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Binding of C5 Protein to P RNA Enhances the Rate Constant for Catalysis for P RNA Processing of Pre-tRNAs Lacking a Consensus G(+1)/C(+72) Pair

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Abstract

The RNA subunit of the ribonucleoprotein enzyme ribonuclease P (RNase P (P RNA) contains the active site, but binding of *Escherichia coli* RNase P protein (C5) to P RNA increases the rate constant for catalysis for certain pre-tRNA substrates up to 1000-fold. Structure-swapping experiments between a substrate that is cleaved slowly by P RNA alone (pre-tRNA^{f-met605}) and one that is cleaved quickly (pre-tRNA^{met608}) pinpoint the characteristic C(+1)/A(+72) base pair of initiator tRNA^{f-met} as the sole determinant of slow RNA-alone catalysis. Unlike other substrate modifications that slow RNA-alone catalysis, the presence of a C(+1)/A(+72) base pair reduces the rate constant for processing at both correct and miscleavage sites, indicating an indirect but nonetheless important role in catalysis. Analysis of the Mg²⁺ dependence of apparent catalytic rate constants for pre-tRNA^{met608} and a pre-tRNA^{met608} (+1)C/(+72)A mutant provides evidence that C5 promotes rate enhancement primarily by compensating for the decrease in the affinity of metal ions important for catalysis engendered by the presence of the CA pair. Together, these results support and extend current models for RNase P substrate recognition in which contacts involving the conserved (+1)G/C(+72) pair of tRNA stabilize functional metal ion binding. Additionally, these observations suggest that C5 protein has evolved to compensate for tRNA variation at positions important for binding to P RNA, allowing for tRNA specialization.

Keywords

RNase P; tRNA; ribonucleoprotein; enzyme specificity; catalysis

Introduction

A high degree of specificity is a hallmark of biological catalysis; however, many enzymes, in particular those involved in RNA metabolism, tolerate significant variations in substrate structure. The spliceosome, for example, must recognize a multitude of different intron/exon junctions and is assisted by a host of protein cofactors.¹ Similarly, elongation factor Tu and the A, P, and E sites of the ribosome must recognize the entire complement of aminoacyl tRNAs.^{2–4} A further example of multiple substrate recognition in tRNA metabolism is ribonuclease P (RNase P), an essential ribonucleoprotein enzyme that processes the 5' end of tRNA precursors.^{5–7} Due to the robust *in vitro* activity and simple structure of the bacterial enzyme, which is composed of only a 400-nt catalytic RNA (P RNA) and a smaller

protein (e.g., the 120-aa C5 in *Escherichia coli*), RNase P is highly amenable to detailed studies of molecular recognition.^{5–8}

Structure–function studies identified conserved sequences flanking the pre-tRNA cleavage site that are recognized by specific structural elements of P RNA (Fig. 1) (e.g., Refs. 6, 9, and 11–14). These sequences include the 3' RCC sequence of tRNA that interacts with L15 in P RNA and the N(–1) nucleobase that interacts with the J5/15 element of the RNA subunit.^{9–11,15–17} An additional recognition element is the closing base pair of the acceptor stem formed by the pairing of nucleotides +1 and +72. Structure–function studies using a range of substrates and functional assays have shown that changes in the identity of these nucleotides have large negative effects on P RNA catalysis and substrate binding affinity (e.g., Refs. 14,18, and 19), but the specific interaction between the (+1)/(+72) G-C base pair (conventional tRNA numbering²⁰) and the ribozyme is still unclear. Recently, a comprehensive analysis of the effects of mutations and functional group modifications at (+1)/(+72) in a model minihelix substrate provided evidence that the N2 amine of G(+1) contributes to ground-state binding affinity, as well as to catalytic rate, and that miscleavage occurs when this residue is mispaired or deleted.¹⁴ Effects of N2 amine deletion can be offset by increasing Mg²⁺ concentration, implicating contacts with this residue in stabilizing functional metal ion binding. Photo-crosslinking experiments position G(+1) adjacent to J5/15 and J18/2 of P RNA in the enzyme–substrate complex.^{13,21} These regions are accordingly positioned adjacent to the substrate cleavage site in models of the P RNA–pre-tRNA complex; however, the functional resolution remains low.

Despite the importance of these determinants for the processing of model substrates by P RNA *in vitro*, they are not universal among the pre-tRNA substrates encountered by the RNase P holoenzyme *in vivo*.^{11,17,22} Inspection of pre-tRNA gene sequences in *E. coli* reveals that a significant number (30%) lack a consensus U necessary for interaction at the N(–1) position, but nonetheless retain a G(+1)/C(+72) pair or other consensus Watson–Crick base pairs at the cleavage site. About 20% have Watson–Crick base pair other than the consensus G-C at the (+1)/(+72) positions, but the majority of these retain a consensus U at N(–1). Comparative kinetic and thermodynamic studies using the *E. coli* RNase P holoenzyme showed that, under optimal multiple-turnover reaction conditions [100 mM NaCl and 17.5 mM MgCl₂ (pH 8.0)], the binding affinity and cleavage rates of different *E. coli* pre-tRNA substrates are essentially uniform, regardless of variations from consensus structure at the cleavage site.²² In contrast, the P RNA subunit alone, under identical reaction conditions, shows lower binding affinities and slower cleavage rates for ‘nonconsensus’ substrates. The apparent contribution of C5 protein to catalysis is greater than 1000-fold for some nonconsensus substrates.

To more fully understand the mechanistic basis for multiple substrate recognition by *E. coli* RNase P, in the present study we sought to identify the specific structures that dictate slow ribozyme catalysis of pre-tRNA^{f-met605} (a substrate for which the C5 protein has a 1000-fold effect on the apparent cleavage rate constant) and to determine for this substrate the mechanistic basis for its dependence on the binding of C5 protein for optimal catalysis. The results unambiguously identify the characteristic C(+1)/A(+72) pair of initiator tRNA^{f-met} as the sole determinant of slow P-RNA-alone cleavage of its precursor. These and other quantitative analyses of (+1)/(+72) mutants in different tRNA ‘backgrounds’ strongly suggest that, under physiological conditions, C5 makes a large contribution to catalysis for >30% of substrates with non-G(+1)/C(+72) pairs. As predicted by the results of Kikovska *et al.*, substrates that lack a consensus G(+1)/C(+72) show a decrease in the apparent affinity of functional metal ion binding.^{14,23} We find that the apparent catalytic effect of the protein for substrates with an A-C cleavage site pair is the offsetting of this negative effect on metal ion interactions.

Results

C5 protein makes a larger contribution to the rate constant for catalysis for the nonconsensus pre-tRNA^{f-met605} substrate (~1000-fold) than for the consensus pre-tRNA^{met608} substrate (~10-fold)

Previous analyses of the contribution of C5 to substrate recognition identified several endogenous *E. coli* pre-tRNAs that are cleaved slowly by P RNA alone, but have catalytic rate constants that are rescued by binding of the P protein subunit. To identify the specific structural features that necessitate protein-assisted catalysis, we analyzed in greater detail the binding thermodynamics and processing kinetics of the precursors of two methionyl tRNAs: pre-tRNA^{met608} and pre-tRNA^{f-met605} (Fig. 2). The pre-tRNA^{met608} substrate is a consensus RNase P substrate for which the protein contributes ~10-fold to the catalytic step of the processing reaction. The pre-tRNA^{f-met605} substrate is a nonconsensus pre-tRNA that is processed slowly by P RNA alone, but reacts >900-fold faster (with a rate constant comparable to that of pre-tRNA^{met608}), when C5 is present in the reaction. Of the 86 nt in both pre-tRNAs, there are 41 individual sequence differences, including position N(-1) and the +1/+72 base pair. Comparative analyses were undertaken to exploit the functional differences between these substrates to gain further insight into the role of C5 in RNase P enzyme function.

An approximate reaction scheme for comparing the processing of pre-tRNA by P RNA alone and the processing of pre-tRNA by the RNase P holoenzyme is a reversible second-order reaction (binding) with equilibrium constant $K_{d,ptRNA}$, which may include contributions from a conformational change after initial substrate binding, followed by an irreversible first-order reaction (catalysis) with rate constant k_c , in turn followed by reversible dissociation of the two products (Scheme 1).

The substrate cleavage step (k_c) is monitored as the single-turnover rate constant at saturating concentrations of the enzyme (e.g., Fig. 3).²⁴⁻²⁶ $K_{d,ptRNA}$ is monitored by slowing the substrate cleavage step (see the text below) to permit the enzyme-substrate complex to accumulate.^{24,27} The bound fraction of radiolabeled pre-tRNA substrate is quantified by the separation of free and bound pre-tRNAs using a gel-filtration spin column, followed by isotopic counting of appropriate fractions. The binding affinity of the two products, tRNA ($K_{d,tRNA}$) and the 5' leader ($K_{d,leader}$), can be assayed in the same manner using radiolabeled product RNAs. Specificity is determined by comparing the cleavage products of radiolabeled substrates on high-resolution sequencing gels. Unless noted otherwise, the substrate and product binding affinities for the two substrates, as well as the rate constants for catalysis, were determined under reaction conditions that are optimal for the multiple-turnover reaction of the RNase P holoenzyme [50 mM Tris (pH 8.0), 17.5 mM M^{2+} , and 100 mM NaCl]. For binding analyses, the metal ion Ca^{2+} is used in place of Mg^{2+} , resulting in a ~1000-fold decrease in cleavage rate, allowing the enzyme-substrate complex to accumulate and the binding reaction to reach equilibrium.^{24,28}

Consistent with previous results,²² the two methionyl tRNAs both bind to P RNA with low but essentially equivalent affinities (~2400 nM and 3400 nM for pre-tRNA^{met608} and pre-tRNA^{f-met605}, respectively) (Table 1). Binding of C5 to P RNA results in large (4000-fold to 11,000-fold) increases in substrate binding affinity for both pre-tRNAs, resulting in essentially equivalent $K_{d,ptRNA}$ values for the binding of the two substrates (~0.5 nM and 0.3 nM for pre-tRNA^{met608} and pre-tRNA^{f-met605}, respectively).

As shown in Scheme 1, RNase P processing generates mature tRNA and 5' leader sequence products that may dissociate in separate steps. Analysis of the binding affinities of the two mature tRNAs for the P RNA subunit alone revealed the same affinity within error of ~150

nM (Sun *et al.*²² and Table 1). The presence of C5 has essentially no effect on tRNA^{f-met605} binding; however, it increases tRNA^{met608} binding affinity by about 15-fold. The binding of both leader sequence products to the P RNA alone is essentially undetectable ($K_{d,leader} > 3000$ nM). The binding affinity of the pre-tRNA^{met608} leader sequence product for the RNase P holoenzyme is also equivalently low ($K_{d,leader} > 1000$ nM). In contrast, the binding of the pre-tRNA^{f-met605} leader is significantly enhanced by the presence of C5 ($K_{d,leader} = 100$ nM) (Table 1). Inclusion of the leader sequence product had no effect on tRNA product binding affinity; similarly, the presence of tRNA did not strongly influence apparent leader affinity (data not shown). Thus, the binding constants for the tRNA and leader products when the corresponding product was also bound to the enzyme ($K_{d,leader}$ and $K_{d,tRNA}$) were not considered further. These data show, as noted previously,²² that the binding affinities of both consensus and nonconsensus substrates for RNase P are essentially uniform. Additionally, weaker binding of nonconsensus tRNA appears to be offset by stronger interactions with the 5' leader sequence.

Single-turnover kinetic assays performed at saturating concentrations of the enzyme were used to compare the rate of cleavage of the bound substrate (k_c) for both the canonical pre-tRNA^{met608} substrate and the noncanonical pre-tRNA^{f-met605} substrate (Fig. 3). As previously reported, the rate constant for cleavage by the P RNA subunit alone is significantly lower for the pre-tRNA^{f-met605} substrate (0.001 min^{-1}) than for the pre-tRNA^{met608} substrate (0.14 min^{-1}).²² The presence of C5 resulted in a ~10-fold increase in k_c for the pre-tRNA^{met608} substrate, while there was a ~1000-fold increase in the rate constant for cleavage of the noncanonical pre-tRNA^{f-met605} substrate. Thus, for both substrates, the protein subunit dramatically enhances the binding affinity for the precursor, while having more modest and specific effects on product binding affinity (Table 1). However, the major difference between these two substrates is the large increase in the rate constant for substrate cleavage for pre-tRNA^{f-met605} when C5 is present (Table 1 and Fig. 3).

Structure-swapping experiments identify the characteristic C(+1)/A(+72) base pair of pre-tRNA^{f-met605} as the sole determinant of slow cleavage by P RNA

The specific structural features responsible for the observed defect of pre-tRNA^{f-met605} in a P RNA reaction are necessarily among the 41 differences in nucleotide identity compared to pre-tRNA^{met608} (Fig. 2). Therefore, we undertook structure-swapping experiments in which the sequences of the fast-reacting pre-tRNA^{met608} are systematically replaced by the sequences of pre-tRNA^{f-met605}. The single-turnover rate constants k_c for these substrates were then compared (Table 2). The nomenclatures and structures of the hybrid substrates in which the structure of pre-tRNA^{f-met605} was systematically substituted by pre-tRNA^{met608} sequences and *vice versa* are shown in Fig. 4.

First, the entire acceptor stems were interchanged to produce the 605stem/608loop and 608stem/605loop substrates, and the defect in k_c for P RNA was found to clearly follow the pre-tRNA^{f-met605} acceptor stem (Table 2). The leader 3' RCCA and the closing (+1)/(+72) base pair were then swapped to produce the 605top/608tRNA and 608top/605tRNA substrates, and the defect in k_c for P RNA was observed to be associated with these elements of pre-tRNA^{f-met605}. Because pre-tRNA^{met608} and pre-tRNA^{f-met605} have the same 3' ACCA sequence, the elements responsible for the catalytic defect must be contained in the 5' leader or in the closing (+1)/(+72) base pair. The 605leader/608tRNA and 608leader/605tRNA substrates were generated by swapping only leader sequences. Interestingly, the pre-tRNA^{met608} leader partially rescued the defect in pre-tRNA^{f-met605} cleavage. Such leader-specific effects on catalysis could be due to differences in ground-state substrate structure. Importantly, replacing the G(+1)/C(+72) in consensus pre-tRNA^{met608} by C(+1)/A(+72) decreases the catalytic rate constant to a level similar to that of nonconsensus pre-

tRNA^{f-met605}, while the catalytic defect in pre-tRNA^{f-met605} can be fully rescued by replacing C(+1)/A(+72) with a G-C base pair (Table 2).

Taken together, these data show that the absence of G(+1)/C(+72) in pre-tRNA^{f-met605} is the major determinant that results in the catalytic defect in the cleavage of pre-tRNA^{f-met605} by P RNA alone and necessitates C5 protein assistance in cleavage. These data are consistent with previous structure–function studies of *Bacillus subtilis* and *E. coli* P RNA substrate recognition, leading to the current model in which specific functional groups of the G(+1)/C(+72) are recognized by RNase P.^{14,18,19,23}

Despite the fact that the (+1)/(+72) G-C base pair is highly conserved, there are examples of *E. coli* pre-tRNAs that have each of the four different Watson–Crick base pairs at the (+1)/(+72) positions. To quantitatively assess the effects of other Watson–Crick base pairs on catalysis by P RNA with and without C5 protein binding in the pre-tRNA contexts used here, we compared the rate constants of the cleavage of pre-tRNA^{met608} with different Watson–Crick base pairs at (+1)/(+72). As shown in Table 2, changing (+1)/(+72) G-C to other Watson–Crick pairs results in a lower apparent catalytic rate constant for cleavage by P RNA alone. U-A and A-U substitutions result in >140-fold and 28-fold reductions, respectively, in k_c , while a C-G substitution reduced the catalytic rate constant by ~3-fold. Similarly, an earlier study has shown that the replacement of (+1)/(+72)G-C with a U-A base pair in the pre-tRNA^{Gln} and pre-tRNA^{TyrSu3} context influences the cleavage efficiency of the P RNA reaction.²³ In contrast, when C5 protein is bound to P RNA, there is relatively little effect of the variation in the (+1)/(+72) base pair on catalysis (at most 3-fold). Thus, the uniformity of the rate constants of holoenzyme cleavage for all of the different structure variants examined suggests that C5 also contributes to efficient catalysis for the numerous other endogenous *E. coli* pre-tRNAs (20%) that contain non-G-C pairs at the cleavage site.

The magnitude of the defect in P RNA catalysis due to the presence of a C(+1)/A(+72) base pair is independent of pre-tRNA context

The data above provide strong support that sequence variation in the G(+1)/C(+72) base pair in the population of *E. coli* pre-tRNAs results in significant defects in P RNA—but not holoenzyme—catalysis necessitating a contribution by C5 protein. However, it is possible that the effects of variation at (+1)/(+72) are restricted to the pre-tRNAs used in the swapping experiments, while in other substrates, the effect might be larger or smaller because of differences in structural context. To test this possibility, we determined the effects of substitution at the (+1)/(+72) pair in two other substrates: pre-tRNA^{leu601}, which contains a noncanonical G(+1)/U(+72) pair and lacks a U at the (–1) position, and *B. subtilis* pre-tRNA^{asp}, an often-used model substrate that contains consensus RNase P recognition elements including G(+1)/C(+72) (pre-tRNA^{Bs_asp}; Fig. 2).

As observed previously, the rate constant for catalysis by the RNase P holoenzyme is essentially insensitive to pre-tRNA identity and structural variation at G(+1)/C(+72) (Table 3). For P RNA alone, a very large defect (~700-fold) in k_c is engendered when a C·A pair is substituted at (+1)/(+72) of the consensus pre-tRNA^{Bs_asp} (k_c decreases from 0.7 min^{–1} to 0.001 min^{–1}). Similarly, a C(+1)/A(+72) substitution in pre-tRNA^{leu601} results in a 50-fold decrease in $k_{obs}^{P RNA}$ compared with a G-C substitution at the (+1)/(+72) base pair (k_c decreases from 0.07 min^{–1} to 0.0013 min^{–1}). Thus, inclusion of a C(+1)/A(+72) pair reduces the single-turnover rate constants for both substrates to ~0.001 min^{–1}.

As illustrated in Tables 2 and 3, the rate constant for P-RNA-catalyzed cleavage is greatest for all of the substrates tested when a consensus G(+1)/C(+72) is present. However, there are some relatively small but detectable pre-tRNA context-dependent effects of mutations at

(+1)/(+72). Notably, *E. coli* pre-tRNA^{met608} and *B. subtilis* pre-tRNA^{Bs_asp} both contain all of the known consensus recognition elements, but pre-tRNA^{Bs_asp} is found to be more sensitive to the C(+1)/A(+72) mutation (Table 3). Additionally, pre-tRNA^{leu601}, which contains a noncanonical G(+1)/U(+72) pair and noncanonical A(−1), is not significantly stimulated by inclusion of the consensus G-C at (+1)/(+72), as would be expected. As shown in Table 3, pre-tRNA^{leu601} with a G(+1)/C(+72) base pair is cleaved 10-fold slower than the consensus pre-tRNA^{Bs_asp} substrate, but only 2-fold slower than the consensus pre-tRNA^{met608}. Thus, the identity of the (+1)/(+72) base pair is clearly a general feature of pre-tRNA that contributes to optimal P RNA catalysis, and the kinetic effects of base substitutions at these positions are largely, but not entirely, independent of pre-tRNA context.

The presence of a C(+1)/A(+72) base pair appears to decrease the rate constants for both accurate cleavage and miscleavage

Given the well-documented effects of (+1)/(+72) base-pair changes on cleavage specificity in other substrates (e.g., Refs. 23 and 29) and the dramatic effects of (+1)/(+72) base-pair variation on P RNA catalysis observed in this study, we mapped precisely the site of RNase P processing for pre-tRNA^{met608}, pre-tRNA^{f-met605}, and the structure variants described above. Comparison of the cleavage products of 5′-³²P-end-labeled substrates to synthetic RNA markers demonstrates that both P RNA and RNase P act exclusively at the correct phosphodiester bond between N(+1) and N(−1) for all of the substrates tested (Fig. 5a; data not shown). This result is distinct from previous analyses by Kikovska *et al.*, which clearly show changes in *E. coli* P RNA specificity for a helical substrate (pATser) and a suppressor pre-tRNA^{23,29} when the G(+1)/C(+72) base pair is mutated. It is possible that our analyses used substrates that are less sensitive to loss of specificity determinants. Indeed, it has been demonstrated previously that miscleavage due to changes in the identity of N(−1) requires the presence of other substrate modifications that slow the rate of processing at the correct phosphodiester bond.^{11,30}

Therefore, to test more rigorously whether a C(+1)/A(+72) pair in pre-tRNA results in any loss of cleavage site fidelity, we sought an experimental system in which the correct and incorrect cleavage complexes are in competition and both correct cleavage and miscleavage are observed. Accordingly, we tested the effects of substituting a C·A for G(+1)/C(+72) in the context of a pre-tRNA^{met608} substrate containing an Rp phosphorothioate (Rp-PS) modification at the phosphate between N(−1) and N(+1). This modification results in the failure to efficiently recruit the catalytic Mg²⁺ to the correct phosphodiester bond and causes miscleavage, primarily between the (−1) and the (−2) residues, providing an experimental window for examining effects on cleavage fidelity.³⁰ As shown in Fig. 5b, the presence of a C(+1)/A(+72) pair in the PS-modified substrate does not result in a large change in the fraction of the substrate that is miscleaved. Predominate cleavage at the correct site can be rescued by adding 5 mM Cd²⁺ to the reactions, suggesting that the substitution in the (+1)/(+72) base pair does not perturb the well-documented “rescue” of miscleavage by adding Cd²⁺, which favors interactions with the cleavage site phosphorothioate and restores the active-site metal ion interactions.^{30–32} For P RNA, minor cleavage products at N(−1) and N(−2) (indicated by an asterisk) may be due to miscleavage for this substrate. For RNase P, the products at N(−2) and N(−3) appear to arise from miscleavage of the phosphorothioate-modified substrate in the presence of Cd²⁺; however, these products do not impact the quantitative analyses (see the text below). These results indicate that, in general, the presence of a cleavage site C(+1)/A(+72) pair has little effect on specificity for either the holoenzyme or RNA-alone reactions.

A more detailed insight into the role of the cleavage site base pair is revealed by considering these results in terms of a simple quantitative mechanism for miscleavage reaction kinetics.

A simple model for the formation of correct and incorrect ribozyme–substrate complexes that result in a mixture of cleavage products under single-turnover conditions involves two parallel pseudo-first-order reactions, as illustrated in Scheme 2, where $E-S_c$ is the enzyme substrate complex that forms with equilibrium constant K_c and gives rise to processing at the correct site, with rate constant k_c generating product P_c ; and $E-S_m$ is a complex that forms with equilibrium constant K_m , generating miscleaved product P_m .^{30,33,34} Any conformational change that occurs after substrate binding would be encompassed in the terms for K_c and K_m . In Scheme 2, the fraction of substrate cleaved at the correct site is a function of the rates of chemical catalysis at both sites (k_c and k_m), as well as the equilibrium terms that describe the partitioning of the substrate into the $E-S_c$ and $E-S_m$ complexes (K_c and K_m). Specificity can be quantitatively expressed in terms of F_c as the fraction of the product resulting from processing at the correct site at infinite time. For this model, if cleavage is rate limiting, F_c is defined according to Eq. (1):

$$F_c = \frac{K_c k_c}{K_c k_c + K_m k_m} \quad (1)$$

Importantly, changes in F_c can arise from differences in the intrinsic rates of cleavage at either of the two sites, differences in binding affinities between the two complexes, or a combination of both.

The observed rate constant for the depletion of the substrate at saturating enzyme concentrations k_{obs} is described by Eq. (2):

$$k_{obs} = \frac{K_c k_c + K_m k_m}{K_c + K_m} \quad (2)$$

where K_c , K_m , k_c , and k_m are equilibrium substrate association and rate constants shown in Scheme 2. For the pre-tRNA^{met608} substrate, an Rp-PS modification at the reactive phosphate results in a large 1000-fold decrease in k_{obs} , as observed previously for an Rp-PS modification in other pre-tRNAs. Importantly, the additional substitution of G(+1)/C(+72) with a C-A pair in the Rp-PS-containing pre-tRNA^{met608} substrate results in an additional large decrease in k_{obs} (data not shown) relative to the Rp-PS modification alone. Therefore, the presence of a C(+1)/A(+72) pair decreases k_c and/or k_m substantially. If a cleavage site C-A pair affects k_c and k_m differentially, then there must necessarily be a significant change in F_c when G(+1)/C(+72) is mutated to C-A in the pre-tRNA^{met608} substrate. As shown in Fig. 5b, replacing G(+1)/C(+72) with C-A in the context of a substrate containing a cleavage site Rp-PS modification changes the fraction of correct cleavage F_c only slightly (from 0.6 to 0.4) in both the holoenzyme reaction and P-RNA-alone reactions. Thus, in general, the G(+1)/C(+72) base pair is a dominant determinant of optimal catalytic rates, but it does not appear to make a large contribution to cleavage fidelity in this context.

In sum, variation from the G(+1)/C(+72) base pair to a CA pair does not affect the specificity of either P RNA or the RNase P holoenzyme. Inclusion of a cleavage site Rp-PS modification does engender miscleavage by decreasing the rate constant for cleavage at the correct phosphodiester bond, as documented previously.^{30–32} The Rp-PS-modified substrate provides an experimental window to more sensitively quantify the effect of cleavage site structural variation on enzyme specificity. However, even in this context, variation from the G(+1)/C(+72) base pair to a CA pair does not affect the specificity of either P RNA or the RNase P holoenzyme. As indicated above, we assume that the decrease observed in k_{obs} when C(+1)/A(+72) is present is due to a corresponding decrease in k_m or k_c , or both. Since F_c is largely unaffected, then the ratio of $K_c k_c$ to $(K_c k_c + K_m k_m)$ must also remain relatively

constant. If k_c alone was decreased, then there would necessarily be a large increase in miscleavage. If both k_c and k_m decrease by the same amount, then there will be no change in the observed F_c ; however, the k_{obs} should decrease proportionally as we have observed. Therefore, the fact that the presence of a C(+1)/A(+72) pair decreases k_{obs} , but has little effect on F_c , strongly suggests that this variation in substrate structure affects the rate constants for catalysis at both correct and incorrect phosphodiester bonds and, in so doing, does not result in a significant loss of cleavage site fidelity.

Increasing monovalent ion concentration enhances P RNA catalysis, but does not specifically rescue catalytic defect due to the presence of a C(+1)/A(+72) pair

Next, we investigated the mechanism by which C5 enhances catalysis for the pre-tRNA^{f-met605} substrate and, by extension, the broad class of endogenous *E. coli* substrates that have non-G-C pairs at the base of the acceptor stem. Binding of C5 to P RNA stabilizes its global structure and influences the conformation of the RNA near the tRNA-binding site.^{35–37} Chemical probing revealed increased cleavage in the presence of protein in J5/15, J18/2, and the P15 loop.³⁶ The (+1)/(+72) pair is proximal to these elements in the enzyme–substrate complex based on photoaffinity crosslinking.^{13,21} Based on these data and metal titration experiments, it was concluded that the protein influences both the conformation and the metal coordination in these regions of P RNA. Buck *et al.* hypothesized that stabilization of a particular RNA conformation at the protein-binding site influences both the substrate binding and the catalytic properties of RNase P, resulting in a reduced Mg²⁺ concentration requirement for the maximal cleavage rate by the holoenzyme compared with the RNA alone.^{35,36} The C5 protein therefore has three important functions that could result in selective transition-state stabilization for substrates containing a C(+1)/A(+72) pair: (1) stabilizing P RNA structure required to position C(+1)/A(+72)-containing substrates in the active site; (2) interacting with the 5' leader sequence to position substrates with a C(+1)/A(+72) pair in the active site; and (3) selectively increasing the affinity of metal ions that are important for the catalysis of C(+1)/A(+72)-containing pre-tRNAs.

First, we considered the possibility that one or more of the small changes in local P RNA structure occurring upon C5 binding could specifically contribute to transition-state stabilization for substrates containing non-G-C pairs at (+1)/(+72). Since the precise interaction with the G(+1)/C(+72) pair is unknown, a direct test of this model is difficult. To explain the substrate-specific effect of the protein on catalysis, we hypothesize that in the RNA-alone reaction, interactions with the G(+1)/C(+72) pair are thermodynamically coupled to a P RNA structure that is more optimal for catalysis; in the absence of this interaction, the structure is unchanged, and catalysis is slow. C5 binding would then stabilize this hypothetical structure in the absence of the G-C contact. To begin testing this proposal, we sought a way to stabilize substrate binding and P structure independently of protein binding. A high concentration of monovalent ions significantly stabilizes P RNA folding, increases substrate binding affinity by several orders of magnitude, and results in only 2-fold to 10-fold increases in k_c .³⁸ To investigate the first possibility, we measured the rate constants for the cleavage of pre-tRNA^{met608} and pre-tRNA^{f-met605} bearing G(+1)/C(+72) or C(+1)/A(+72) under standard high monovalent ion conditions for the RNA-alone reaction (1 M NH₄Cl and 17.5 mM MgCl₂).

If a high concentration of salt can replace the protein subunit with respect to specifically enhancing catalysis for noncanonical substrates such as pre-tRNA^{f-met605}, then this result would imply that the protein merely offsets unfavorable electrostatic interactions. Alternatively, if a significant difference in k_c is observed when G(+1)/C(+72) is changed to CA regardless of ionic strength, then a more specific mechanism in terms of conformation or kinetic mechanism would be indicated. As shown by the data in Table 4, catalytic rates are significantly faster for all of the substrates tested in high monovalent metal ion (10-fold to

100-fold); however, the ratio of the rate constant for the substrate containing G(+1)/C(+72) to the rate constant for the substrate containing C(+1)/A(+72) is maintained at both high and low ionic strengths. The k_c for the P RNA cleavage of pre-tRNA^{f-met605} increases ~40-fold from 0.0011 min⁻¹ to 0.044 min⁻¹ upon changing from 100 mM NaCl to 1 M NH₄Cl. However, the k_c for P RNA cleavage of pre-tRNA^{met608} increases similarly (~60-fold) with increasing ionic strength. Therefore, the ratios of the rate constants for these two substrates (k_c , pre-tRNA^{met608}/ k_c , pre-tRNA^{f-met605}) are 130 and 190 at 100 mM NaCl and 1 M NH₄Cl, respectively. For the pre-tRNA^{met608}CA substrate, k_c increases almost 100-fold at the higher ionic strength, but there remains the same ~40-fold difference between the rate constant for this substrate and the rate constant for the native pre-tRNA^{met608}. Despite its large contribution to catalysis, the absence of the G(+1)/C(+72) base pair only caused a slight increase in K_d (P RNA) (<4-fold) for these substrates (Table 4). Thus, stabilization of P structure and binding affinity by increasing ionic strength does improve the rate constant for catalysis by P RNA alone, suggesting that some activating changes in structure have occurred. However, these effects are not specific to the pre-tRNA^{f-met605} substrate and therefore suggest that C5 does not act to selectively stabilize the transition state for the substrate containing a C(+1)/A(+72) pair by altering P RNA structure.

Evidence that the contribution of C5 to catalysis does not involve interactions with the 5' leader sequence

One of the main functional properties of P protein is contacting the 5' leader sequence directly.^{39,40} Although we showed that swapping leader sequences did not offset the effects of a C(+1)/A(+72) pair, interactions with the leader could help dock the substrate in the active site. Such interactions may be more important for substrates such as pre-tRNA^{f-met605} that lack other substrate recognition elements like the G(+1)/C(+72) pair. Direct interactions of P protein with nucleotides proximal to the cleavage site in the 5' leader in *B. subtilis* RNase P are indicated by photocrosslinking and affinity cleavage studies.^{39,40} Time-resolved fluorescence resonance energy transfer experiments also demonstrate that nucleotides (-4) to (-7) of the 5' leader contact the P protein in *B. subtilis* RNase P.⁴¹

The RNase P protein from *E. coli* also enhances pre-tRNA binding affinity, consistent with a direct interaction with the leader sequence (Ref. 22; see the text above). Since the *E. coli* and *B. subtilis* proteins are interchangeable both *in vitro* and *in vivo*,^{22,42} it is likely that they form the same interactions with the substrate. To test more directly whether nucleotides -4 to -7 are contacted by the *E. coli* protein, we compared the nucleotides in tRNA protected from hydroxyl radical cleavage by either P RNA alone or the RNase P holoenzyme (Fig. 6). In this experiment, a population of radiolabeled *B. subtilis* pre-tRNA^{asp}, the same substrate used to investigate protein interactions in *B. subtilis* RNase P, was incubated with an excess of *E. coli* P RNA or RNase P to form the enzyme-substrate complex. The pre-tRNA residues protected from solvent were determined by exposure to hydroxyl radicals and analysis of the nucleotides subjected to cleavage by this reagent on high-resolution sequencing gels.

A protection factor (κ) was calculated by dividing normalized band intensities from samples in which the substrate and the enzyme were incubated together by the intensities in control reactions in which the enzyme was absent. The larger is the κ value, the greater is the degree of protection from solvent. Average κ values for nucleotides in the leader sequence and flanking the RNase P cleavage site are plotted in Fig. 6. Protection is clearly observed at nucleotides -2 through +3 for P RNA alone, as well as for other nucleotides in the D-loop, as observed previously;⁴³ however, there is essentially no protection of nucleotides -4 through -7. The presence of C5 enhances the degree of protection at the same nucleotides protected by P RNA alone. This effect is likely due to a slower substrate dissociation rate constant for the holoenzyme relative to the RNA alone. In addition, there are holoenzyme-

specific protections clearly observed at nucleotides -6, -5, and -4, with enhanced protection at nucleotide -3. These results are consistent with the model for leader sequence interactions with C5 based on the *B. subtilis* enzyme in which only nucleotides that are 3–4 nt distal from the cleavage site in the leader sequence form direct interactions with the protein subunit.^{39–41} Additionally, the enhanced protection correlates with phosphorothioate interferences observed at the -2 position for minimal substrates.⁴⁴

If these interactions are essential for enhancing the rate of catalysis, then the pre-tRNA^{f-met605} substrate with a shortened leader will react slowly with both P RNA alone and the RNase P holoenzyme. Alternatively, if the enhancement of catalysis is independent of these direct contacts, then the protein should increase the rate of catalysis for the pre-tRNA^{f-met605} substrate, regardless of leader length. Therefore, we determined k_c for pre-tRNA^{met608} and pre-tRNA^{f-met605} substrates in which the 5' leader sequence was truncated to 2 nt with the sequence 5'-GA-3' (referred to as 5'-GA-pre-tRNA^{met608} and 5'-GA-pre-tRNA^{f-met605}, respectively). A comparison of the k_c values for these substrates and for pre-tRNA^{met608} and pre-tRNA^{f-met605} substrates with longer (10 nt) leaders is shown in Table 5. We observe that truncation of the leader sequence has no effect on the rate constant for cleavage of the pre-tRNA^{met608} by either P RNA or RNase P. Interestingly, decreasing the leader length in the 5'-GA-pre-tRNA^{f-met} substrate increases by 15-fold the observed k_c for cleavage by P RNA compared to the same substrate with a 10-nt leader. Nonetheless, the reaction is 150-fold slower than the reaction of this same substrate with RNase P. This result demonstrates that despite the truncation of the leader sequence to prevent a direct contact between C5 and nucleotides -3 to -6, the protein subunit retains the ability to enhance catalysis to levels equal to that observed with the same pre-tRNA with a 10-nt leader (Table 5). We conclude therefore that the mechanism by which C5 enhances catalysis is independent of direct contacts with the leader sequence.

C5 contributes to the catalytic step for pre-tRNA^{f-met605} by increasing the affinity of metal ions that are important for catalysis

Previous studies have established a role for the protein subunit of *B. subtilis* RNase P in reducing the metal ion concentrations required for binding and catalysis.^{38,45} Furthermore, with the use of the consensus substrate pre-tRNA^{met608}, kinetic analysis and quantitative measurements of the thiophilic metal ion rescue of a cleavage site phosphorothioate modification provided evidence that binding of C5 protein enhances the chemical step of the reaction, in part, by a 3-fold to 5-fold increase in the cooperative binding affinity of active-site metal ions.³¹ These observations raise the possibility that the effect of variation in (+1)/(+72) base-pair identity on catalysis is related to metal ion binding affinity, and that the C5 protein may enhance the rate of catalysis of substrates bearing a nonconsensus C(+1)/A(+72) base pair by increasing metal ion affinity.

To address these questions, we evaluated the Mg^{2+} concentration dependence of k_c for pre-tRNA^{met608} and pre-tRNA^{met608}CA with both P RNA alone and the *E. coli* RNase P holoenzyme. As shown in Fig. 7, for the RNase P holoenzyme reactions, the rate constants for pre-tRNA^{met608} and pre-tRNA^{met608}CA are within 3-fold throughout the $[Mg^{2+}]$ titration range. This result is important because it demonstrates that the catalytic uniformity observed for the holoenzyme reaction rates at 17.5 mM Mg^{2+} described above is not the result of saturating Mg^{2+} concentrations. Additionally, the holoenzyme reactions appear noncooperative when fitted to the Hill equation:

$$k_c = \frac{k_{c,\max} [\text{Mg}]^n}{(K_{d,\text{Mg}})^n + [\text{Mg}]^n}$$

where $k_{c,\max}$ is k_c at saturating Mg^{2+} concentration, $K_{d,\text{Mg}}$ is the apparent binding dissociation constant, and n is the Hill number. Fitting the data for both substrates reacted with the RNase P holoenzyme gives $n \approx 1$, with similar apparent $K_{d,\text{Mg}}$ values for both substrates. The $K_{d,\text{Mg}}(\text{holo})$ for pre-tRNA^{met608} is ~ 53 mM, and the $K_{d,\text{Mg}}(\text{holo})$ for pre-tRNA^{met608} CA is ~ 39 mM. Thus, the lack of a (+1)/(+72) G-C base pair has little effect on the apparent Mg^{2+} binding affinities of holoenzyme-pre-tRNA complexes for these substrates and under the reaction conditions tested here.

A comparison of these results to the corresponding Mg^{2+} dependence for the P-RNA-alone reaction reveals several important observations. First, it is clear that, regardless of a G-C or C-A at the (+1)/(+72) base pair, the binding of C5 protein to P RNA increases (~ 10 -fold) the apparent affinity of divalent metal ions essential for catalysis, consistent with previous observations using the consensus substrate pre-tRNA^{met608},³¹ as well as previous studies of *B. subtilis* RNase P.^{38,45} Second, at higher Mg^{2+} concentrations, the P-RNA-alone reaction can reach catalytic rates comparable with those of holoenzyme reactions. This observation is also in agreement with the idea that the effects of C5 protein are associated with enhanced Mg^{2+} binding affinities, instead of a direct contribution of amino acid functional groups. Third, the apparent Mg^{2+} binding affinity is sufficiently low that it is difficult to estimate $K_{d,\text{Mg}}(\text{P RNA})$ and $k_{c,\max}(\text{P RNA})$ for either substrate, limiting a direct quantitative comparison between $K_{d,\text{Mg}}(\text{P RNA})$ for the binding of pre-tRNA^{met608} and pre-tRNA^{met608} CA. The key limitation is that it is not possible to achieve saturating concentrations of Mg^{2+} due to the precipitation of RNA at high Mg^{2+} concentrations.

In evaluating the G(+1)/C(+72) base pair's contribution to $K_{d,\text{Mg}}$, the assumption that changes at the (+1)/(+72) base pair do not significantly influence the cooperativity of Mg^{2+} binding is made necessarily. If the cooperativity is the same for P RNA and RNase P reactions, the ratio between the k_{obs} of substrates bearing G-C and the k_{obs} of substrates bearing C-A can be expressed as:

$$\frac{k_c(\text{GC})}{k_c(\text{CA})} = C \frac{\left((K_{d,\text{Mg}} \text{CA})^n + [\text{Mg}]^n \right)}{\left((K_{d,\text{Mg}} \text{GC})^n + [\text{Mg}]^n \right)}$$

where C is the ratio of $K_{c,\max}(\text{GC})$ to $K_{c,\max}(\text{CA})$ and is constant, and $K_{d,\text{Mg}} \text{CA}$ and $K_{d,\text{Mg}} \text{GC}$ are the apparent Mg^{2+} binding affinities for the substrates containing C(+1)/A(+72) and G(+1)/C(+72), respectively. At infinite Mg^{2+} concentration, the observed $k_c(\text{GC})/k_c(\text{CA})$ will approach $K_{c,\max}(\text{GC})/K_{c,\max}(\text{CA})$ (i.e., C). At concentrations of Mg^{2+} below $K_{d,\text{Mg}} \text{CA}$ and $K_{d,\text{Mg}} \text{GC}$, the ratio of the rate constant for the two substrates will approach the ratio $K_{d,\text{Mg}} \text{CA}/K_{d,\text{Mg}} \text{GC}$ as the Mg^{2+} concentration approaches zero. If $K_{d,\text{Mg}} \text{CA}$ and $K_{d,\text{Mg}} \text{GC}$ are equal, then $K_{d,\text{Mg}} \text{CA}/K_{d,\text{Mg}} \text{GC}$ will be unity, and the ratio $k_c(\text{GC})/k_c(\text{CA})$ will be constant regardless of Mg^{2+} concentration. However, the difference between $k_c(\text{GC})$ and $k_c(\text{CA})$ for P RNA diminishes significantly as $[\text{Mg}^{2+}]$ increases; thus, the $k_c(\text{GC})/k_c(\text{CA})$ ratio decreases from approximately 40 to 2 as $[\text{Mg}^{2+}]$ increases from 17.5 mM to 160 mM. This phenomenon is observed only when $K_{d,\text{Mg}} \text{CA}$ is significantly greater (at least 40-fold) than $K_{d,\text{Mg}} \text{GC}$, suggesting that the catalytic defect for the P RNA

reaction with the (+1)C/(+72)A-containing substrate is most likely to be related to an increase in the apparent $K_{d,Mg}$ for P RNA.

Discussion

Conserved sequences flanking the pre-tRNA cleavage site are clearly key determinants for recognition and catalysis by the P RNA subunit *in vitro* (e.g., Refs. 9, 11, 16, 18, 23, 30, 46, and 47). Yet, inspection of the pre-tRNA pool in *E. coli* reveals that many endogenous pre-tRNAs do not contain these conserved sequences. Such structural variation at the cleavage site of pre-tRNA has a dramatic influence on the substrate binding affinity and cleavage rate for reactions catalyzed by the P RNA subunit alone.²² Binding of C5 protein to P RNA, however, results in a much higher binding affinity and less sensitivity to substrate mutations that affect the P-RNA-alone reaction.^{22,35,48–50} Indeed, binding of C5 protein ‘rescues’ the detrimental effects of the natural structural variation at the pre-tRNA cleavage site, resulting in essentially uniform apparent affinities and cleavage rates with endogenous substrates.²² For some nonconsensus substrates such as pre-tRNA^{f-met605}, the apparent effect of C5 on the cleavage step is very large. This observation suggests an important role of C5 protein in multiple substrate recognition.

To better understand the mechanistic basis for multiple substrate recognition by RNase P, we aimed to identify the structural features of individual pre-tRNAs that limit optimal P RNA cleavage rates and to determine the mechanistic basis for the protein's large positive contribution to rate enhancement. In this work, we identified the (+1)/(+72) base pair as a major determinant of optimal P RNA catalysis by utilizing structure swapping between a consensus pre-tRNA^{met608} and a nonconsensus pre-tRNA^{f-met605}. The catalytic effects of this determinant are largely independent of pre-tRNA context. In all tRNA backgrounds tested, addition of C5 “rescued” the effect of a suboptimal C(+1)/A(+72) base pair. Importantly, an analysis of the Mg²⁺ dependence of catalytic rate constants indicates that the binding of C5 protein to P RNA results in a profound increase in the apparent affinity of divalent metal ions essential for catalysis. In this regard, it is important to consider that the observed effects obtained at 17.5 mM Mg²⁺ are expected to be exacerbated at lower physiological Mg²⁺ concentrations.^{22,45} Therefore, despite the fact that the presence of a leader sequence is not necessary for the protein effects on the rate constant observed, under *in vivo* conditions of lower metal ion concentration, direct contacts between the protein and the leader sequence may regain importance relative to what is observed under the conditions used in this study.

Two previous studies of *E. coli* tRNA^{f-met} maturation *in vivo* provide an important contrast to the data presented here and the conclusions drawn from them. Meinel and Blanquet showed that over-expression of pre-tRNA^{f-met} in *E. coli* results in accumulation of an unprocessed precursor that is attributable to the presence of C(+1)/A(+72). Over-expression of RNase P or inclusion of a 5′ leader sequence from the native gene improved the fraction of the overexpressed precursor observed.⁵¹ The positive effect of the leader sequence was proposed to arise from the presence of a G residue 2 nt upstream of the cleavage site. However, subsequent *in vitro* studies by this group using purified RNase P holoenzyme further showed that the identity of (+1)/(+72) had little effect on the processing of pre-tRNA,⁵² as we have also observed. Additionally, the identity of the nucleotide at the −2 position in the leader had little effect on multiple-turnover cleavage by the RNase P holoenzyme. Thus, our results correspond to the *in vitro* results reported previously using a purified holoenzyme rather than a reconstituted holoenzyme, which provides a good check on accuracy. However, both sets of data are difficult to rationalize with the results from overexpression of pre-tRNA^{f-met} *in vivo*. Potentially, the overexpression of the substrate results in interactions with the enzyme that are nonproductive or that lead to altered

productive substrate binding modes. As for the contribution of leader sequences to processing, there is emerging evidence that such is indeed the case and may reflect interactions with the protein subunit. Tight-binding leader sequences appear to compensate for weak-binding tRNA portions of the substrate, and comparative sequence analyses indicating sequence specificity may underlie these effects. Additionally, the presence of the pre-tRNA^{met} leader sequence, in part, rescued the defect in catalytic rate engendered by the presence of a C(+1)/A(+72) pair. Thus, there is accumulating evidence that idiosyncratic sequences in the leaders of pre-tRNAs can have important effects on binding affinity and processing efficiency. Yet, the precise ‘code’ for these determinants and their thermodynamic contribution to substrate binding affinity and catalysis remain to be elucidated.

Because the G(+1)/C(+72) base pair is highly conserved in pre-tRNAs, the recognition of this element has been studied in some detail. Using pre-tRNA^{Gln}, pre-tRNA^{Tyr}Su3, and a model RNA hairpin substrate, Kikovska *et al.* showed that replacement of (+1)/(+72) G-C with U-A affected ground-state binding and cleavage rates under multiple-turnover and single-turnover conditions for P RNA alone.²³ A follow-up study provided evidence that the exocyclic amine (2NH₂) of (+1)G is particularly critical for cleavage site recognition, ground-state binding, and catalysis, but in a substrate-dependent manner. Based on metal titration data, it was suggested that a modification of 2NH₂ at position (+1) G can influence the charge distribution—and thus the Mg²⁺ binding affinity—of active-site metal ions.¹⁴

The results reported here are essentially consistent with these studies with respect to the important role of the G(+1)/C(+72) pair in P RNA catalysis. However, we observed only a small contribution of G(+1)/C(+72) to ground-state binding in this study (Table 1), while a relatively large effect was observed previously. One possible explanation is that, as indicated in the previous studies,²³ the influence of the (+1)/(+72) base pair on ground-state binding may vary in a substrate-dependent manner, and the substrates used in this study could be less sensitive to the structural perturbation in this base pair with respect to ground-state binding. Interestingly, our study provides evidence that the impact of G(+1)/C(+72) on P RNA catalysis is largely substrate independent (Table 3), while substrate dependence was observed previously. This inconsistency might be due to the comparatively lower divalent metal ion concentration used here (17.5 mM *versus* >40 mM Mg²⁺ used in the analyses by Kikovska *et al.*), since the influence of the G(+1)/C(+72) base pair on catalysis will diminish as the divalent metal ion concentration increases.

The data presented here suggest that the G(+1)/C(+72) base pair plays an important role in Mg²⁺ binding, and this idea is also supported by at least two other reports. As mentioned above, a comprehensive structure–function study by Kikovska *et al.* provided evidence that a 2′-amino modification at position G(+1) influenced the charge distribution and Mg²⁺ binding at the cleavage site.¹⁴ In addition, using precursor tRNAs carrying a single 2′-deoxy or 2′-OCH₃ modification at G(+1), Cuzic and Hartmann demonstrated that the 2′-hydroxyl at (+1) G is proximal to the M²⁺ cluster at the phosphates of A67 to U69 in helix P4 of *E. coli* RNase P RNA.⁵³ Indeed, photocrosslinking positions U69 in helix P4 within 4 nt of G(+1).⁵⁴ However, local structural perturbation at the active site is also likely to weaken the affinity of metal ions in the vicinity. As large polyanions, RNAs attract numerous metal ions, allowing for close packing of the phosphodiester backbone. The environment of the RNase P active site with pre-tRNA bound will contain numerous ions whose binding may contribute to transition state stabilization without directly contacting the reactive phosphate.⁵⁵ Thus, assigning a direct role for G(+1)/C(+72) in recruiting Mg²⁺ is still problematic. Nonetheless, together with previous reports, the data shown here underscore the linkage between cleavage site structure and binding of metal ions in the transition state.

The results presented here, in particular those relating to pre-tRNA^{f-met605}, provide insights into the coevolution of the functions of the RNA and protein subunits of RNase P. In primordial biology, all pre-tRNAs may have contained consensus sequences flanking the P RNA cleavage site, so that P RNA alone was sufficient to recognize and catalyze the maturation of all substrates. However, during evolution, tRNA sequences may have varied to permit functional specialization such as initiation of translation or recognition by the ribosome. For example, a G(+1)/C(+72) base pair in pre-tRNA^{met} would have been necessarily changed to a C·A for efficient translational initiation and recognition by methionyl-tRNA *N*-formyl transferase. Mutations in P RNA could be a way to accommodate this sequence drift necessary for tRNA specialization. However, such variation in P RNA would likely influence the processing of all pre-tRNAs. Thus, the protein subunit may have been an evolutionary solution for offsetting the effects of such drift in tRNA sequences. Indeed, the modern protein can modulate molecular recognition to enhance substrate binding by contacting the leader sequence and can facilitate catalysis by increasing apparent Mg^{2+} binding affinities. These data suggest that, at some level, the protein has evolved to accommodate substrate structure variation.

This study further identified the G(+1)/C(+72) base pair as a major determinant of optimal catalytic rates by P RNA acting in a largely substrate-independent manner and provided evidence that the C5 protein subunit can offset structure variation in these positions by enhancing apparent Mg^{2+} binding affinities. However, it is not clear whether the protein subunit uses additional mechanisms to enhance the rate of catalysis. The robust catalytic activity of *E. coli* P RNA with consensus substrates argues that the protein does not contribute functional groups to active-site formation and does not interact directly with the substrate phosphate. This perspective is consistent with recent models for the RNase P mechanism for pre-tRNA processing based on kinetic analyses and structure probing studies that invoke a conformational change involving docking of the substrate into the active site of RNase P.^{19,30,56} Further studies are required to address whether the docking step exists and, if so, how the binding of C5 protein may affect this step.

Materials and Methods

RNA synthesis

E. coli P RNA, *B. subtilis* pre-tRNA^{asp}, and *E. coli* pre-tRNAs were generated by *in vitro* transcription, as previously described.³⁰ Hybrid substrates were transcribed directly from linear PCR product DNAs. The (+1 Rp) PS-modified pre-tRNA was generated by ligation of a (−5, +6) 11-nt oligo (Dharmacon) and a (+7, +74) 3′ fragment of pre-tRNA^{met} generated by *in vitro* transcription. The Rp and Sp stereoisomers were separated and purified by reverse-phase HPLC, as described previously.⁵⁷ For kinetic and thermodynamic studies, pre-tRNAs were 5′-end-labeled with 150 μCi of [γ -³²P]ATP and T4 polynucleotide kinase after dephosphorylation by alkaline phosphatase.

Determination of substrate and product binding affinity

The apparent dissociation constants for pre-tRNAs were determined using a gel-filtration spin column, as previously described.^{24,27} Binding reactions were performed in 50 mM Tris (pH 7.5), 100 mM NaCl or NH₄Cl, and 17.5 mM CaCl₂. For holoenzyme reactions, 0.005% Triton X-100 was included. The binding experiments were performed in Ca²⁺ rather than in the optimal metal ion Mg^{2+} in order to suppress substrate cleavage. Gel electrophoresis was used to confirm that minimal substrate cleavage occurred during the course of the assay. P RNA (a series of increasing concentrations, 1 nM–3 μM) and 5′-³²P-end-labeled pre-tRNA were renatured separately in the absence of divalent metal ion by first incubating at 95 °C for 3 min, and then decreasing the temperature to 37 °C at 0.2 °C/s, and incubating at that

temperature for additional for 10 min. For holoenzyme reactions, C5, which was purified using the Impact expression system (NEB),³⁷ was added to the same concentration as that of P RNA, and the incubation continued for an additional 10 min. The appropriate concentration of the divalent metal ion was added, and the incubation continued for 10 min. Then the substrate and the enzyme were mixed and incubated for an additional 10 min at 37 °C before the sample was loaded to spin columns (Costar) containing 0.7 ml of packed G100 Sephadex equilibrated in the appropriate reaction buffer. Columns were spun for 15 s at 1200g. Enzyme–substrate complexes in the column flow-through were recovered, and the fraction of bound RNA was determined by Cerenkov scintillation counting. The fractions of bound substrate were fitted to a standard binding isotherm:

$$\frac{\text{cpm [ES]}}{\text{cpm[ES]}_{\text{max}}} = \frac{1}{1 + K_d / [\text{E}]}$$

where cpm[ES] is the number of counts recovered in the bound fraction, cpm[ES]_{max} is the number of counts recovered in the bound fraction at saturating enzyme concentration, [E] is the concentration of the enzyme in the binding reaction, and K_d is the apparent dissociation constant. The dissociation constants reported are the average of at least three independent determinations, each with <30% error in the curve fit to the primary data.

Kinetic analyses

Single-turnover kinetic analyses were performed essentially as described previously.⁵⁸ Rate constants were measured under the following conditions: 50 mM 4-morpholineethanesulfonic acid (Mes) for pH 5.3–6.5 (or 50 mM Pipes for pH 6.5–7.5 and 50 mM Tris for pH >7.5), 100 mM NaCl, 17.5 mM MgCl₂ (or concentrations indicated in the text), and 0.005% Triton X-100 for holoenzyme reactions. P RNA (0.1–5 μM) and 5′-³²P-end-labeled pre-tRNA (1–4 nM) were renatured separately, as described above, for thermodynamic analyses. Reaction volumes were typically 40 μl or less. MgCl₂ was added to the desired concentration, and incubation at 37 °C continued for 10 min. For holoenzyme reactions, C5 was added to the same concentration as that of P RNA, and the incubation continued for an additional 10 min. Enzyme and substrate were mixed together to start the reaction, and aliquots (~2 μl) were removed at predetermined intervals and quenched with an equal volume of ethylenediaminetetraacetic acid (EDTA) at least twice the concentration of the divalent metal ion in the reaction. To these samples was added an equal volume of a denaturing gel loading buffer containing 80% formamide and trace amounts of bromophenol blue and xylene cyanol dyes. Reaction products were resolved by denaturing PAGE (15%). The conversion of substrate into product was quantified by PhosphorImaging using a Molecular Dynamics system and ImageQuant software (GE). The data were plotted *versus* time using KaleidaGraph software (Synergy) and fitted to a single-exponential function:

$$F_{\text{react}} = A - Be^{-kt}$$

where F_{react} is the fraction of substrate converted into product at time t . The constant A is the extent of the reaction at $t = \infty$, B is the amplitude of the exponential, and k is the first-order rate constant. The rate constants reported are the average of at least three independent determinations, each with <30% error in the curve fit to the primary data. To ensure a saturating enzyme concentration, we used at least two enzyme concentrations to measure the catalytic rates. When no detectable change in the observed rate constant can be observed as the enzyme concentration doubled, the enzyme concentration is considered to be saturating.

Hydroxyl radical protection analyses

P RNA, holoenzyme, mock enzyme, and substrate mixtures were each assembled in a total volume of 100 μ l. The RNA and protein concentrations in these mixtures were 2.7-fold above the concentration indicated for the cleavage reactions, and the concentrations of the other components were 1.3-fold greater than indicated, so that upon mixing of substrate and enzyme and subsequent addition of cleavage reagents, the final concentration of the reagents would be 100 mM NaCl, 17.5 mM CaCl_2 , 50 mM Mes (pH 6.0), 25 nM 5' ^{32}P pre-tRNA (or control), and 200 nM holoenzyme or 1000 nM P RNA (or control).

Specifically, RNA, Mes, and NaCl were mixed in a total volume of ~ 90 μ l. For mock reactions, a mass of poly(A) RNA equivalent to that of P RNA was added. RNAs were folded by heating all mixtures to 95 $^{\circ}\text{C}$ for 3 min, followed by 10 min at 37 $^{\circ}\text{C}$. At this point, CaCl_2 was added, and the mixture was allowed to incubate for an additional 10 min at 37 $^{\circ}\text{C}$. C5 protein (or C5 storage buffer for the mock holoenzyme reaction) was added to bring the total volume to 100 μ l. This mixture was incubated for an additional 10 min at 37 $^{\circ}\text{C}$.

The hydroxyl radical cleavage reaction was performed in microfuge tubes containing 4.5 μ l of the substrate mixture that was mixed with 4.5 μ l of either ribozyme, holoenzyme, or mock enzyme mixture at 37 $^{\circ}\text{C}$. On the walls of each tube, three droplets of the following reagents were carefully spotted so that they did not touch one another and they remained suspended equidistantly above the substrate mixture in the bottom of the tube. All tubes were held at 37 $^{\circ}\text{C}$ in a programmable heating block during this process:

First droplet: 1 μ l of a 20 mM ferrous ammonium sulfate and 25 mM EDTA solution (which was prepared immediately prior to use)

Second droplet: 1 μ l of 0.7% H_2O_2

Third droplet: 1 μ l of 60 mM Na ascorbate.

Control reactions that contained the ferrous ammonium sulfate/EDTA solution (and 2 μ l of water) were performed. After the reactions had been prepared in this manner, the tubes were carefully transferred to a microfuge without disturbing the drops. The reaction was initiated by quickly spinning to mix. The tubes were transferred back to 37 $^{\circ}\text{C}$ and incubated for 10 min. The reaction was quenched by the addition of a 9- μ l mix containing 5 μ l of 100 mM thiourea, 3 μ l of 0.5 M EDTA, and 1 μ l of 10 mg/ml tRNA. The volume was then increased to 100 μ l with the addition of water. Mixtures were extracted with phenol/chloroform, recovering a constant volume from each tube, and then again with chloroform, again recovering a constant volume from each tube. NaCl was added to 0.3 M, and RNA was precipitated by the addition of 3 vol of 100% ethanol. RNA was pelleted by centrifugation. Air-dried pellets were dissolved in 10 μ l of 10 mM EDTA; to this, 10 μ l of dye-free Formamide GLB was added [formamide, 10 mM EDTA, and 20 mM Tris (pH 8.0)]. Cleavage products were resolved by denaturing (urea) PAGE on a 15% gel. Partial Ribonuclease T1 digests of the substrate were used as markers. RNA was visualized using PhosphorImager screens and quantified using ImageQuant.

Band intensities were quantified for each position in the desired range of each substrate (–10 to +35 with respect to the RNase P cleavage site) in all reactions by PhosphorImager analysis. A protection value was calculated by dividing the band intensities at each position in a control (no enzyme) reaction by those in the experimental reaction (containing P RNA or holoenzyme). This operation normalizes for position-dependent variations in sensitivity to hydroxyl radical cleavage. To further normalize for lane-to-lane variations in sample loading or reaction extent, we required the following additional manipulations of the data. Protection values that were within 2 SD of the mean for the range of positions being considered were

averaged. The protection value for each position within a given lane was divided by this average protection value to yield the fully normalized protection value κ , where a κ value of 1 reflects no protection.

Analysis of the metal ion dependence of k_c

RNase P or P RNA reactions were performed with radiolabeled pre-tRNA^{met608} and pre-tRNA^{met608}CA, essentially as described above, in the presence of Mg²⁺ at concentrations indicated in the text or figures. The observed k_c was then plotted as a function of Mg²⁺ concentration and fitted to a form of the Hill equation:

$$k_c (\text{observed}) = \frac{k_{\max} [\text{Mg}]^n}{(K_{\text{Mg}})^n + [\text{Mg}]^n}$$

where $k_c(\text{observed})$ is the observed rate constant measured at each Mg²⁺ concentration ([Mg]), $k_{\max}$ is the rate constant at saturating Mg²⁺ concentration, K_{Mg} is the apparent dissociation constant, and n is the Hill coefficient.

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Abbreviations used

RNase P	ribonuclease P
Rp-PS	Rp phosphorothioate
Mes	morpholineethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid

Fig. 1.

Interactions between pre-tRNA and RNase P at the site of processing. (a) Cartoon diagram of the RNase P enzyme-substrate complex. P RNA is depicted as a series of cylinders that indicate the positions of individual helices. The C5 protein subunit is shown as a sphere of approximate size relative to that of P RNA. The pre-tRNA substrate is shown as a black ribbon, with the leader sequence shown as a broken line. The site of processing by RNase P is indicated by an arrow. The nucleobase residues that are identified as important determinants for substrate recognition are shown as circles. (b) Details of the interactions between P RNA and the pre-tRNA cleavage site. The 5' R(73)C(74)C(75) 3' sequence located at the 3' terminus of pre-tRNA pairs with G292, G293, and U294 in the L15 region of P RNA; this interaction helps to align the correct phosphodiester bond in the active site.^{9,10} A pyrimidine (predominantly a U) is present 5' to the cleavage site in about 80% of *E. coli* pre-tRNAs, and this residue contacts A248 in the J5/15 region of P RNA.¹¹

Fig. 2. Sequence and secondary structure of *pre-tRNA^{met608}*, *pre-tRNA^{f-met605}*, *pre-tRNA^{Bs_asp}*, and *pre-tRNA^{leu601}*. Circled nucleotides in the *pre-tRNA^{f-met605}* structure indicate positions of sequence differences between *pre-tRNA^{f-met605}* and *pre-tRNA^{met608}*. The +1/+72 base pair is boxed, and the mutations at these positions made in the context of each pre-tRNA are indicated to the right of each secondary structure.

Fig. 3.

Comparison between the catalytic rate constant for the processing of consensus pre-tRNA and the catalytic rate constant for the processing of nonconsensus pre-tRNA. Representative single-turnover kinetic data for the cleavage of $pre-tRNA^{met608}$ (a) and $pre-tRNA^{f-met605}$ (b) by *E. coli* RNase P holoenzyme (filled symbols) and *E. coli* P RNA alone (open symbols). Reactions were performed at saturating enzyme concentrations (for holoenzyme, 1 μ M; for P RNA alone, 10–40 μ M) in 50 mM Mes (pH 5.75), 100 mM NaCl, and 17.5 mM $MgCl_2$. For holoenzyme reactions, 0.005% Triton X-100 was also included.

Fig. 4.

The secondary structure of swapped pre-tRNAs produced by switching corresponding parts between pre-tRNA^{f-met605} and pre-tRNA^{met608}. Filled dots represent portions from pre-tRNA^{met608}; open dots represent portions from pre-tRNA^{f-met605}.

Fig. 5.

Analysis of the effects of C(+1)/A(+72) pair *versus* G(+1)/C(+72) pair on P RNA and RNase P specificity. (a) Comparison of the P RNA and RNase P cleavage products of pre-tRNA^{met608} and pre-tRNA^{met608}CA to synthetic RNA standards. Substrates used or standards are indicated above each lane. Reactions containing either RNase P or P RNA are indicated by a plus (+) symbol above the lane. The position of the cleavage product resulting from processing at the correct tRNA 5' end between N(+1) and N(-1) is indicated by the symbol N(+1) to the right of each gel image. (b) Comparison of the P RNA and RNase P cleavage products of pre-tRNA^{met608} and pre-tRNA^{met608}CA, with or without a cleavage site Rp-PS modification. The reactions containing the pre-tRNA^{met608} and pre-tRNA^{met608}CA substrates are designated with GC and CA, respectively, above the

appropriate lanes. Reactions in which versions of the substrates contain a cleavage site Rp-PS modification are indicated by GC-Rp and CA-Rp for the modified pre-tRNA^{met608} and pre-RNA^{met608}CA substrates, respectively. Reactions containing either Mg²⁺ or Cd²⁺ are indicated by a plus (+) symbol above the lane. The position of the cleavage product resulting from processing at the correct phosphodiester bond is indicated by (N(+1)), while the cleavage product resulting from inaccurately processing one nucleotide 5' to the correct site is indicated by (N(-1)). Incubation times were proportionally longer for the slower-reacting phosphorothioate-modified substrates in the absence of Cd²⁺. For P RNA, minor cleavage products at N(-1) and N(-2) (indicated by an asterisk) may be due to miscleavage for this substrate. For RNase P, the products at N(-2) and N(-3) appear to arise from miscleavage of the phosphorothioate-modified substrate.

Fig. 6.

Hydroxyl radical protection analysis of pre-tRNA^{Bs_asp} binding to *E. coli* P RNA and RNase P holoenzyme. (a) Compiled protection factors (κ) for nucleotides -10 to +35 for binding to P RNA (filled symbols) and RNase P holoenzyme (open symbols). Points represent averages from three independent trials, and standard deviations from these averages are shown by error bars. The κ values were calculated as described in Materials and Methods. (b) Compiled protection data for nucleotides -10 to +35 shown on the secondary structure for pre-tRNA. Each nucleotide is displayed as a circle. The data for protection by P RNA are indicated by filled circles, and the data for RNase P holoenzyme are indicated by open circles as in (a). The diameter of each circle is proportional to the κ value for that nucleotide

position. The range of κ values encompassed by each size of the circle is indicated to the right of the graph in (a).

Fig. 7. Mg^{2+} concentration dependence of k_c for the cleavage of pre-tRNA^{met608} (squares) and pre-tRNA^{met608} CA (circles) by P RNA (open symbols) and by the RNase P holoenzyme (filled circles). The data are fitted to a variation in the Hill equation, as described in Materials and Methods. Individual data points represent the averages from three independent trials, and error bars indicate standard deviations from the average.

Scheme 1.

Reaction scheme for pre-tRNA processing by RNase P.

Scheme 2.
Parallel reaction scheme for correct and miscleavage.

Table 1
Comparison of binding thermodynamics and cleavage kinetics for the processing of pre-tRNA^{met608} and pre-tRNA^{f-met605} by P RNA and RNase P holoenzyme

Substrate (enzyme)	$K_{d,ptRNA}$ [nM]	k_c [min ⁻¹]	$K_{d,leader}$ [nM]	$K_{d,tRNA}$ [nM]
pre-tRNA ^{met608} (P RNA)	1200±170	0.14±0.05	>3000	150±40
pre-tRNA ^{met608} (RNase P)	0.5±0.4	1.30±0.18	>1000	10.7±3.0
pre-tRNA ^{f-met605} (P RNA)	3390±340	0.0011±0.0001	>3000	190±50
pre-tRNA ^{f-met605} (RNase P)	0.3±0.2	1.0±0.3	100±20	200±20

Data from Sun *et al.*²²

Table 2
Comparison of k_c values for the processing of structure variants of pre-tRNA^{met608} and pre-tRNA^{f-met605} by P RNA and RNase P holoenzyme

	$k_c(\text{P RNA}) [\text{min}^{-1}]$	$k_c(\text{RNase P}) [\text{min}^{-1}]$	$k_c(\text{RNase P})/k_c(\text{P RNA})$
pre-tRNA ^{met608}	0.14±0.05	1.3±0.2	9
pre-tRNA ^{f-met605}	0.0011±0.0001	1.0±0.3	910
608Stem/605loop	0.19±0.06	1.8±0.2	9
605Stem/608loop	0.0014±0.0002	1.2±0.2	860
608Top/605tRNA	0.22±0.02	2.7±0.1	12
605Top/608tRNA	0.0015±0.0004	0.9±0.1	600
608Leader/605tRNA	0.023±0.002	3.0±0.5	130
605Leader/608tRNA	0.25±0.02	1.1±0.1	4
pre-tRNA ^{f-met605} GC	0.29±0.02	1.3±0.1	4
pre-tRNA ^{met608} CA	0.0033±0.0004	2.0±0.3	610
pre-tRNA ^{met608} UA	<0.001	1.8±0.2	>1500
pre-tRNA ^{met608} AU	0.005±0.001	1.3±0.3	260
pre-tRNA ^{met608} CG	0.05±0.01	3.9±0.3	78

Table 3
Comparison of k_c values for the processing of +1/+72 base-pair mutants of pre-tRNA^{leu601} and pre-tRNA^{Bs_asp} by P RNA and RNase P holoenzyme

	$k_c(\text{P RNA}) [\text{min}^{-1}]$	$k_c(\text{RNase P}) [\text{min}^{-1}]$
pre-tRNA ^{leu601}	0.031±0.001	2.4±0.2
pre-tRNA ^{leu601} GC	0.07±0.01	4.5±0.5
pre-tRNA ^{leu601} CA	0.0013±0.0002	3.5±0.2
pre-tRNA ^{Bs_asp}	0.7±0.2	3.5±0.4
pre-tRNA ^{Bs_asp} CA	0.0010±0.0003	1.3±0.2

Table 4
Comparison of k_c values for the processing pre-tRNA^{met608}, pre-tRNA^{f-met605}, and (+1)/+72) mutants by P RNA in the presence of 100 mM NaCl and 1 M NH₄Cl

	$k_c(\text{P RNA})$ (100 mM) [min ⁻¹]	$k_c(\text{P RNA})$ (1 M NH ₄ Cl) [min ⁻¹]	$K_{d,\text{ptRNA}}(\text{P RNA})$ (1 M NH ₄ Cl) [μM]
pre-tRNA ^{met608}	0.14±0.05	8.4±0.6	1.0±0.2
pre-tRNA ^{f-met605}	0.0011±0.0001	0.044±0.01	2.4±0.2
pre-tRNA ^{met608} CA	0.0033±0.0004	0.31±0.09	1.4±0.3
pre-tRNA ^{f-met605} GC	0.29±0.02	4.3±0.8	0.7±0.2

Table 5
Rate and equilibrium constants for the cleavage of pre-tRNA^{met608} and pre-tRNA^{f-met605} with 10-nt and 2-nt leader sequences

	P RNA		RNase P	
	k_c [min ⁻¹]	$K_{d,ptRNA}$ [nM]	k_c [min ⁻¹]	$K_{d,ptRNA}$ [nM]
pre-tRNA ^{met608} 10 nt	0.14±0.05	2380±400	1.3±0.2	0.5±0.4
5' -GA-pre-tRNA ^{met608}	0.28±0.05	760±200	1.2±0.2	0.5±0.2
pre-tRNA ^{f-met605} 10 nt	0.0011±0.0001	3390±340	1.0±0.3	0.3±0.2
5' -GA-pre-tRNA ^{f-met605}	0.017±0.06	>5000	2.5±0.5	1.6±0.06