\text{cat}}K_{m}\) ionic strengths, and also at a higher ionic strength in order to gain information as to whether kinetic effects of removal of CCA nucleotides were due to conformational differences or to the loss of specific H-bond contacts. Effects due to a conformational misfit between the enzyme and substrate tend to be suppressed by high ionic strength (23), whereas effects due to loss of specific H-bonds are expected to be insensitive to that condition. This test is practical only for the RNase P RNAs of \textit{E. coli} and \textit{B. subtilis}. The optimal salt concentration for the \textit{T. maritima} RNA (and other thermophilic RNase P RNAs; 18) is equivalent to the high ionic strength used with the other RNAs. The results of the kinetic analyses are summarized in Table I.

The \(K_{m}\) values reflect ribozyme—substrate binding energy and thus identify the contribution of the deleted tRNA nucleotides to the interaction. The RNase P reaction rate is limited by the rate of release of the product (6), so an increase in \(K_{m}\) also results in an increase in \(k_{\text{cat}}\). As shown in Table I, the removal of successive nucleotides from the 3' end of the pre-tRNA results in an incremental increase in \(K_{m}\) with each of the RNase P RNAs at optimal ionic strength. This indicates some contribution of each nucleotide to the RNase P RNA—pre-tRNA interaction. At optimal ionic strength, the overall contribution of the 3'-terminal CCA to the interaction is 2–3 kcal/mol (\(\Delta\Delta G\) for tRNA\textsuperscript{ACCA}), an appreciable fraction (~20%) of the total binding energy. This result is seen only with the RNase P RNA alone reaction. Consistent with previous results (10), we find that the \(K_{m}\) of the holoenzyme for the substrate is not significantly affected by the absence of the CCA (data not shown).
Figure 3. Identification of crosslink sites in RNase P RNA by primer extension. tRNA-hexyl azide (non-radiolabeled) was crosslinked with RNase P RNA. Purified crosslinked RNAs were recovered from denaturing polyacrylamide gels (Materials and Methods) and annealed with 5'-end-labeled primers complementary to RNase P RNA. Primer extensions by reverse transcriptase were carried out as detailed (Materials and Methods), and products were resolved in an 8% denaturing polyacrylamide sequencing gel. Crosslinked species for each tRNA are numbered as 1, 2, 3 corresponding to the designations in Figure 2A and B. Primer extensions on RNase P RNA with deoxyribonucleotides to identify sequence are indicated as C, U, A, G. As a control (lane N), primer extension was carried out with RNase P RNA which was UV irradiated in the absence of tRNA. The arrows indicate the primary crosslinking site indicated by reverse transcriptase stop. Pre-tRNA\^Ap of \textit{B.subtilis} and mat-tRNA\(^{\text{Asp}}\) of \textit{E.coli} were employed. (A) Primer extension of \textit{E.coli} RNase P RNA. (B) Primer extension of \textit{B.subtilis} RNase P RNA.

Assays carried out at high ionic strength indicate that much of the contribution of the CCA to the binding of pre-tRNA by the RNase P RNAs is conformational. For instance, in the case of the \textit{E.coli} RNase P RNA, removal of the entire CCA terminus results in a loss of 2.0 kcal/mol of binding energy (tRNA vs. tRNA\(\Delta\)CCA) at optimal ionic strength, but a loss of only 1.4 kcal/mol at high salt. Removal of the 3'-terminal A results in a loss of 0.8 kcal/mol at optimal ionic strength, but no loss (~0.3 kcal/mol) at high salt. One interpretation of these measurements is that the A does not contribute a specific contact, rather, stacking of the A on the adjacent C residues stabilizes the conformation of the latter for an optimal fit to the binding site of the ribozyme. Removal of one or both of the C residues, however, causes loss of binding energy at optimal and high ionic strength, indicative of specific contacts.

In order to test the base specificity of the contribution of the two C residues to binding, we made mutants in which the C residues, individually or together, were converted to other bases. At optimal ionic strength, replacement of either C residue results in loss of binding energy nearly equivalent to that contributed by the entire CCA sequence (Table I). At high ionic strength, nucleotides other than C can contribute slightly to binding, but there is a clear preference for C. The amount of energy involved in the interaction, ~0.5–1 kcal/mol (at high ionic strength), would be consistent with the formation of 1–2 H-bonds (24). Remarkably, the presence of a 3'-terminal arylazide, a photoaffinity agent (below), alone can substitute entirely for the presence of CCA in binding to the ribozyme (Table I). We have no information on whether or not the arylazide interacts with RNase P RNA in the same manner as the C residues. The interaction with the arylazide might be by H-bonding or by stacking.

Preparation of photoaffinity-labeled tRNA

A photoaffinity approach was used to map the path of the CCA on RNase P RNA. The 3'-ends of mutant precursors were modified in order to attach a photoaffinity agent, as outlined in Figure 1 and detailed in Materials and Methods. First, the ribose cis-diol at the 3'-terminus of the tRNA was oxidized by treatment with sodium periodate to create the dialdehyde (25). The oxidized tRNA was then alkylated under reducing conditions with either ethylene diamine or hexyl diamine (26). Finally, a primary amine-specific photoaffinity agent was reacted with the alkylated tRNAs (19). In order to monitor the extent of the reactions, a portion of the RNA from each step of the procedure was resolved by electrophoresis in an 8% denaturing polyacrylamide sequencing gel (Figure 1 inset). The results indicate that the chemical modifications of the tRNA are essentially quantitative.

Conditions for crosslinking RNase P RNA and pre-tRNA

Figure 2 shows examples of crosslinking analyses that illustrate some parameters of the reaction. Isotopically labeled pre-tRNAs, modified to contain the photoagent, were crosslinked to the three types of RNase P RNA and the products were resolved by gel electrophoresis. The electrophoretic patterns of conjugate species formed by the three RNase P RNAs are similar. In the case of the \textit{E.coli} RNA (Figure 2A), three main conjugate bands are evident and the pattern varies to some extent between the pre-tRNAs with different 3'-termini (below). As seen with the \textit{B.subtilis} RNA (Figure 2B), no conjugate bands occur in the absence of RNase P RNA; the crosslinked species corresponding to \textit{E.coli} bands 1 and 2 are not resolved.

Crosslinking was performed at high monovalent salt concentration to encourage binding, but Ca\(^{2+}\) was substituted...
for Mg$^{2+}$ and pH was reduced to 6.0. Under these conditions, tRNA binds as efficiently to the ribozyme as under the optimal reaction conditions, but the rate of cleavage is reduced ~10$^6$-fold (27, 28). Thus, the crosslinked species identified in Figures 2A and 2B are complexes of RNase P RNA and pre-tRNA substrate, not mat-tRNA product (Figure 2A and B). This is documented in Figure 2C, the results of an experiment in which two isotopically labeled pre-tRNA substrates containing the photoagent were irradiated in the presence of RNase P RNA from *T. maritima* and either Mg$^{2+}$ or Ca$^{2+}$, and the products resolved.
crosslinking in the template, producing a band in the gel that is not present if the template is irradiated in the absence of the crosslinking agent (lanes N). Primary crosslink sites (the nucleotide 5' to the gel band) are indicated in the figures. The crosslink sites of tRNAs containing the ethyl diamine connection to the photoagent were identified with the same method (data not shown). In the cases of the B. subtilis and T. maritima RNAs, the sites of crosslinking to the hexyl azide-containing tRNAs were identical to ethyl azide-containing tRNAs. In the case of the E. coli RNA, the tRNA–hexyl azide crosslinked to residues 285–287, whereas the tRNA–ethyl azide did not (Figure 4). This is consistent with the orientation of the CCA sequence on RNase P RNA (below) and the greater length of the hexyl connector.

It is evident from Figure 3 that pre-tRNA and the various deletion-mutant tRNAs crosslink to a different, but overlapping, suite of nucleotides in RNase P RNA. All these results, and those obtained in similar experiments with the T. maritima RNase P RNA, with both the hexyl diamine and ethyl diamine connectors, are summarized in Figure 4 and discussed further below (Discussion).

We also examined the crosslinking pattern of mat-tRNA<sup>Phe</sup> from E. coli with the RNase P RNAs of E. coli and B. subtilis. Crosslinking sites were, with one exception, the same for mat-tRNA<sup>Phe</sup> and pre-tRNA<sup>Asp</sup> (Figure 3 above), indicating the generality of the results to other tRNAs and the general equivalence of the 3'-termini of mat-tRNA and pre-tRNA in binding to the ribozyme. The exception is that the mat-tRNA<sup>Phe</sup> crosslinks to nucleotide 333 in the E. coli (but not the B. subtilis) RNase P RNA, whereas the pre-tRNA<sup>Asp</sup> does not crosslink efficiently at that site. It is possible that, in the case of the E. coli RNase P RNA, the leader sequence occludes crosslinking from the native 3'-end; the deletion mutants all crosslink to nucleotide 333. In the case of the B. subtilis RNA, the sites of crosslinking to pre-tRNA<sup>Asp</sup> and mat-tRNA<sup>Phe</sup> are the same. Taken together, the results indicate, consistent with other results, that the pre-tRNA and the mat-tRNA interact in the same way with RNase P RNA. The holoenzyme, however, is reported to bind pre-tRNA significantly better than mat-tRNA (29). Perhaps the protein moiety plays a role in fine discrimination.

**DISCUSSION**

As previously observed, the 3'-terminal CCA of tRNA contributes to binding by RNase P RNA (7, 10, 13) and to catalysis (30). In the present study, with RNase P RNAs from three different organisms, removal of the CCA resulted in an increase in <i>K<sub>m</sub></i> of ~30–60-fold at optimal ionic strength. Much of this contribution of the CCA to binding is due to conformational effects rather than specific contacts between the CCA and the RNase P RNA. This is indicated by the observations (Table I) that under reaction conditions of high monovalent ionic strength the difference in <i>K<sub>m</sub></i> of the ribozyme for pre-tRNA containing or lacking the CCA is only about 10-fold. The high ionic strength probably screens some of the electrostatic repulsion resulting from an unfavorable fit between the ribozyme and substrate lacking CCA. The influence of CCA in fostering the proper fit between the two RNAs could be on the tRNA or on the RNase P RNA. The CCA moiety of tRNA is a stacked helix (31), which could stabilize or subtly influence the conformation of the adjacent tRNA acceptor stem in which RNase P cleaves. In that case, in the absence of the CCA the substrate would be distorted and so interact with the ribozyme less favorably than does the CCA-containing tRNA. Alternatively, the interaction

![Diagram of RNase P RNA and tRNA interaction](image.png)

**Figure 5.** Orientation of the 3'-terminal CCA of tRNA in complex with RNase P RNA. The orientation of the CCA (highlighted) of tRNA on the internal loop of the E. coli RNase P RNA indicated by the crosslinking results (text) is shown.
of CCA might engender a small conformational change in the ribozyme that improves the fit with tRNA. The same subtle changes may be affected by the RNase P protein, since the holoenzyme does not manifest a requirement for the CCA for optimal binding.

These studies with the substitution mutants show that the interaction of the CCA with RNase P RNA is strongly dependent upon the sequence. The main contribution of the 3'-terminal A to the interaction seems to be conformational, however, the C residues together contribute ~1 kcal/mol to binding energy at high salt, indicating that specific contacts are formed. The energetic contribution of the two C residues would correspond to about the free energy contributed by one nucleotide in a triple helical array (32). Crosslinking experiments locate the binding site of CCA in an internal loop composed of nucleotides 254-259/291-295 (E.coli numbering). Results with the deletion mutants orient the interaction of CCA on the internal loop in RNase P RNA. As diagrammed in Figure 5, the CCA sits parallel to nucleotides 254-259 and antiparallel to nucleotides 290-295 in the internal loop. Chemical footprinting (33), mutational (34) and other crosslinking (22) studies are consistent with this location and orientation of the CCA association. Chemical and enzymatic susceptibility data suggest that this loop is an irregular helix (33, 35), so perhaps one or both of the C residues forms a triple helix with the RNase P RNA. Some nucleotides outside the loop also crosslink to the 3'-end of the tRNA (and to the 5'-end; 22), indicating their proximity in the tertiary structure of the ribozyme.

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