Participation of the 3′-CCA of tRNA in the Binding of Catalytic Mg^{2+} Ions by Ribonuclease P†

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ABSTRACT: Ribonuclease P (RNase P) contains a catalytic RNA that cleaves precursor tRNA (pre-tRNA) to form the mature 5′-end of tRNA. Previous kinetic analyses with mutant pre-tRNAs indicated that both C residues of the invariant 3′-terminal CCA form specific interactions with RNase P RNA that contribute to the energetics of substrate binding (*1*, *2*). In the present study, we have used single-turnover kinetic analysis to investigate whether specific changes in the 3′-terminal CCA influence the rate of the chemical step through which enzyme-bound substrate is converted to product (k_2). At optimal ionic strength (1.0 M NH_4Cl, 25 mM $MgCl_2$), deletion or substitution of the 3′-proximal C residue (C<u>C</u>A) reduced the rate of the chemical step of cleavage (k_2) by 60-fold. Similar changes to the 5′-proximal C residue (<u>C</u>CA) or the 3′-terminal A residue (CC<u>A</u>) reduced k_2 only a few fold. Each mutant substrate exhibited weakened affinity for Mg^{2+}, as measured by Hill plots, and the severity of these defects correlated with the observed reductions in k_2. Furthermore, elevated concentrations of Mg^{2+} partially, but not completely, suppress the k_2 defects caused by deletion or substitution of the 3′-proximal C residue. We conclude that the 3′-CCA of pre-tRNA, particularly the 3′-proximal C residue, comprises part of the catalytic pocket formed in the pre-tRNA−RNase P complex and participates in the binding of Mg^{2+} ions that are essential for catalysis by RNase P RNA.

All organisms and organelles that synthesize tRNA require the activity of ribonuclease P, the endonuclease that cleaves 5′-leaders from pre-tRNAs, to form the mature 5′-ends of tRNA (see *3*−*6* for a review). In bacteria, RNase P is composed of a large RNA subunit (∼400 nucleotides) and a small protein subunit (∼120 amino acids; *7*−*9*). In vitro and at high ionic strength, the RNA alone can function as an RNA enzyme (ribozyme) that cleaves pre-tRNA in the absence of protein (*10*). Both monovalent and divalent cations play critical roles in the reaction of RNase P RNA. Such diverse experimental approaches as intermolecular cross-linking, Fe-EDTA modification−interference, and gel filtration demonstrate that monovalent cations are necessary for both proper folding of the ribozyme and substrate binding (*11*−*13*). It is likely that monovalent cations function primarily as counterions that screen ionic repulsion between phosphate groups (*14*, *15*).

While divalent cations (preferably Mg^{2+} or Mn^{2+}) also enhance substrate binding and folding of RNase P RNA (particularly at low ionic strength; *12*, *13*), they are additionally essential for catalytic activity (*16*−*21*). Multiple Mg^{2+} ions are required for the optimal activity of RNase P RNA, and catalysis exhibits a cooperative dependence upon Mg^{2+} concentration (*21*). Both the 2′-OH group of the 5′-proximal C at the 3′-end of tRNA (<u>C</u>CA) and the 2′-OH group adjacent to the cleaved phosphodiester bond of tRNA are critical for catalysis, and it has been proposed that these groups are involved in metal binding (*21*, *22*). Additionally, the 5′-phosphate of residue A67 of the *E. coli* RNase P RNA has been implicated specifically in binding Mg^{2+} ions involved in catalysis, by manganese rescue of a kinetic defect introduced by phosphorothioate substitution at this site (*23*).

RNase P acts on all species of tRNA, so it is expected that sequences and/or structures common to all pre-tRNAs are the features recognized by RNase P. Analysis of deletion constructs has revealed that RNase P RNA recognizes its substrate primarily through interactions with the coaxial helix formed by the acceptor- and T-stems (*24*). The results of intermolecular cross-linking and modification−interference experiments have provided further support for this conclusion (*11*, *25*−*30*). Only two well-conserved sequence elements are found in the acceptor- and T-stems: the 3′-terminal CCA and the GUUCG sequence of the T-loop. Nucleotides in the T-arm contribute to substrate recognition, but their exact functions have not been elucidated (*28*, *30*).

The role of the 3′-CCA, however, has been explored more fully, and this element clearly is important in the action of RNase P. In vitro, the K_M of bacterial RNase P RNA for tRNA lacking CCA, or tRNA in which CCA has been mutated, is 10−20-fold higher than for native tRNA (*1*, *2*, *31*−*35*). Detailed kinetic analysis of pre-tRNA substrates bearing deletions or nucleotide substitutions in the 3′-CCA

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suggests that the 3′-terminal A makes only a minor, sequence-independent contribution to substrate binding that is dispensable at elevated ionic strength (*1*). In contrast, both C residues of the 3′-CCA contribute to optimal binding in a sequence-specific manner. Deletion or modification of either residue, particularly the 3′-proximal C (CCA), weakens substrate binding to a much greater extent than does deletion of the 3′-A. A binding site for 3′-CCA has been identified within the J15/J16 internal loop of the bacterial RNase P RNA, by a combination of chemical modification−protection assay, compensatory−mutational analysis, and intermolecular cross-linking (*1*, *2*, *36*). Kirsebom and Svard (*2*) have proposed that the 3′-CCA associates through Watson−Crick base pairs with residues of the J15/J16 loop. In contrast, Easterwood and Harvey (*37*) have proposed a more complex set of tertiary interactions between the 3′-CCA and the structure formed by non-Watson−Crick pairings within the J15/J16 loop.

Previous mutational analyses of the function of the tRNA 3′-CCA in the RNase P reaction primarily measured the binding affinity of RNase P RNA for the mutant substrates (i.e., K_D or K_M effects; *1*, *2*). Under the steady-state conditions used in such experiments, the catalytic rate, k_{cat}, is limited by the rate of product release, and so the effects of alterations in 3′-CCA on the actual rate of catalysis could not be assessed (*15*, *21*, *38*, *39*). We have now analyzed how the structure of the 3′-CCA affects k_{cat} under single-turnover reaction conditions, which more closely reflect the rate of catalysis by RNase P. Our results indicate that, in addition to enhancing substrate binding, the 3′-CCA, in particular the 3′-proximal C residue, also specifically functions in catalysis by contributing to the binding of catalytic Mg^{2+} ions.

MATERIALS AND METHODS

Preparation of RNA by Runoff Transcription. Unlabeled RNase P RNAs were prepared by runoff transcription in vitro of plasmid DNA with T7 RNA polymerase (20 mM sodium phosphate, pH 7.7, 8 mM $MgCl_2$, 2 mM DTT, 5 mM spermidine, 1 mM each rNTP, 37 °C, overnight). RNAs were purified by electrophoresis through 8 M urea, 4% polyacrylamide gels in TBE (90 mM Tris−borate, 9 mM EDTA, pH 8), viewed by UV shadow, excised, and eluted into 40 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, and 2 mM DTT. Radiolabeled tRNAs were prepared by addition of [α-^{32}P]GTP (3000 Ci/mmol, Amersham Corp.) to a transcription mixture in which the unlabeled GTP concentration was lowered to 0.1 mM. RNA concentrations were determined by incorporation of isotopic label or by UV spectrophotometry.

Determination of Kinetic Parameters. Uniformly radiolabeled *B. subtilis* pre-tRNA^{Asp} and variants thereof were as described in Oh and Pace (*1*). Kinetic assays were performed in 16.5 mM PIPES/44 mM Tris, pH 6, 0.05% NP-40, 0.1% SDS, 20 nM substrate, and monovalent salt concentrations as indicated, at 37 °C. RNase P RNAs and substrates were preincubated separately in reaction buffer at 37 °C (the reaction temperature) for 5−10 min, and then mixed to start the reaction. Pseudo-first-order cleavage rate constants (k_{obs}) were determined from plots of $\ln(S_0/S_t)$ vs time, where S_0 is the initial substrate concentration and S_t is the substrate remaining at a given time point. The concentration of enzyme used in these experiments ranged from 200 to 4000 nM. Reactions were quenched by addition of 2.5 volumes of cold ethanol, sodium acetate to 0.3 M and EDTA to twice the $MgCl_2$ concentration of the reaction. Reaction products were resolved by electrophoresis in 8% denaturing polyacrylamide gels. The relative intensities of the appropriate RNA bands were measured using a phosphorimager (Molecular Dynamics).

Determination of M^{2+} Binding Constants. Conditions for steady-state reactions were 20 nM pre-tRNA, 1.0 nM *E. coli* RNase P RNA, 50 mM HEPES/NaOH, pH 8, 0.05% NP-40, 0.1% SDS, NH_4Cl as indicated, and 1−300 mM $MgCl_2$ or 1−100 mM $MnCl_2$. For holoenzyme reactions, pre-tRNAs were incubated with 1.0 nM *E. coli* RNase P RNA and 1.0 nM *E. coli* RNase P protein in a reaction buffer consisting of 50 mM HEPES/NaOH, pH 8, 0.05% NP-40, 100 mM NH_4Cl, and 1−100 mM $MgCl_2$. Conditions for single-turnover reactions were 20 nM pre-tRNA, 250 nM *E. coli* RNase P RNA, 16.5 mM PIPES/44 mM Tris, pH 6, 0.05% NP-40, 0.1% SDS, 1.0 M NaCl, and 1−600 mM $MgCl_2$ or 1−300 mM $MnCl_2$. Reactions were quenched by the addition of 2.5 volumes of cold ethanol. Reaction products were resolved by electrophoresis in 8% denaturing polyacrylamide gels and quantitated by phosphorimaging (Molecular Dynamics). Apparent M^{2+} binding constants were calculated from Hill plot parameters (*40*).

RESULTS

Role of the 3′-End of Pre-tRNA in Catalysis. The RNase P reaction is described in the kinetic Scheme 1, where E is RNase P RNA, S is pre-tRNA, P is mature tRNA, and the asterisk indicates complex formation:

Scheme 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*S \xrightarrow[(k_{chem})]{k_2} E^*P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

RNase P RNA binds substrate and products equally well ($k_1 \approx k_{-3}$), so the product, mature tRNA, is released only slowly. During multiple-turnover reactions catalyzed by the RNase P ribozyme, product release rather than substrate cleavage is rate-limiting (*15*, *21*, *38*, *39*). Consequently, k_{cat} reflects only the off-rate of product release and does not address the rate of the chemical step, k_2. To determine the role of the 3′-end of pre-tRNA in the catalytic event, mutant substrates altered in the native 3′-CCA were analyzed under single-turnover conditions (i.e., [E] ≫ [S]), in which the product release step is inconsequential and the chemical step is examined directly. At saturating enzyme concentrations, essentially all substrate is bound to the ribozyme, and so the rate of product formation is characterized by simple, first-order kinetics; under these conditions, the reaction rate constant k_2 reflects the rate at which the enzyme−substrate complex catalytically resolves into the enzyme−product complex. In the case of the native substrate, k_2 of the ribozyme exhibits a first-order dependence on [OH^-] throughout the range of pH 6−8; the native RNase P RNA reaction at pH 8 proceeds at 200−300 min^{-1}, but at pH 6 only 2−3 min^{-1}, amenable to manual sampling (*21*). In these experiments, therefore, reactions were carried out at pH 6 in order

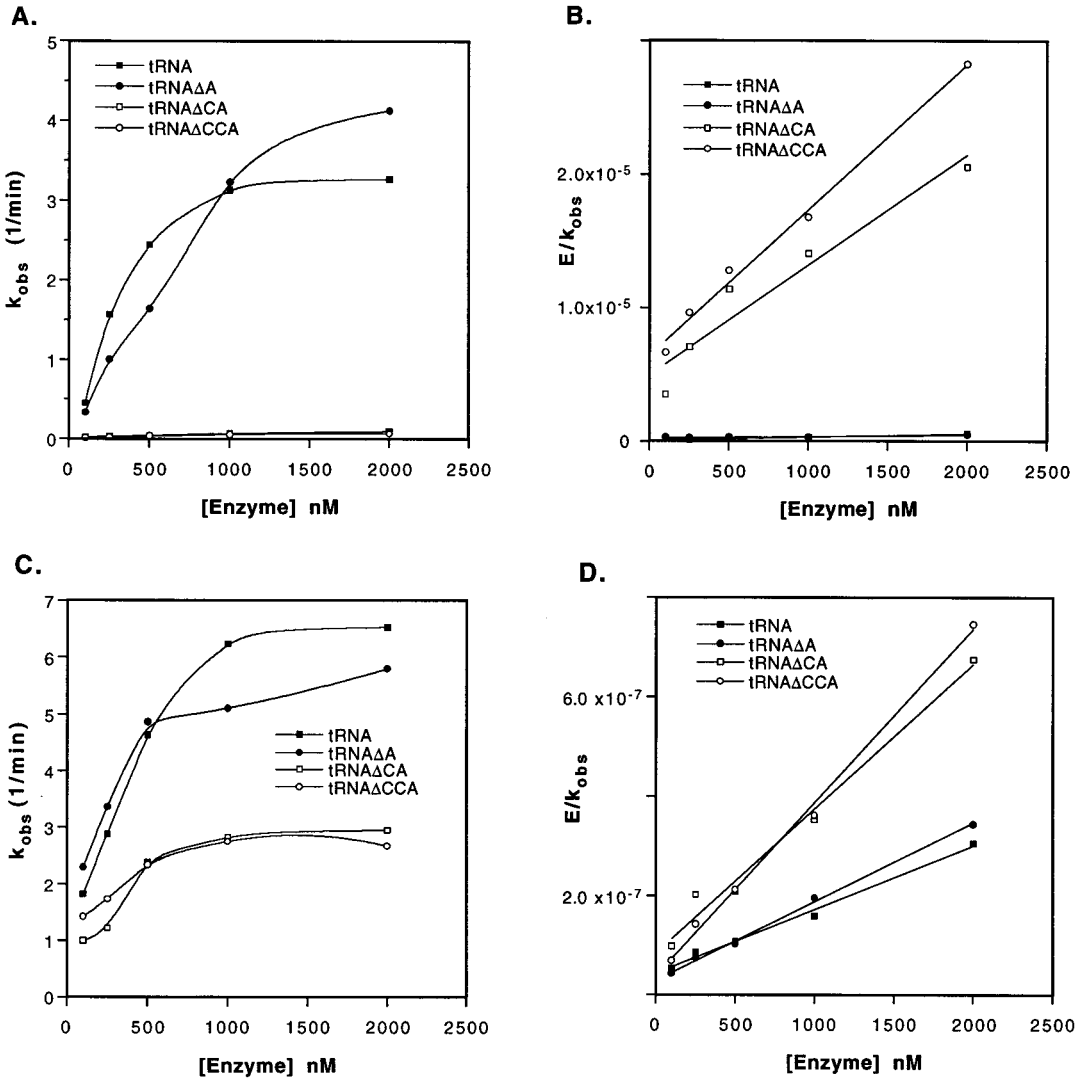

FIGURE 1: Measurement of the cleavage reaction rate (k_2) with mutant pre-tRNAs. The value of k_2 at pH 8.0 was extrapolated from the k_{cat} of the single-turnover reaction at pH 6.0. Reaction conditions were 20 nM pre-tRNA, 0.05% NP-40, 0.1% SDS, and 16.5 mM PIPES/44 mM Tris, pH 6.0, with *E. coli* RNase P RNA and monovalent salt as indicated. Optimal ionic strength reactions (A and B) contain 1.0 M NaCl, 25 mM $MgCl_2$, and high ionic strength reactions (C and D) contain 2.0 M NaCl, 50 mM $MgCl_2$. Rate constants (k_{obs}) were measured from pseudo-first-order decay reactions (Materials and Methods). The data were linearized on a Hanes−Woolf plot from which k_{cat} at pH 6.0 was derived from slope^{-1} (B, D). Note that the coordinates of the *y*-axes differ in the plots.

to slow the reaction sufficiently that the chemical step of the reaction could be measured accurately.

A series of pre-tRNA substrates differing solely in 3′-CCA structure (*1*) were subjected to k_2 determinations. Since suboptimal salt concentrations can magnify the effects of 3′-CCA mutations on multiple-turnover reactions (*1*, *32*), we determined single-turnover rate constants (k_2) over a range of monovalent and divalent salt concentrations. For each substrate, first-order cleavage rate constants were measured over a range of enzyme concentrations (e.g., Figure 1A,C). Values of k_2 were then extracted from Hanes−Wolff plots (e.g. Figure 1B,D and Table 1). As summarized in Table 1, removal of the 3′-terminal A from the substrate (tRNAΔA in Table 1) results in diminution of the catalytic rate, but full activity is recovered at high ionic strength. The suppression by high ionic strength of the influence of the 3′-A on reaction rate demonstrates that the 3′-A has no specific role in the catalytic site. In contrast, removal or mutation of the CC moiety of the 3′-CCA results in diminished catalytic rates that are not fully restored at high ionic strength (Figure 1, Table 1). These results indicate that the structure of the 3′-end of pre-tRNA, especially the identity of the 3′-proximal C residue (Table 1), directly influences the chemical step of the RNase P reaction. Either deletion or substitution of the 3′-proximal C residue produced equivalent effects on catalysis, indicating the specific involvement of that base moiety in catalytic activity.

Elevated concentrations of Mg^{2+} also partially suppress the k_2 deficiencies that are exhibited by the mutant pre-tRNAs (Table 1). It is unlikely that the ability of Mg^{2+} to suppress the defects caused by 3′-CCA mutations is explained solely by ionic strength effects, because at 1.0 M NaCl the increase in Mg^{2+} concentration (from 25 to 50 mM) only slightly alters the ionic strength of the reaction (from 1.1 to 1.2 M). Consequently, the suppression of k_2 defects by elevated Mg^{2+} suggests that the binding of catalytically important Mg^{2+} ions is disrupted in substrates with mutated 3′-CCA. To test this hypothesis more directly, we measured the apparent binding affinities of ribozyme complexes containing either native or 3′-end disrupted substrates for Mg^{2+}, and Mn^{2+}, under either

Table 1: Effect of 3′-CCA Structure on Rate of RNase P RNA Catalysis (k_2) in Single-Turnover Reactions

	$k_2 \pm$ SD (min^{-1})[a]					
[NaCl] (M):	1.0		2.0		3.0	
[$MgCl_2$] (mM):	25	50	25	50	25	50
substrates						
pre-tRNA	1.3 ± 0.4	1.2 ± 0.4	2.0 ± 0.4	1.3	1.5 ± 0.03	1.2
pre-tRNAΔA	0.7 ± 0.3	1.1 ± 0.4	2.4 ± 0.9	1.3	1.3 ± 0.2	1.1
pre-tRNAΔCA	0.02 ± 0.01	0.1 ± 0.06	0.4 ± 0.2	0.5	0.2 ± 0.04	0.4 ± 0.08
pre-tRNAΔCCA	0.02 ± 0.01	0.2 ± 0.07	0.2 ± 0.05	0.5	0.2 ± 0.02	0.4 ± 0.04
pre-tRNACUA	0.02 ± 0.0	0.3	nd[b]	0.5	0.3 ± 0.03	0.4 ± 0.04
pre-tRNAUCA	0.3 ± 0.1	0.5	nd	1.1	0.8 ± 0.2	0.8 ± 0.3

[a] Measured at pH 6. [b] nd: not determined.

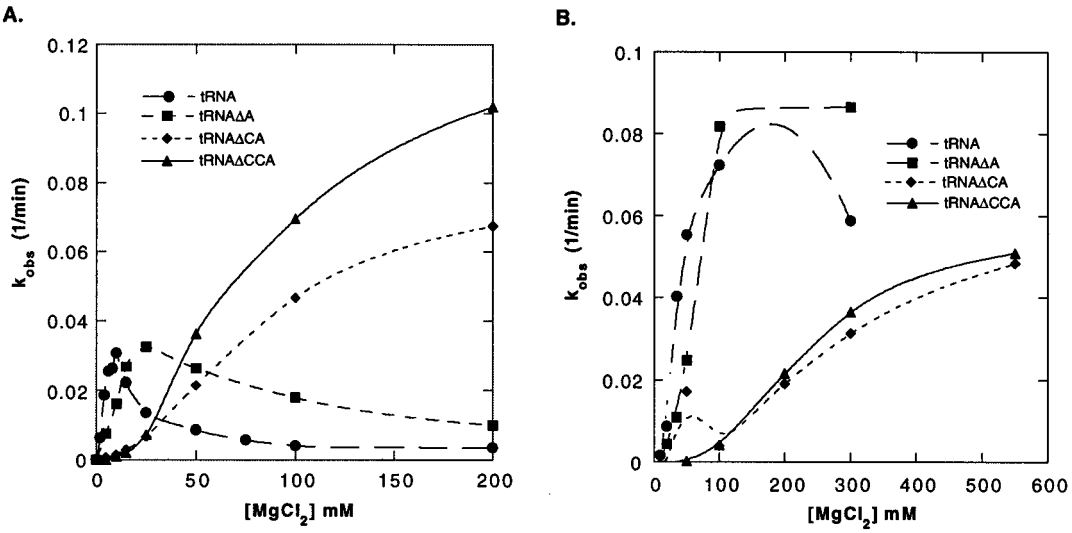

FIGURE 2: Titration of Mg^{2+} in reactions of 3′-CCA mutant pre-tRNAs with RNase P RNA. The conditions of both steady-state (A) and single-turnover (B) reactions are detailed under Materials and Methods, with [Mg^{2+}] as indicated in the figure. Pseudo-first-order reaction rate constants (k_{obs}) are depicted as a function of [Mg^{2+}]. Note that the coordinates of the *x*- and *y*-axes differ in the plots.

Table 2: Effect of 3′-CCA Sequence on Divalent Metal K_D^{app} Values

	multiple-turnover				single-turnover	
divalent:	Mg^{2+}			Mn^{2+}	Mg^{2+}	Mn^{2+}
monovalent:[a]	1.0 M	2.0	0.1[b]	1.0	1.0	1.0
substrates						
pre-tRNA	3 mM	2	3	0.4	41	6
pre-tRNAΔA	8	6	5	0.8	77	17
pre-tRNAΔCA	51	25	6	4	240	18
pre-tRNAΔCCA	47	20	5	4	312	17
pre-tRNACUA	45	nd[c]	6	4	nd	nd
pre-tRNAUCA	15	nd	5	nd	nd	nd

[a] Multiple-turnover reactions were in NH^{4+}. Single-turnover reactions were in Na^+. [b] Holoenzyme reaction. [c] nd: not determined.

multiple-turnover (Figure 2A, Table 2) or pre-steady-state conditions (Figure 2B, Table 2). We also tested the influence of the protein moiety of RNase P on the divalent cation requirement for catalysis (Table 2). The RNase P protein has long been recognized to suppress many mutations in the RNA moiety of the holoenzyme and to relax the preference of the enzyme for substrates containing the native 3′-CCA. The data were analyzed by Hill plots (Figure 3 and data not shown; *40*), from which the cooperativity and apparent binding constants for divalent metals are deduced (Table 2).

At 1.0 M NH_4^+, deletion of the 3′-A or 3′-CA residues resulted in incremental increases in K_D^{app} for Mg^{2+} (3-fold vs 17-fold, respectively), while removal of 3′-CCA produced no additional effect on magnesium binding affinity. The results of assays of the base substitution mutants were consistent with those of the deletion mutants. Higher ionic strength (2 M NH_4^+) enhanced the Mg^{2+} affinity of complexes containing all substrates; however, the relative effects of the 3′-CCA mutations were generally similar to those observed at lower ionic strength (1 M NH_4^+). Although the structure of the 3′-end of pre-tRNA significantly affected the K_D^{app} for Mg^{2+}, the number of cooperatively bound Mg^{2+} ions that result in maximum activity, as measured from the slopes of Hill plots (Figure 3), were not notably different among the various substrates. As reported previously (*21*, *41*), multiple Mg^{2+} (minimum three) ions are required for optimal activity in the *E. coli* RNase P RNA reaction.

In single-turnover reactions, the K_D^{app} for Mg^{2+} was incrementally increased by removal of two nucleotides from the 3′-end, but no further increase occurred by removal of the entire 3′-CCA (Figure 2B, Table 2). In general, these results are consistent with the data obtained from multiple-turnover reaction conditions, although the effects of 3′-CCA mutations were not as drastic under single-turnover conditions as under multiple-turnover conditions. K_D^{app} values obtained in single-turnover experiments with the RNA-alone reaction were somewhat higher (5−6-fold) than in multiple-turnover assays. We attribute the differences in these values to the different monovalent ions used in the two cases (Na^+ for single-turnover vs NH_4^+ for multiple-turnover reactions);

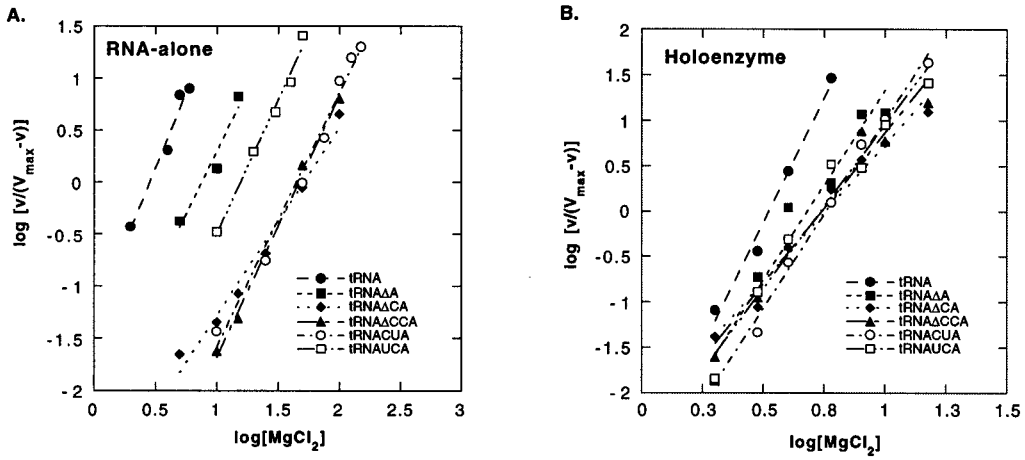

FIGURE 3: Measurement of apparent Mg^{2+}-binding constants in RNA-alone and holoenzyme reactions with mutant pre-tRNAs. Conditions for RNA-alone (A) and holoenzyme (B) reactions are detailed under Materials and Methods. First-order reaction rates were measured under either steady-state or single-turnover (data not shown) conditions. The data were linearized by a Hill plot. The dissociation constant of Mg^{2+} was obtained from the intercept of the *x*-axis where $Y\{\log[v/(V_{max} - v)]\} = 0$ and the slope (~3) of the plot indicates the number of cooperative Mg^{2+}-binding sites.

Na^+ is slightly less effective than NH_4^+ in supporting catalysis by RNase P, but is necessary under low-pH conditions because of the buffering capacity of NH_4^+ (K^+ is excluded because of the inclusion of SDS in RNA-only reactions). The RNase P protein has no effect on K_D^{app} of the enzyme−substrate complex for Mg^{2+} in the case of the native substrate, but strongly suppresses the effect on K_D^{app} of removal or alteration of CCA.

Accurate Cleavage by RNase P RNA of 3′-Modified tRNAs. RNase P RNA exhibits remarkable specificity for cleavage at the correct phosphodiester bond, and it has been shown that pre-tRNAs modified at the 5′-end also are accurately cleaved by RNase P RNA (*21*). However, since some ribozyme mutants reportedly cleave some pre-tRNAs inaccurately (*4*), we tested whether 3′-end modifications would induce inaccurate cleavage. Uniformly labeled pre-tRNAs were incubated with the ribozyme under optimal ionic conditions (1.0 M NH_4Cl, 25 mM $MgCl_2$), and the product RNAs were separated on 8% polyacrylamide sequencing gels, which separates 5′-leader sequences with sufficient resolution to detect single-nucleotide differences in size. The length of the 5′-leader sequence was identical to that of the native pre-tRNA for all of the deletion and substitution pre-tRNA substrates used in this study (data not shown). Identical experiments were performed at other ionic strengths to determine whether specificity might be altered under less-than-optimal conditions. No aberrant cleavage was observed for any of the mutant substrates under any set of conditions.

DISCUSSION

The structure of the 3′-end of pre-tRNA has long been known to affect the activity of bacterial RNase P (*1*, *2*, *31*−*34*, *42*) (Figure 4). For instance, mutations that block the activity of the CCA-adding enzyme of *E. coli* (*cca*$^-$) cause the accumulation of 5′-immature tRNA, suggesting that, at least in vivo, an intact 3′-CCA is required for processing by the RNase P holoenzyme (*42*). Paradoxically, the in vitro activity of the RNase P holoenzyme is not dramatically affected by the presence or absence of 3′-CCA (*1*, *31*). It is possible that the differences between in vivo and in vitro results reflect differences in either the stoichiometries of

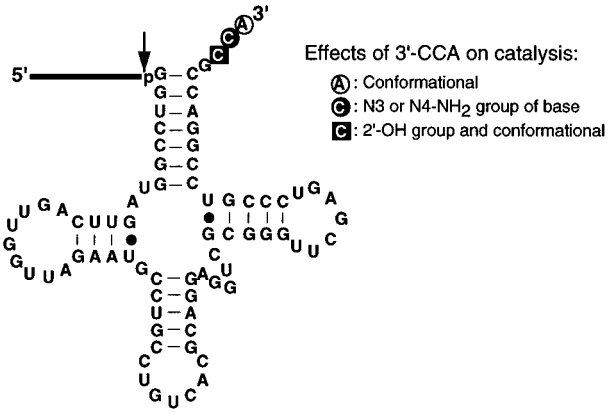

FIGURE 4: Secondary structure of in vitro transcript pre-$tRNA^{Asp}$ of *B. subtilis*. The cleavage site by RNase P RNA is indicated by the arrow. The 5′-leader sequence is indicated as a thick line. The 5′- and 3′-ends of RNA are indicated. Functional groups potentially involved in catalysis by RNase P RNA are highlighted.

enzymes and substrates in the respective systems or the interplay of competing biosynthetic pathways in vivo.

In contrast to the holoenzyme, RNase P RNA shows clear preference for the 3′-CCA for optimal activity in vitro. As shown here and previously (*1*, *2*, *31*−*35*), deletion or substitution of nucleotides within the 3′-CCA decreases the catalytic efficiency (k_{cat}/K_M) of RNase P RNA in multiple-turnover reactions, primarily by elevating K_M. As one example (*1*), deletion of all three nucleotides from pre-$tRNA^{Asp}$ increases K_M by 30−60-fold, depending on the species of RNase P RNA assayed; this corresponds to a loss of ca. 2−2.5 kcal/mol of binding energy. Based on a gel-shift assay, a pre-$tRNA^{Gly}$ substrate lacking the 3′-CCA was reported to bind *E. coli* RNase P RNA with an apparent K_D 600-fold higher than that of native substrate, representing a loss of ca. 4 kcal of binding energy (*35*). Thus, different lines of evidence show that perturbation of the 3′-CCA structure results in weakened interactions between substrate and ribozyme.

The spatial proximity of the 3′-CCA to the scissile bond in the folded tRNA suggests that the 3′-CCA structure could play a role in catalysis in addition to its facilitation of substrate binding. Indeed, the 2′-hydroxyl group of the 3′-

distal C residue (3′-CCA) has been proposed to coordinate a Mg^{2+} ion that participates in catalysis (*22*). However, previous kinetic analyses of substrates with modified 3′-ends were carried out primarily under multiple-turnover conditions, where k_{cat} is limited by product release, rather than the chemical step of catalysis per se. To explore more directly how the structure of the 3′-CCA of pre-tRNA affects the rate of catalysis by RNase P RNA, we used single-turnover kinetic assays, in which the ribozyme is initially saturated with substrate, to measure the rate of the chemical step (k_2). Single-turnover kinetic assays were conducted under low-pH (pH 6) reaction conditions, where k_2 of the native reaction is limited by the availability of nucleophilic hydroxide ions (*21*). While the differences in k_2 values reported here reflect changes in the rate of chemistry, the basis for the change is not defined. The mutations might disrupt transition-state structure or other steps in the reaction pathway such as conformational rearrangements that lead to the transition state.

Our results indicate that, in addition to its previously adduced role in substrate binding, the 3′-CCA of pre-tRNA is also required for optimal catalysis by bacterial RNase P RNA. Both deletions and nucleotide substitutions within the 3′-CCA result in decreased k_2 values. Deletion of the 3′-terminal A residue results in only a slight decrease in the catalytic rate under standard reaction conditions (1 M NaCl, 25 mM $MgCl_2$), and is fully suppressed by elevated monovalent ionic strength. This suggests that the role of the 3′-terminal A is structural, not catalytic. A similar phenotype is seen in multiple-turnover reactions. The compensation for deletion of the 3′-A by high-salt conditions suggests that loss of this residue disturbs the overall conformational fit between substrate and ribozyme RNAs, thus leading to unfavorable intra- or intermolecular electrostatic interactions. These unfavorable appositions are screened by elevated ionic strength, which probably tightens the structure of the complex. Mutation of the 3′-distal C residue (tRNACCA → tRNAUCA) also produced only a minor decrease in k_2 (ca. 4-fold); however, this defect could not be wholly remedied by elevated ionic strength conditions. Hardt et al. (*35*) have reported similar results in an analysis of the mutant substrates tRNAACA and tRNAGCA. The cytosine at this position therefore may contribute a base-specific functionality that is required for optimal catalytic activity.

The most drastic effects on k_2 were observed when the 3′-proximal C residue was either substituted (tRNACUA) or deleted (tRNAΔCA, tRNAΔCCA). Under standard reaction conditions (1 M NaCl, 25 mM $MgCl_2$), these substrates were cleaved at rates approximately 60-fold slower than was native substrate. This loss of catalytic activity can be attributed, at least in part, to the loss of an interaction between the 3′-proximal C residue and a Mg^{2+} ion that participates in catalysis, based on two lines of reason. First, RNase P activity could be restored by elevating the Mg^{2+} concentration of reactions involving the substrates with altered 3′-proximal C residues (Figure 2). Since the range over which Mg^{2+} was raised in these experiments did not appreciably change the ionic strength of the reactions and similar effects are seen with Mn^{2+} at lower concentrations, rescue of activity is probably due to specific interactions with divalent cation, rather than nonspecific electrostatic effects. Second, the apparent affinity (K_D^{app}) of the enzyme−substrate complex for the divalent cations was reduced upon either mutation or deletion of the 3′-proximal C residue, relative to that of native substrate; qualitatively similar results were obtained under both single- and multiple-turnover conditions.

Since replacement of the 3′-proximal C residue with uridine produces as severe a defect in catalysis as deletion of that residue, loss of the base moiety, per se, is probably responsible for the catalytic defect. Thus, either or both the N3- or 4-amino group of the 3′-proximal C is probably involved in coordinating metal-bound water molecules through hydrogen-bonding. We are surprised that deletion of the 3′-CA produced as severe an effect on k_2 as did deletion of the entire 3′-CCA, considering the previously proposed function of the 2′-OH group of the 3′-distal C residue in binding Mg^{2+} (*22*). Clearly, the loss of both the 3′-proximal C residue, which we propose interacts with Mg^{2+}, and the 2′-OH group of the 3′-distal C residue does not result in a phenotype that is more severe than the two individual defects. The absence of an additive phenotype suggests that these functional groups (i.e., the 2′-OH group of the 3′-distal C and either the N3 or the 4-NH_2 group of the 3′-proximal C) participate in the same basic interaction, for instance by participation in a cooperative network that includes the catalytic Mg^{2+}-hydrate ions.

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