

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

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Received July 15, 1994; Revised and Accepted August 25, 1994

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 (k_{cat}/K_m) ionic strength (1 M NH_4^+ /25 mM Mg^{2+}), K_m increases incrementally 3- to 10-fold upon stepwise removal of each nucleotide from the 3'-end. At high ionic strength (2 M NH_4^+ /50 mM Mg^{2+}), which suppresses conformational effects, removal of the 3'-terminal A had little effect on K_m , 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 agreement with previous studies, we find that the RNase P holoenzyme *in vitro* does not discriminate between tRNAs containing or lacking CCA. The structural elements of the three RNase P RNAs in proximity to the 3'-end of tRNA were examined by photoaffinity crosslinking. Photoagent-labeled tRNAs with 3'-terminal CCA, only CC or C, or lacking all these nucleotides were covalently conjugated to the three RNase P RNAs by irradiation and the sites of crosslinks were mapped by primer extension. The main crosslink sites are located in a highly conserved loop (probably an irregular helix) that is part of the core of the RNase P RNA secondary structure. The crosslinking results orient the CCA of tRNA with respect to that region of the RNase P RNA.

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 vitro*, the K_m 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).

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 RNAs 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 CCGGACCGCCAGGTCTTCCATCC-TGCA and its complement TGGCGGTCCAGAAGGTAGG were used to create *FokI*, *BbsI* and *BstNI* restriction sites at the 3'-end of the *B. subtilis* tRNA^{Asp} gene. The vector plasmid

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pDW152 (16) was digested with the restriction enzymes *BspEI* 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* DH5 α F' and the constructs were confirmed by sequencing. A plasmid containing the desired sequences was named pDW152BF. The plasmid DNA digested with *BstNI*, *BbsI* 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 cytosine 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 or 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 I. Kinetic parameters of RNase P RNA

Substrate	<i>E. coli</i>				<i>B. subtilis</i>				<i>T. maritima</i>				
	K _m ± SD	k _{cat} ± SD	ΔG	ΔΔG	K _m ± SD	k _{cat} ± SD	ΔG	ΔΔG	K _m ± SD	k _{cat} ± SD	ΔG	ΔΔG	
Optimal	tRNA	12 ± 9	0.2 ± 0.1	-11.2	0.0	11 ± 7	0.7 ± 0.3	-11.3	0.0	17 ± 9	0.7 ± 0.4	-11.5	0.0
Salt	tRNAΔA	48 ± 10	0.8 ± 0.2	-10.4	0.8	30 ± 17	2.2 ± 0.5	-10.7	0.6	75 ± 30	2.0 ± 0.5	-10.5	1.0
	tRNAΔCA	180 ± 40	1.0 ± 0.1	-9.6	1.6	330 ± 280	7.8 ± 5.0	-9.2	2.1	370 ± 110	12 ± 5	-9.5	2.0
	tRNAΔCCA	350 ± 40	2.1 ± 1.0	-9.2	2.0	710 ± 220	10 ± 4.4	-8.7	2.6	970 ± 140	27 ± 12	-8.9	2.6
	tRNAΔA	380 ± 50	4.6 ± 0.5	-9.1	2.1	690 ± 490	21 ± 14	-8.7	2.6	540 ± 140	15 ± 5	-9.3	2.2
	tRNAΔCA	510 ± 200	3.0 ± 0.9	-8.9	2.3	ND	ND	ND	ND	ND	ND	ND	ND
	tRNAΔCA	360 ± 30	5.5 ± 0.9	-9.1	2.1	520 ± 50	18 ± 2.4	-8.9	2.4	770 ± 160	23 ± 6	-9.0	2.5
	tRNAΔCA	400 ± 220	3.4 ± 2.9	-9.1	2.1	1130 ± 430	11 ± 7.7	-8.4	2.9	370 ± 75	2.5 ± 0.7	-9.5	2.0
	tRNAΔCA	370 ± 100	2.2 ± 1.0	-9.1	2.1	510 ± 160	5.3 ± 4.1	-8.9	2.4	820 ± 480	3.1 ± 1.0	-9.0	2.5
	tRNAΔCA	180 ± 90	3.7 ± 2.5	-9.6	1.6	330 ± 150	4.2 ± 2.9	-7.8	3.5	290 ± 60	5.5 ± 0.7	-9.7	1.8
	tRNAΔCA	180 ± 40	0.4 ± 0.2	-9.6	1.6	ND	ND	ND	ND	ND	ND	ND	ND
High Salt	tRNA	19 ± 12	0.05 ± 0.01	-11.0	0.0	15 ± 5	0.6 ± 0.4	-11.1	0.0				
	tRNAΔA	11 ± 3	0.1 ± 0.02	-11.3	-0.3	20 ± 9	1.5 ± 0.1	-10.9	0.2				
	tRNAΔCA	71 ± 13	1.7 ± 0.2	-10.1	0.9	50 ± 15	5.8 ± 3.0	-10.4	0.7				
	tRNAΔCCA	160 ± 50	5.0 ± 3.0	-9.6	1.4	160 ± 40	15 ± 5	-9.6	1.5				
	tRNAΔCA	100 ± 30	3.3 ± 1.2	-9.9	1.1	90 ± 10	13 ± 1	-10.0	1.1				
	tRNAΔCA	70 ± 40	0.9 ± 0.03	-10.1	0.9	ND	ND	ND	ND				
	tRNAΔCA	50 ± 10	1.8 ± 0.4	-10.4	0.6	80 ± 30	11 ± 1	-10.0	1.1				
	tRNAΔCA	120 ± 66	3.2 ± 1.8	-9.8	1.2	170 ± 17	11 ± 7	-9.6	1.5				
	tRNAΔCA	110 ± 44	3.0 ± 1.7	-9.9	1.1	130 ± 24	8.2 ± 6.2	-9.8	1.3				
	tRNAΔCA	180 ± 160	6.9 ± 8.8	-9.6	1.4	150 ± 63	9.6 ± 5.3	-9.7	1.4				
tRNAΔCA	60 ± 10	0.9 ± 0.2	-10.2	0.8	ND	ND	ND	ND					
Phenylazide attached													
High Salt	tRNA	36	0.2	-10.6	0.0	21	0.8	-10.9	0.0				
	tRNAΔA	27	0.2	-10.7	-0.1	45	2.3	-10.4	0.5				
	tRNAΔCA	25	0.7	-10.8	0.1	42	4.4	-10.5	0.4				
	tRNAΔCCA	61	1.9	-10.2	0.4	28	3.2	-10.7	0.2				

K_m (nM)

k_{cat} (1/min)

ΔG (kcal/mol)

ΔΔG = -ΔG(tRNA- mutant tRNA)

The optimal (k_{cat}/K_m) condition for the *E. coli* and *B. subtilis* RNase P RNAs was 1.0 M NH₄Cl, 25 mM MgCl₂, 0.1% SDS, 0.05% Nonidet P-40 (NP40) and 50 mM HEPES/NaOH, pH 8.0, and the same for the *T. maritima* RNA except in containing 3.0 M NH₄Cl, 50 mM MgCl₂. The high salt reaction conditions for *E. coli* and *B. subtilis* RNAs were 2.0 M NH₄Cl, 50 mM MgCl₂, 0.1% SDS, 0.05% NP40 and 50 mM HEPES/NaOH, pH 8.0. Cleavage reactions were carried out and analyzed as described in Materials and Methods. K_m and k_{cat} were obtained from Lineweaver–Burk plots. The values shown are the mean of 3–10 repetitions. ΔG ($-RT \ln K_m - 1$) was calculated from the mean of K_m , and $\Delta \Delta G$ was obtained by the subtraction of ΔG for pre-tRNA from ΔG for experimental tRNAs. tRNA with the CCA sequence is designated tRNA, and tRNAs lacking A, CA, and CCA are respectively indicated as tRNA Δ A, tRNA Δ CA and tRNA Δ CCA. Mutagenized nucleotides in the 3'-terminal sequences of tRNA are underscored.

hydrolysis and chromatographic resolution of the released nucleotides (17).

Kinetic assays

Uniformly labeled ($[\alpha\text{-}^{32}\text{P}]\text{GTP}$) pre-tRNA^{Asp} of *B. subtilis* 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 nM RNase P RNA and in salt concentrations as indicated. Assays in the optimum (k_{cat}/K_m) salt condition for RNase P RNAs of *E. coli* and *B. subtilis* were carried out at 1.0 M NH_4Cl and 25 mM MgCl_2 at 37°C. Assays of *E. coli* and *B. subtilis* RNase P RNA under 'high salt' conditions were carried out in 2.0 M NH_4Cl and 50 mM MgCl_2 at 37°C. *T. maritima* RNase P RNA was assayed at 50°C in 3.0 M NH_4Cl and 50 mM MgCl_2 (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_3 at 37°C for 1 h. NaBH_4 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 photoagent (*N*-hydroxysuccinimidyl 4-azidobenzoate in DMSO) and 50 mM HEPES/NaOH, pH 9.0, at room temperature for 1 h in the dark (19). Modified tRNAs were precipitated in ethanol, dried *in vacuo*, resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored in the dark.

Formation of RNase P RNA-tRNA cross-links

Crosslinking reactions were performed in 2.0 M NH_4Cl , 0.1% SDS, 0.05% NP-40 and with either 25 mM CaCl_2 in 16.5 mM PIPES/44 mM Tris, pH 6.0, or 25 mM MgCl_2 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_2 (–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 *E. coli* RNase P RNA were: TTACC-TGGCACCCTGCC (60R), CTCTTACCSCACCNTTTCACCT (174RN), GGGTGGAGTTTACCGTGC (225R), and AAGTGGACTAAATG (370R). For *B. subtilis* RNase P RNA: CTTCGCTAGGWACGAACACT (80R), AAATTTGGGW-WWCTCGCTCGA (235R), and GTGGTCTAACGTTCTGT (382R) were used. For *T. maritima* RNase P RNA: RTAA-GCCGGRTTCTGT (347R), CCGGGCTGCAGGAATC (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_2 , 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% bromphenol 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.

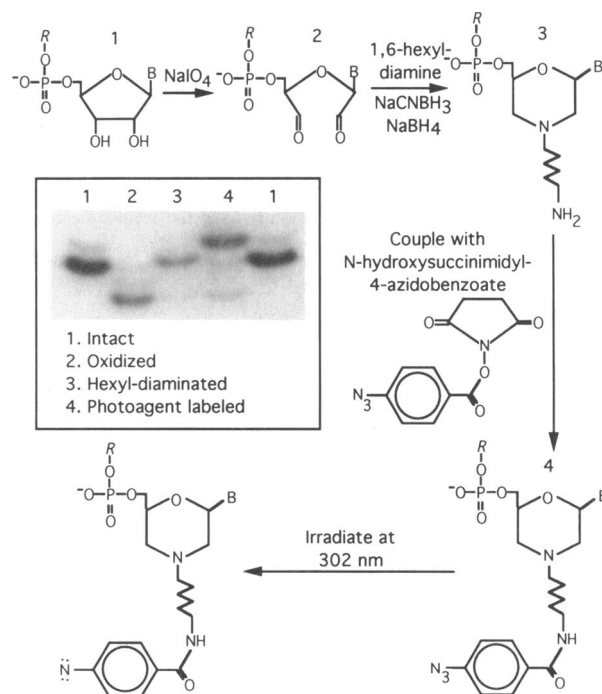


Figure 1. Preparation of 3'-photoagent-labeled tRNA. R and B designate the tRNA and base of the tRNA 3'-end, respectively. As detailed in Materials and Methods, the ribose *cis*-diol at the 3'-end of tRNA obtained by run-off transcription was oxidized with sodium periodate, modified with an alkyl diamine under reducing conditions, then *N*-hydroxysuccinimidyl-4-azidobenzoate was coupled to the alkyl diamine-attached-tRNA. A portion of labeled tRNA product from each step was resolved by electrophoresis in an 8% polyacrylamide gel containing 8 M urea and visualized by autoradiography (inset).

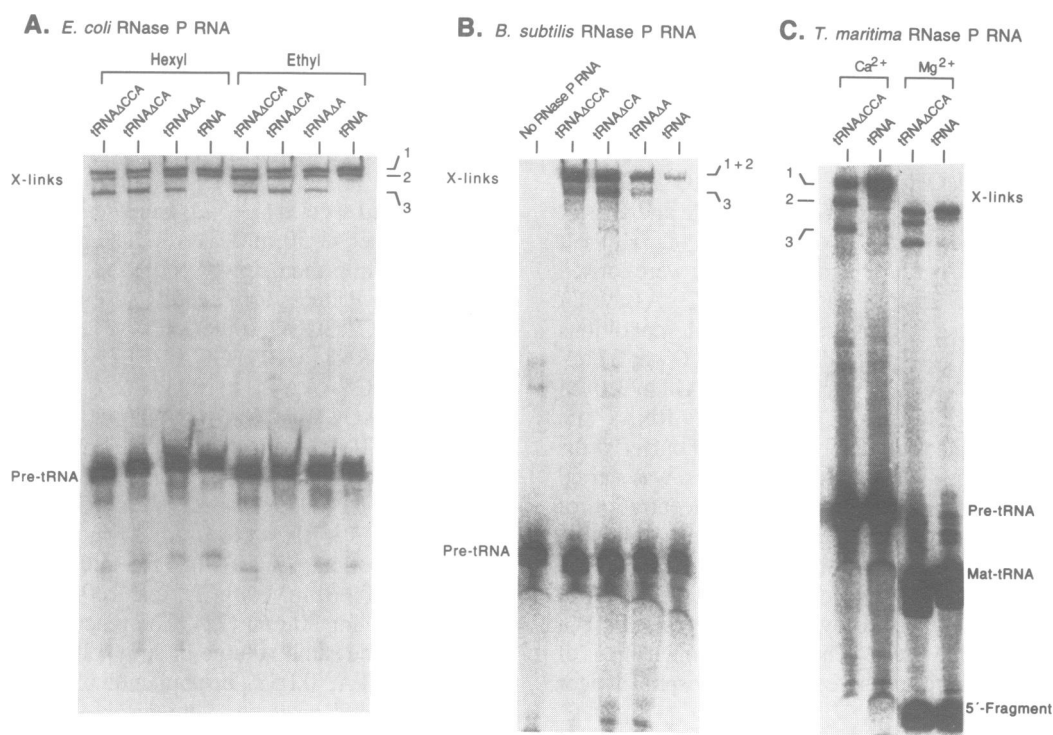


Figure 2. Some properties of tRNA crosslinking to three types of RNase P RNA. (A) Effect of CCA and crosslink length with *E. coli* RNase P RNA. *E. coli* RNase P RNA in crosslinking reaction buffer, containing 2.0 M NH_4Cl , 25 mM CaCl_2 , 0.05% NP40, 0.1% SDS, and 16.5 mM PIPES/44 mM Tris, pH 6.0 (28), was incubated at 37°C for 10 min and mixed with either tRNA–ethyl azide or tRNA–hexyl azide (uniformly ^{32}P -labeled), also preincubated in the same reaction buffer. The mixture was further incubated at 37°C for 10 min. The final concentrations of RNase P RNA and tRNA containing the azido group were 400 and 60 nM, respectively. Samples were irradiated for 30 min at 302 nm. RNAs were resolved in a 4% denaturing polyacrylamide gel and the results were analyzed with a Phosphorimager (Molecular Dynamics). (B) *B. subtilis* RNase P RNA–tRNA crosslinks. Crosslinking reactions were carried out with *B. subtilis* RNase P RNA and tRNA–ethyl azide as described above. The reaction was performed in 25 mM CaCl_2 to suppress processing of pre-tRNA. As a control (lane No RNase P RNA), tRNA–ethyl azide (uniformly ^{32}P -labeled) was irradiated without RNase P RNA. The results were analyzed with a Phosphorimager. (C) Crosslink patterns of pre-tRNA to *T. maritima* RNase P RNA in Mg^{2+} or Ca^{2+} . *T. maritima* RNase P RNA was preincubated at 50°C for 15 min, then mixed with uniformly labeled ($[\alpha\text{-}^{32}\text{P}]\text{GTP}$) tRNA–hexyl azide. The final concentrations of tRNA and RNase P RNA were 60 and 400 nM, respectively. The two tRNAs tested in this experiment are as indicated. The crosslinking reactions contained 3.0 M NH_4Cl , 0.05% NP40, 0.1% SDS and, as indicated, either 25 mM CaCl_2 in 16.5 mM PIPES/44 mM Tris, pH 6.0, or 25 mM MgCl_2 in 50 mM HEPES/NaOH, pH 8.0. Irradiation was carried out on ice for 30 min, products were resolved by electrophoresis in a 4% polyacrylamide gel containing 8 M urea and the results were analyzed with a Phosphorimager.

RESULTS

Kinetic analyses with mutant pre-tRNAs

As detailed in Materials and Methods, a transcription vector containing the gene for *B. subtilis* pre-tRNA^{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 *in vitro* transcription (Materials and Methods), contained the normal CCA (pre-tRNA), or lacked A (pre-tRNA Δ A), CA (pre-tRNA Δ CA), or CCA (pre-tRNA Δ CCA). 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_{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 *E. coli* and *B. subtilis*. The optimal salt concentration for the *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_{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 Δ CCA), an appreciable fraction ($\sim 20\%$) 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).

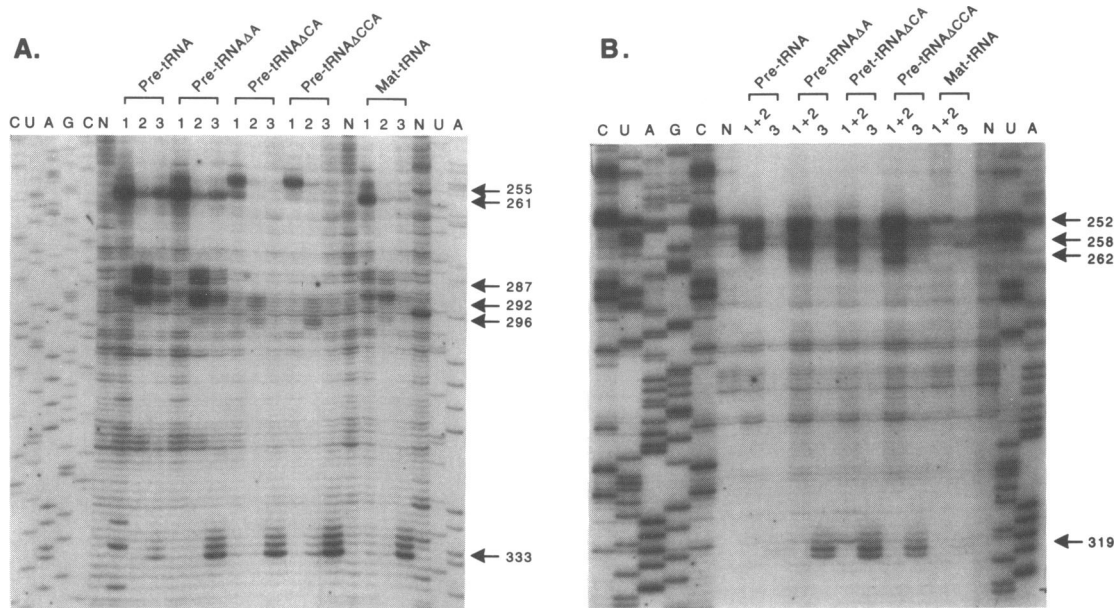


Figure 3. Identification of crosslink sites in RNase P RNA by primer extension. tRNA-hexyl azide (non-radiolabeled) was crosslinked with RNase P RNA. Purified crosslinked RNAs were recovered from denaturing polyacrylamide gels (Materials and Methods) and annealed with 5'-end-labeled primers complementary 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 dideoxynucleotides 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^{Asp} of *B. subtilis* and mat-tRNA^{Phe} of *E. coli* were employed. (A) Primer extension of *E. coli* RNase P RNA. (B) Primer extension of *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 *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 Δ 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 *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 *B. subtilis* RNA (Figure 2B), no conjugate bands occur in the absence of RNase P RNA; the crosslinked species corresponding to *E. coli* bands 1 and 2 are not resolved.

Crosslinking was performed at high monovalent salt concentration to encourage binding, but Ca²⁺ was substituted

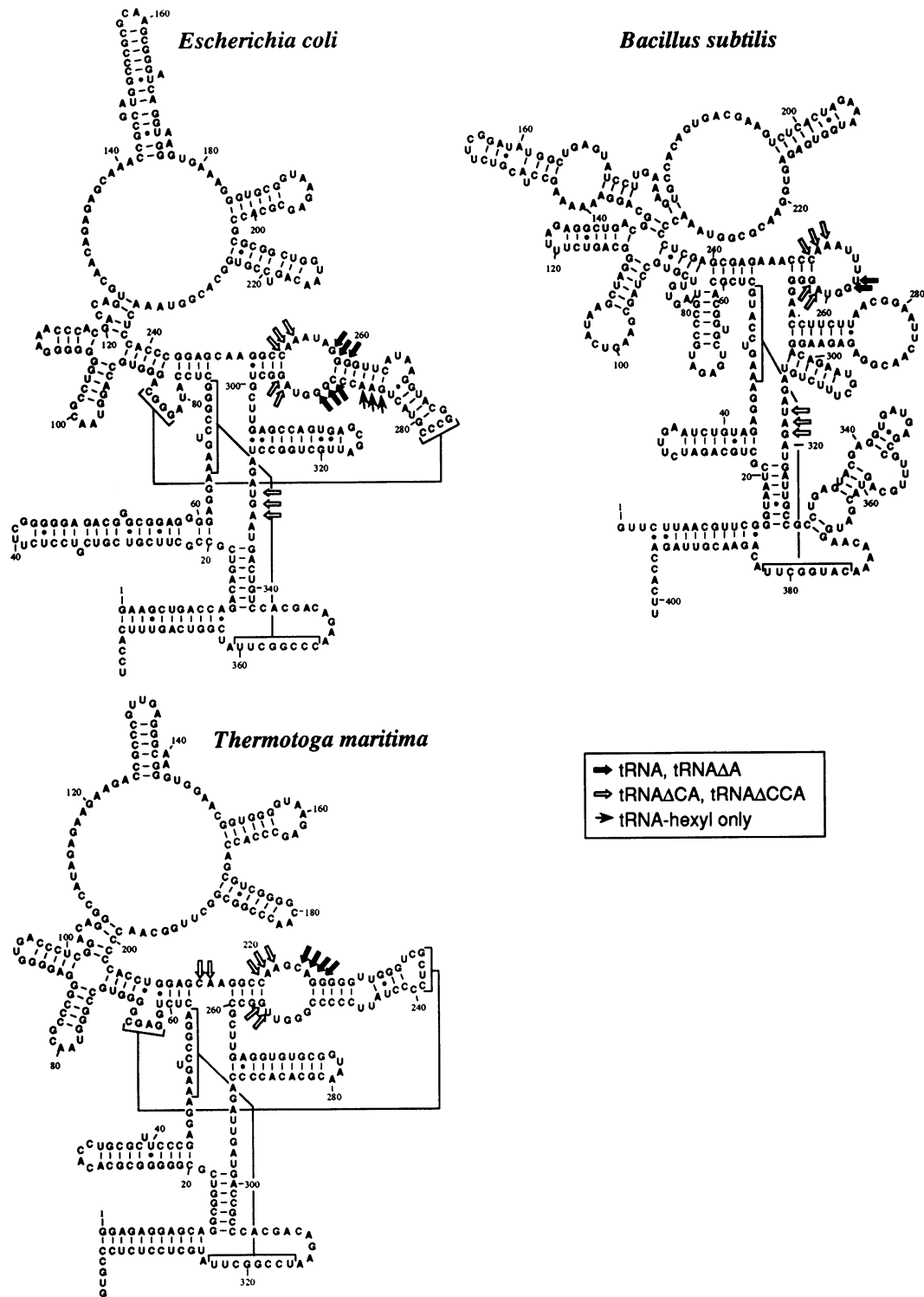


Figure 4. Interaction sites of RNase P RNA with the 3'-end of tRNA. Crosslink sites of the indicated RNase P RNAs and tRNAs as designated in the box are shown in the secondary structures of the RNase P RNAs by the use of arrows.

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 $\sim 10^6$ -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

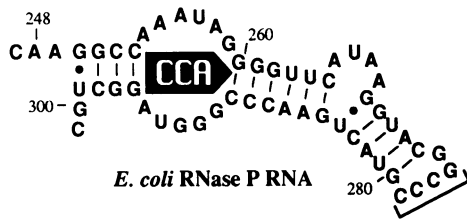


Figure 5. Orientation of the 3'-terminal CCA of tRNA in complex with RNase P RNA. The orientation of the CCA (highlighted) of tRNA on the internal loop of the *E. coli* RNase P RNA indicated by the crosslinking results (text) is shown.

by gel electrophoresis. It is evident from the autoradiogram that in the presence of Mg^{2+} all or most of the labeled pre-tRNA is cleaved by the RNase P RNA, resulting in the formation of mat-tRNA and the precursor-specific fragment. In contrast, little reaction occurred in the presence of Ca^{2+} . The more rapid electrophoretic migration of the conjugates produced in the presence of Mg^{2+} indicates that those conjugates contain mature tRNA, whereas those formed in the presence of Ca^{2+} contain pre-tRNA. The similarity of the patterns and distributions of conjugates formed in the presence of Mg^{2+} or Ca^{2+} indicates that the precursor and mature tRNAs interact equivalently with RNase P RNA. Indeed, crosslinking of mat-tRNA to the RNase P RNA resulted in the same conjugates observed upon crosslinking pre-tRNA in the presence of Mg^{2+} (data not shown). Irradiation of the RNase P RNA with pre-tRNA and pre-tRNA Δ CCA, however, results in a different distribution of conjugate bands in Figure 2C. This difference and the comparable results seen in the other panels, with the other RNase P RNA, indicate that the 3' termini of tRNA and tRNA Δ CCA crosslink to different sites in the RNase P RNAs (shown below).

Figure 2A also compares crosslinking results obtained using modified pre-tRNAs containing linkers of different length between the 3'-ends of the RNAs and the azido crosslinking group. Modification with hexyl diamine or ethyl diamine results in crosslinks of 14 or 9 Å, respectively. As seen in the figure, the pattern of crosslinking was the same for the two types of crosslinker arms. Similar comparisons were seen with the RNase P RNAs from *B. subtilis* and *T. maritima*. In each case, use of the ethyl diamine crosslinker arm resulted in a somewhat higher crosslinking efficiency (less than two-fold) than that of the longer, hexyl diamine arm.

Analysis of crosslink sites by primer extension

Nonradioactive native and deletion-mutant tRNAs containing the photoagent connected by either hexyl or ethyl diamine were crosslinked to each of the three types of RNase P RNA by UV irradiation. Individual crosslinked species equivalent to those seen in Figure 2 were isolated from 4% denaturing polyacrylamide gels, and the sites of crosslinking were determined by primer extension using reverse transcriptase (RT). Three conjugate bands were analyzed in the cases of the *E. coli* and *T. maritima* RNAs (as seen in Figures 2A and 2C) and two in the case of the *B. subtilis* RNase P RNA (Figure 2B).

Figures 3A and 3B show primer extension analyses of, respectively, the *E. coli* and *B. subtilis* RNase P RNA conjugates with tRNAs containing the hexyl diamine coupling to the phenyl azide. RT halts one nucleotide prior to (3' to) the actual site of

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 tRNA 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^{Phe} 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^{Phe} and pre-tRNA^{Asp} (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^{Phe} crosslinks to nucleotide 333 in the *E. coli* (but not the *B. subtilis*) RNase P RNA, whereas the pre-tRNA^{Asp} 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^{Asp} and mat-tRNA^{Phe} 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 K_m 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 K_m 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

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.

ACKNOWLEDGEMENTS

We are very grateful to Dr Jim M. Nolan for his help in this project and for reviewing this manuscript carefully. We thank Dr David S. Waugh (Hoffmann-La Roche) for the gift of *FokI* enzyme used in this study, and Dr Bernadette Pace for T7 RNA polymerase. We also thank Ms Mary Anne Rubio and Ms Suzanne Schwartz for help in preparation of this manuscript. This research was supported by NIH grant GM 34527 to N.R.P.

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