The Protein Component of *Bacillus subtilis* Ribonuclease P Increases Catalytic Efficiency by Enhancing Interactions with the 5′ Leader Sequence of Pre-tRNA^{Asp} †

Sharon M. Crary, S. Niranjanakumari, and Carol A. Fierke*

Department of Biochemistry, Box 3711, Duke University Medical Center, Durham, North Carolina 27710

Received March 17, 1998; Revised Manuscript Received April 29, 1998

ABSTRACT: Ribonuclease P (RNase P) is a ribonucleoprotein complex that catalyzes the formation of the mature 5′ end of tRNA. To investigate the role of the protein component in enhancing the affinity of *Bacillus subtilis* RNase P for substrate (Kurz, J. C., Niranjanakumari, S., Fierke, C. A. (1998) *Biochemistry 37*, 2393), the kinetics and thermodynamics of binding and cleavage were analyzed for pre-tRNA^{Asp} substrates containing 5′ leader sequences of varying lengths (1−33 nucleotides). These data demonstrate that the cleavage rate constant catalyzed by the holoenzyme is not dependent on the leader length; however, the association rate constant for substrate binding to holoenzyme increases as the length of the leader increases, and this is reflected in enhanced substrate affinity of up to 4 kcal/mol. In particular, the protein component of RNase P stabilizes interactions with nucleotides at −2 and −5 in the 5′ leader sequence of the pre-tRNA substrate. A 1 nucleotide leader decreases substrate affinity ≥15-fold compared to tRNA^{Asp} due to ground-state destabilization of the enzyme−substrate complex. This destabilization is overcome by increasing the length of the leader to 2 nucleotides due to P RNA−pre-tRNA contacts that are stabilized by the P protein. The affinity of RNase P holoenzyme (but not RNA alone) for pre-tRNA^{Asp} is further enhanced with a substrate containing a 5 nucleotide leader. These data indicate that novel direct or indirect interactions occur between the 5′ leader sequence of pre-tRNA^{Asp} and the protein component of RNase P.

In prokaryotic genomes, particularly in *Bacillus subtilis*, tRNAs are often located as precursor tRNA (pre-tRNA) sequences in polycistronic gene clusters (*1*−*3*). These pre-tRNAs are processed by a number of endo- and exonucleolytic enzymes to produce mature tRNA (*4*). Cleavage of pre-tRNA, catalyzed by ribonuclease P (RNase P),[1] to form the mature 5′ end of tRNA can be the first event that occurs in this maturation process (*5*, *6*). RNase P is composed of a ∼400 nucleotide RNA component and a 15 kDa protein component, both of which are required for efficient pre-tRNA processing in vivo (*5*, *7*−*9*). However, the RNA component of the bacterial RNase P (P RNA) is catalytic alone in vitro (*9*−*13*). That is, P RNA can recognize and bind a pre-tRNA substrate and catalyze hydrolysis of a specific phosphodiester bond, producing mature tRNA with a 5′ phosphate. The protein component of RNase P (P protein) decreases the concentration of mono- and divalent cations required for optimal activity (*10*, *11*) by specifically enhancing the affinity of the holoenzyme for pre-tRNA substrates (*14*, *15*).

RNase P binds and cleaves multiple pre-tRNA sequences in vivo, suggesting that the main molecular recognition elements are the conserved secondary and tertiary structures of tRNA, rather than a specific primary sequence. A variety of experiments involving modification-interference analysis (*16*−*22*), phylogenetic comparative mutational analysis (*23*−*28*), and cross-linking techniques (*29*−*31*) have elucidated regions of the bacterial RNase P RNA that interact with the substrate. Interactions between the enzyme and substrate are localized to certain conserved sequences in the RNase P RNA, including the central P4 helix, the loop after P15, and the J11/12. The P RNA recognizes the helix formed by the acceptor stem of the tRNA, as has been directly demonstrated by the ability of P RNA to catalyze cleavage of truncated model substrates (*32*−*34*). Additionally, P RNA contacts other regions of tRNA, including the T stem-loop (*17*, *21*, *22*, *26*, *35*), the 3′ CCA sequence (*16*, *31*, *36*), and the 5′ leader sequence (*16*, *37*, *38*). Nonetheless, the conformation of the RNase P RNA is relatively equivalent with either bound substrate or product (*29*, *30*), and P RNA actually binds pre-tRNA more weakly than tRNA (*39*, *40*).

In contrast, for holoenzyme RNase P the pre-tRNA substrate is bound significantly tighter than tRNA due to the presence of the protein component (*15*). At low salt concentrations (10 mM $MgCl_2$, 100 mM NH_4Cl), addition of the protein component of *B. subtilis* RNase P enhances the affinity of pre-tRNA^{Asp} 10^4-fold while the affinity of tRNA^{Asp} increases less than 10-fold. Since there are no absolute consensus sequences for the 5′ leader sequences in *B. subtilis* polycistronic tRNA clusters (*1*−*3*), it is likely that the increased affinity of pre-tRNA caused by the protein

† Supported by National Institutes of Health Grant GM 55387. S. M.C. was supported in part by NIH Training Grant GM08558.

* To whom correspondence should be addressed. Phone (919) 684-2557. Fax: (919) 684-8885.

[1] Abbreviations: low salt/Mg^{2+} buffer, 50 mM MES, 50 mM Tris, 10 mM $MgCl_2$, 100 mM NH_4Cl, pH 6.0; low salt/Ca^{2+} buffer, 50 mM MES, 50 mM Tris, 10 mM $CaCl_2$, 100 mM NH_4Cl, pH 6.0; high salt/Ca^{2+} buffer, 50 mM MES, 50 mM Tris, 25 mM $CaCl_2$, 1 M NH_4Cl, pH 6.0; E, P RNA or RNase P holoenzyme; EDTA, (ethylenedinitrilo)-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; P RNA, RNA component of RNase P; P protein, protein component of RNase P; RNase P, ribonuclease P; S or pre-tRNA^{Asp}, precursor tRNA^{Asp}; TE, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0; Tris, tris(hydroxymethyl)-aminomethane; PCR, polymerase chain reaction; PEI, polyethylenimine.

S0006-2960(98)00613-8 CCC: $15.00
Published on Web 06/11/1998

Table 1: Association and Cleavage Rate Constants and Dissociation Constants of Truncated pre-tRNA^{Asp} Substrates Catalyzed by RNase P Holoenzyme or RNase P RNA Alone[a]

substrate 5′ leader length	5′ leader sequence[b]	k_{obs}[c] (μM^{-1} s^{-1}), holoenzyme low salt/Mg^{2+}	k_2 (s^{-1}), holoenzyme low salt/Mg^{2+}	K_D (nM), holoenzyme low salt/Ca^{2+}	K_D (nM), P RNA low salt/Ca^{2+}	K_D (nM), P RNA high salt/Ca^{2+}
33	...UACCCAAAAU	6[d]	0.33[d]	0.4[d]	4000[d]	26 ± 6
10	GCCAAAACAU	8.7 ± 0.6	0.27 ± 0.02			
5	GACAU	6 ± 1	0.27 ± 0.05	≤0.5		
4	GCAU	0.25 ± 0.02	0.27 ± 0.04	19 ± 3		
3	GAU	0.95 ± 0.07	0.28 ± 0.03	28 ± 8		
2	GU	0.11 ± 0.01	0.24 ± 0.03	19 ± 5	2500 ± 1000	23 ± 3
1	U	0.004 ± 0.001	>0.01[e]	3000 ± 1000	>20000	41 ± 8
0				200 ± 30	560 ± 100	2.1 ± 0.3

[a] Single-turnover data ([E]/[S] > 5; [S] = 0.2−10 nM) measured in low salt/Mg^{2+} (50 mM Tris, 10 mM $MgCl_2$, 100 mM NH_4Cl, pH 6.0). Concentration of RNase P holoenzyme was the following: for 10-tRNA and 5-tRNA, [E] = 1−1000 nM; for 4-tRNA, 3-tRNA, and 2-tRNA, [E] = 2−15000 nM; and for 1-tRNA, [E] = 0.5−1 μM. All rate constants, except for 1-tRNA, were determined using three-dimensional fits of the appearance of product over time for various enzyme concentrations. The association rate constant for 1-tRNA was calculated from a linear fit of the observed rate constants versus enzyme concentration. Dissociation constants measured at 37 °C in low salt/Ca^{2+} (50 mM MES, 50 mM Tris, 10 mM $CaCl_2$, 100 mM NH_4Cl, pH 6.0) or high salt/Ca^{2+} (50 mM MES, 50 mM Tris, 25 mM $CaCl_2$, 1 M NH_4Cl, pH 6.0), as described in the legend to Figure 2. The concentration of holoenzyme was higher than the substrate ([E]/[S] > 5; [S] = 0.2−10 nM) as follows: for 5-tRNA, [E] = 0.5−16 nM; for 4-tRNA, 3-tRNA, and 2-tRNA, [E] = 0.5−200 nM; and for tRNA, [E] = 20−1000 nM. The concentration of P RNA used was for 2-tRNA, [E] = 1−20 μM; and for tRNA, [E] = 0.6−3.5 μM. [b] All substrates with leaders ≤14 nucleotides have the tRNA^{Asp} sequence with a truncated 5′ leader sequence, as shown, and contain at least one guanosine at the 5′ end. The sequence of the entire pre-tRNA^{Asp} 5′ leader sequence is GGGAGACCGGAAUUCGAGCUCGGUACCCAAAAU. [c] The observed rate constant at low enzyme concentrations reflects k_1 for all substrates, except possibly 1-tRNA (see text). [d] Taken from Kurz et al., 1998. [e] No curvature was observed in a plot of k_{obs} versus [E] up to 1 μM.

component is due to recognition of the presence of a leader rather than any particular sequence specificity. To investigate the differentiation between substrate and product, we analyzed the dissociation constants and association and cleavage rate constants for pre-tRNA^{Asp} substrates with 5′ leader sequences varying in length (1−33 nucleotides), using techniques developed for the elucidation of a detailed kinetic scheme for the cleavage of pre-tRNA^{Asp} catalyzed by P RNA (*39*, *41*). The transient kinetic and thermodynamic binding experiments with *B. subtilis* RNase P holoenzyme demonstrate that the protein subunit of RNase P increases the affinity and specificity for pre-tRNA^{Asp} substrates with 5′ leaders longer than 4 nucleotides.

MATERIALS AND METHODS

RNA and Protein Preparation. All RNAs were prepared by in vitro transcription from linearized plasmids with T7 RNA polymerase (*42*) purified from an overexpressing strain provided by W. Studier (*43*). P RNA was transcribed as previously described (*39*). Plasmid pDW152, a gift from the laboratory of Dr. Norman Pace, was used as a template for polymerase chain reaction (PCR) to amplify the DNA for pre-tRNA^{Asp} substrates with various 5′ leader lengths. These DNA fragments were then subcloned into pUC18 (USB) behind a T7 RNA polymerase promoter. Substrates with leaders ranging from 0 to 14 nucleotides were then transcribed from these plasmids (Table 1). These substrates are referred to as *N*-tRNA, where *N* is the number of nucleotides in the 5′ leader. All substrates (except 1-tRNA) begin with guanosine for the in vitro transcription reaction. For substrates with leaders ≤14 nucleotides, the nucleotides at positions −1 through −7 are identical to the leader sequence for pre-tRNA^{Asp} found in the *trrnE* gene cluster in *B. subtilis* (*3*). The plasmid for an additional substrate, GG-tRNA, containing guanosines at positions −1 and −2 was also constructed. Substrates were transcribed in the presence of 1 mM each of ATP, CTP, and UTP, 0.4 mM GTP, and 4 mM guanosine (Sigma) to produce RNA containing a 5′ hydroxyl group (*44*). This RNA was subsequently labeled at the 5′ hydroxyl by incubation with γ-^{32}P-ATP and T4 polynucleotide kinase (New England Biolabs). To prepare a substrate with one uracil nucleotide 5′ of the cleavage site (1-tRNA), 4 mM 5′ end-labeled UpG (Sigma) was included in the transcription reaction (*42*, *45*). The concentration of 1-tRNA was determined from the specific activity of the purified RNA.

Recombinant P protein was induced in BL21(DE3)pLysS *Escherichia coli* (*46*) containing plasmid pPWT-1 with the P protein gene under the control of a T7 RNA polymerase promoter by the addition of isopropyl-thio-β-D-galactoside and purified by the denaturing purification method using two CM-sepharose columns, as previously described (*15*). Holoenzyme was reconstituted immediately prior to use by incubation of refolded P RNA with an excess concentration of protein at 37 °C.

Single-Turnover Experiments. Single-turnover experiments were performed under conditions of excess enzyme concentration ([E]/[S] > 5; [E] = 1 nM to 20 μM; [S] = 0.1−10 nM) at 37 °C and pH 6.0. This lower pH decreases the cleavage rate constant so that all the time points could be acquired manually (*45*). Transient kinetic experiments were performed in low salt/Mg^{2+} buffer (50 mM MES, 50 mM Tris, 100 mM NH_4Cl, 10 mM $MgCl_2$, pH 6.0). All RNAs were denatured at 95 °C for 3 min in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), diluted into reaction buffer, and then incubated at 37 °C for 10 min. Holoenzyme was reconstituted by addition of P protein to the P RNA solution and incubated for 5−10 min. Reactions were initiated by the addition of substrate to enzyme and were quenched by dilution into an equal volume of 0.2 M EDTA, pH 8.0. Reactions with substrates containing leaders shorter than 7 nucleotides were spotted on polyethylenimine (PEI) cellulose thin-layer chromatography plates (Merck), dried, soaked in methanol for five minutes to remove interfering salts, re-dried, and then developed in 1 M LiCl (*47*). Reactions with substrates containing leader sequences longer than 6 nucle-

otides were analyzed on a 6% polyacrylamide gel containing 7 M urea. The separated reactants and products were visualized and quantified using a PhosphorImager (Molecular Dynamics).

Measurement of Dissociation Constants. An excess concentration of RNase P holoenzyme or P RNA was incubated with substrate ([E]/[S] > 5; [E] = 0.5 nM to 20 μM; [S] = 0.2−10 nM), for 10−20 min at 37 °C in low salt/Ca^{2+} buffer (50 mM MES, 50 mM Tris, 10 mM $CaCl_2$, 100 mM NH_4Cl, pH 6.0) or high salt/Ca^{2+} buffer (50 mM MES, 50 mM Tris, 25 mM $CaCl_2$, 1 M NH_4Cl, pH 6.0). The substitution of calcium for magnesium decreases the cleavage rate constant about 10^4-fold (*40*) while maintaining the folded RNA structure (*37*). Bound and unbound substrate were separated by passage through G-75 gel filtration resin using spin column chromatography (*39*, *48*, *49*). The fraction of substrate bound was determined as the fraction of total radioactivity that is found in the eluate, as measured by Cerenkov scintillation counting.

Data Analysis. Single-turnover data were fit with both KaleidaGraph (Synergy Software) and Systat (Systat, Inc.). All reported errors are the asymptotic standard errors. For reactions of holoenzyme and substrate, the time course for appearance of product over time was fit to a mechanism of two consecutive irreversible first-order reactions (eq 1) (*15*, *39*, *50*). A precise determination of the rate constants for substrate association (k_1) and cleavage (k_2) can be obtained by simultaneously fitting several data sets of the appearance of product (P; defined as product/(substrate + product)) over time (t) measured at different concentrations of enzyme [E]. This method provides an accurate method for determining values for k_2 by extrapolating from conditions at which cleavage is not the sole rate-contributing step. Alternatively, at enzyme concentrations either 10-fold above or 10-fold below the enzyme concentration at which $k_1[E] = k_2$, the data can be approximated by a single-exponential fit (eq 2) indicating the value of either k_1 at low [E] or k_2 at high [E]. For reactions of P RNA alone with substrate, the time course for the appearance of product was fit to the equation for a single-exponential decay (eq 2). Under these conditions, the bound enzyme−substrate complex equilibrates rapidly with free enzyme and substrate before it is converted to enzyme and product (*15*).

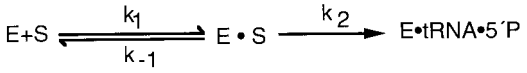
$$[P]_t = [\text{pre-tRNA}]_0 \times \left[1 + \frac{1}{k_1[E] - k_2}(k_2 e^{-k_1[E]t} - k_1[E]e^{-k_2 t})\right] \quad (1)$$

$$[P]_t = [P]_\infty (1 - e^{-k_{obs}t}) \quad (2)$$

Thermodynamic binding data were fit with a standard binding isotherm (eq 3) in which the fraction of substrate or product bound ($[E{\cdot}L]/[L]_{total}$) was plotted against the concentration of enzyme ([E]). Since the column procedure does not completely separate free and bound ligand, the fraction of bound ligand was determined as ($fraction_{cpm\ bound} - fraction_{cpm\ bound\ with\ no\ enzyme}$)/($fraction_{cpm\ bound\ at\ endpoint} - fraction_{cpm\ bound\ with\ no\ enzyme}$).

$$[E{\cdot}L]/[L]_{total} = 1/(1 + K_D/[E]_{total}) \quad (3)$$

Scheme 1



RESULTS

Previous kinetic and thermodynamic experiments indicate that the protein component of RNase P increases the affinity of pre-$tRNA^{Asp}$ enormously (10^4-fold) while only modestly increasing the affinity of tRNA (≤10-fold) (*15*), indicating that the protein component of RNase P enhances interactions with the substrate leader sequence. To further understand the effect of the protein component on the affinity of pre-$tRNA^{Asp}$, we have prepared substrates with 5′ precursor fragments varying in length from 1 to 33 nucleotides (Table 1). Kinetic and thermodynamic analyses of RNase P-catalyzed cleavage of these substrates allow determination of the minimum 5′ leader length required for optimal activity.

Transient kinetic experiments with RNase P allow the direct observation of both substrate association (k_1, Scheme 1) and cleavage (k_2, Scheme 1) (*39*). As observed for pre-$tRNA^{Asp}$ with a 33 nucleotide leader sequence (*15*, *39*), under single-turnover conditions the reaction of RNase P holoenzyme with substrates containing a leader ≥2 nucleotides follows a mechanism of two consecutive irreversible first-order reactions. For this mechanism, the cleavage rate constant is faster than the dissociation rate constant ($k_2 > k_{-1}$, Scheme 1) so that substrate binding is essentially irreversible. Therefore, under single-turnover conditions substrate association is the rate-limiting step at low enzyme concentration ($k_1[E] < k_2$) while the cleavage step becomes rate-limiting at high enzyme concentration ($k_1[E] > k_2$) (Figure 1). At intermediate concentrations of enzyme ($k_1[E] = k_2$), a lag in the formation of product due to the transient accumulation of ES is observed (Figure 1); this behavior is diagnostic of a mechanism with two irreversible first-order reactions in which product is formed in the second step (*50*).

Substrate Association Rate Constant Depends on the Leader Length for RNase P Holoenzyme. At low concentrations of RNase P holoenzyme, the time course for the appearance of $tRNA^{Asp}$ from pre-$tRNA^{Asp}$ substrates fits a single pseudo-first-order exponential decay, and the observed rate constants are linearly dependent on the RNase P concentration indicating that substrate association, k_1, is the rate-limiting step (Figure 1). The rate constant for the association of substrate with RNase P holoenzyme at pH 6.0 is $6 \pm 2 \times 10^6$ M^{-1} s^{-1} for substrates with a 5′ leader of 5−33 nucleotides (Table 1), in agreement with the value measured for pre-$tRNA^{Asp}$ at pH 8 (*39*). However, the association rate constant for 4-tRNA is decreased 20-fold to $2.5 \pm 0.2 \times 10^5$ M^{-1} s^{-1}. Similarly, the association rate constants for 3-tRNA and 2-tRNA are 6-fold and 54-fold slower than pre-tRNA with a 33 nucleotide leader (Figure 1; Table 1). To confirm that these observed rate constants for substrates with shorter leader sequences still reflect the association step, we demonstrated that $k_{obs}/[E]$ decreases <2-fold as the pH is lowered from 8 to 6 (data not shown), consistent with the pH independence of substrate binding (*15*, *45*). For 1-tRNA, the observed single-turnover rate constant for product formation is linearly dependent on the concentration of enzyme up to 1 μM and the calculated

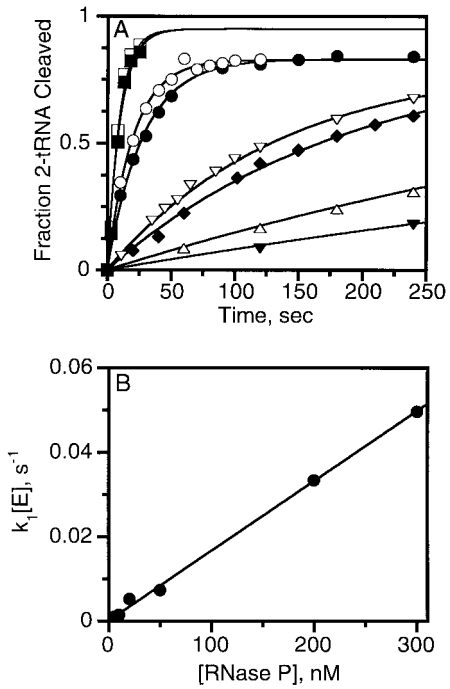


FIGURE 1: Single-turnover measurements of RNase P-catalyzed cleavage of 2-tRNA at varied enzyme concentrations. 5′ End-labeled 2-tRNA (1−10 nM) was mixed with RNase P holoenzyme (25−300 nM) and incubated in low salt/Mg^{2+} buffer at 37 °C. Reactions were stopped by dilution into an equal volume of 0.2 M EDTA, the products were separated on PEI-TLC plates, and cleavage was visualized and quantified using a PhosphorImager. (A) At low (<300 nM) and high (≥3 μM) concentrations of RNase P, the time course for the appearance of product follows a single exponential [5 nM (▼), 10 nM (△), 20 nM (◆), 50 nM (▽), 200 nM (●), 300 nM (○), 3 μM (□), 5 μM (■)]. The data are fit using eq 1. This single-exponential fit of the appearance of product over time yields an estimate for a value of the cleavage rate constant, k_2 (0.22 ± 0.02 s^{-1}). A three-dimensional fit of all of the time courses for the appearance of product at various enzyme concentrations to a mechanism of two consecutive first-order reactions (eq 2) gives a more accurate value for k_2 (0.27 ± 0.05). (B) The slope of a plot of the observed rate constants at low enzyme versus the concentration of RNase P holoenzyme allows for an estimation of the association rate constant, k_1 (1.7 ± 0.3 × 10^5 M^{-1} s^{-1}). This rate constant (k_1 = 1.0 ± 0.1 × 10^5 M^{-1} s^{-1}) was also determined from a fit of the time courses for appearance of product for all of the enzyme concentrations to a mechanism of two consecutive first-order reactions (eq 2).

second-order rate constant (4.1 ± 0.1 × 10^3 $M^{-1}s^{-1}$) is decreased 1500-fold compared to the association rate constant for substrates with longer 5′ leaders. Furthermore, the kinetic mechanism for this substrate may be altered to rapid equilibrium binding, and hence, the 1500-fold decrease could reflect a decrease both in substrate affinity and in the cleavage rate constant, rather than solely a decrease in the association rate constant.

Cleavage Rate Constant is Independent of the Leader Length. At high concentrations of RNase P (k_1[E] ≥ 10k_2), the time course for appearance of product is described by a single first-order exponential decay that is independent of the concentration of RNase P and reflects k_2, the rate constant for substrate cleavage (eq 2; Scheme 1). For wild-type pre-tRNA^{Asp}, k_2 has been measured as 0.33 s^{-1} at pH 6.1 with 10 mM $MgCl_2$, 100 mM NH_4Cl (*15*). The cleavage rate constant varies less than 10% for substrates with a leader of 2−33 nucleotides (Table 1), indicating that the length of the 5′ leader is not important for stabilizing the transition state for substrate cleavage. The cleavage rate constant for pre-tRNA with a single-nucleotide leader may decrease (k_2 ≥ 0.01 s^{-1}), but the actual value is difficult to determine because this substrate binds so poorly.

Additionally, the cleavage rate constant for a substrate with a leader sequence composed of two guanosines, GG-tRNA, is decreased to 3.9 ± 0.1 × 10^{-3} s^{-1}, demonstrating that the cleavage step is not entirely independent of the 5′ leader sequence. While decreasing the length of the leader to 2 nucleotides does not affect the value of k_2, changing the nucleotide at position −1 from U to G decreases the cleavage rate constant 69-fold. The efficiency of pre-tRNA cleavage catalyzed by RNase P has previously been observed to depend on the sequence at the −1 position (*23*, *51*).

Catalytic Efficiency is Modestly Dependent on Leader Length for RNase P RNA Alone. Unlike RNase P holoenzyme, the dissociation rate constant for pre-tRNA^{Asp} from the enzyme−substrate complex is rapid compared to the cleavage rate constant (k_{-1} > k_2, Scheme 1) (*15*). Thus, under single-turnover conditions, the observed second-order rate constant at low concentrations of P RNA reflects both the substrate affinity and the cleavage rate constant (k_2/K_D), rather than the association rate constant (k_1). For the 2-tRNA substrate, this second-order rate constant decreases only 3-fold (400 ± 30 M^{-1} s^{-1}) compared to the value of 1400 M^{-1} s^{-1} determined for pre-tRNA^{Asp} under identical conditions (*15*) (Table 1). Thus, truncation of the 5′ leader sequence has a modest effect on pre-tRNA processing catalyzed by P RNA alone. However, we cannot determine whether removal of the leader sequence specifically decreases the association rate constant.

Substrate Affinity is Dependent on the 5′ Leader Length for RNase P Holoenzyme. To identify the effect of the 5′ leader length on the affinity of RNase P holoenzyme for substrate, we measured the dissociation constants for a variety of pre-tRNA^{Asp} substrates with RNase P (Figure 2). For these experiments we substituted 10 mM $CaCl_2$ for 10 mM $MgCl_2$ to decrease the cleavage rate constant, while maintaining the correct P RNA folding and binding properties (*15*, *37*, *40*, *52*). These thermodynamic measurements of the substrate dissociation constants demonstrate that truncation of the 5′ leader decreases the affinity of the holoenzyme for substrate (Table 1). The dissociation constant for substrates with leader lengths greater than 4 nucleotides is <0.5 nM while the K_D for substrates with leaders of 2, 3, or 4 nucleotides is about 50-fold weaker (Figure 2). Interestingly, 1-tRNA has a K_D of 3 ± 1 μM; thus holoenzyme binds 1-tRNA 15-fold less tightly than product tRNA and 160-fold less tightly than 2-tRNA (Table 1).

For comparison, we also measured the affinity of P RNA for the truncated substrates (Table 1). As previously observed, the affinity of P RNA for pre-tRNA^{Asp} is significantly reduced (10^4-fold) compared to the holoenzyme under these conditions. Furthermore, these data demonstrate that P RNA does not significantly discriminate between substrates with varied leader lengths until the leader is only 1 nucleotide; the K_D for 2-tRNA is 2.5 ± 1 μM compared to 4 μM for pre-tRNA^{Asp} (*15*). However, the affinity of P RNA for 1-tRNA is decreased ≥8-fold compared to 2-tRNA and ≥30-fold compared to product tRNA (Table 1). This destabilization of substrate binding to P RNA can be partially

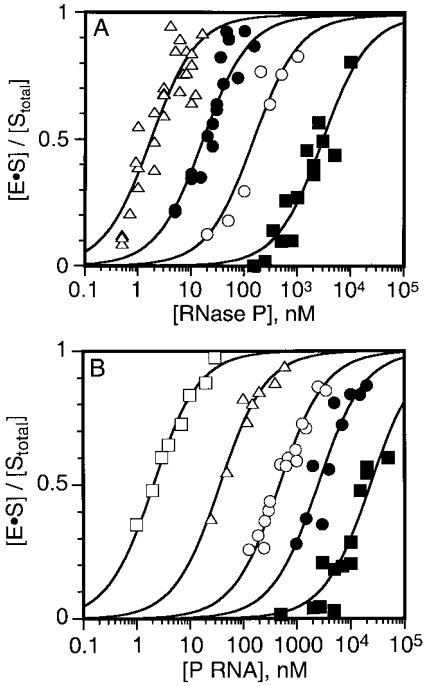


FIGURE 2: Measurement of the dissociation constants describing the affinity of RNase P holoenzyme or P RNA for various truncated substrates and product tRNA. 5′ End-labeled substrate (≤1 nM) was incubated with excess enzyme (0.5−500 nM) in low salt/Ca^{2+} buffer or high salt/Ca^{2+} buffer at 37 °C before bound and unbound substrate were separated by passage through a gel filtration spin column. Dissociation constants were calculated from a fit of the data to eq 3. (A) Affinity of RNase P holoenzyme for substrates and product in low salt/Ca^{2+} buffer [tRNA (○), 1-tRNA (■), 2-tRNA (●), 5-tRNA (△)]. (B) Affinity of P RNA for substrates and product in low salt/Ca^{2+} buffer [tRNA (○), 1-tRNA (■), 2-tRNA (●)) or high salt/Ca^{2+} buffer (tRNA (□); 1-tRNA (△)].

ameliorated by the addition of cations; in 25 mM $MgCl_2$, 1 M NH_4Cl, the K_D for 1-tRNA is enhanced 480-fold and this value is only 20-fold weaker than that of tRNA binding to P RNA alone under these conditions (Table 1). Furthermore, the K_D for dissociation of either 2-tRNA or pre-tRNA from a complex with P RNA ($K_D \sim 25$ nM) is decreased about 100-fold by the addition of high salt.

DISCUSSION

Kurz and colleagues (*15*) have previously demonstrated that the RNase P holoenzyme binds pre-$tRNA^{Asp}$ with a significantly higher affinity than $tRNA^{Asp}$. Since this is not observed for P RNA alone (*39*), the increased affinity for substrate indicates that the holoenzyme interacts more favorably with the 5′ leader. To investigate the effect of the P protein on substrate specificity, we measured rate and equilibrium constants for various 5′ truncated substrates binding to RNase P, in the presence and absence of the protein component at relatively low salt conditions (10 mM $MgCl_2$, 100 mM NH_4Cl). While P RNA is minimally active at such low salt, these experiments allow for a direct comparison to the holoenzyme reaction so that changes in rate constants due solely to differences in the salt concentration can be ruled out. To highlight any purely electrostatic effects, additional binding studies were performed with P RNA alone at high salt (25 mM $CaCl_2$, 1 M NH_4Cl). An important result from these transient kinetic data is that the rate constant for substrate cleavage catalyzed by the holoenzyme does not change significantly as the leader is lengthened from 2 to 33 nucleotides; however, the association rate constant for substrate binding to holoenzyme does increase as the length of the leaders increases, and this is reflected in enhanced substrate affinity of up to 4 kcal/mol (Figure 3).

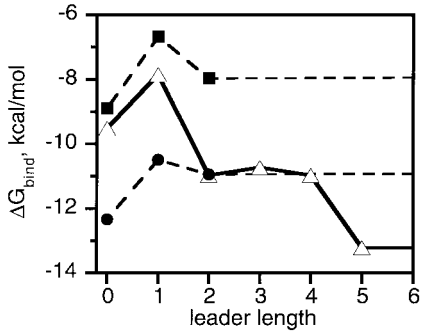


FIGURE 3: The free energy (ΔG_{bind}) for the association of pre-$tRNA^{Asp}$ with RNase P depends on the leader length for both RNase P holoenzyme (△) and P RNA in either low salt/Ca^{2+} buffer (■) or high salt/Ca^{2+} buffer (●)). The free energy is calculated as $-RT \ln(1/K_D)$ where $T = 37$ °C using the dissociation constants listed in Table 1.

Sequence Dependence. A comparison of the *B. subtilis* pre-tRNA leader sequences reveals little or no sequence conservation, except at the −1 position where U is preferred, although A and C, but rarely G, are also observed (*1*−*3*). Consistent with this observation, our data demonstrate that while a substrate with as little as a 2 nucleotide leader has a wild-type cleavage rate constant (Table 1), this efficient cleavage rate constant is not totally independent of the sequence of the 5′ leader. For example, a substrate with a 2 nucleotide leader in which the nucleotide at the −1 position has been changed to a guanine (GG-tRNA) has a maximal cleavage rate constant that is at least 50 times slower than the substrate with the wild-type sequence (GU-tRNA). Previously, Tallsjö and colleagues (1996) demonstrated that the G at position −1 in pre-$tRNA^{His}$ is essential for cleavage site selection by *E. coli* RNase P RNA, likely due to the formation of a base pair with C_{73} which alters the interaction between this base and U_{294} of P RNA. Similarly, in our pre-$tRNA^{Asp}$ construct, the U at position −1 can base pair with the G at position 74, immediately prior to the 3′CCA sequence. It is possible that the disruption of this base pair when the −1 U is mutated to G causes the observed decrease in the cleavage rate constant possibly due to misalignment of the substrate at the cleavage site (*25*, *51*). Thus, although the cleavage rate constant for pre-$tRNA^{Asp}$ is not dependent on the length (2−33 nucleotides), pre-tRNA cleavage can be affected by the sequence of the 5′ leader sequence.

Ground-State Destabilization of 1-tRNA. Although it is not an efficient substrate, 1-tRNA is bound and cleaved by the *B. subtilis* P RNA, the *E. coli* RNase P RNA (*45*), and the *B. subtilis* RNase P holoenzyme in vitro. Interestingly, the affinities of the holoenzyme and P RNA for 1-tRNA at low salt (10 mM $MgCl_2$, 100 mM NH_4Cl) decrease by 1.7 and >2.2 kcal/mol, respectively, compared to product $tRNA^{Asp}$. At high salt (25 mM $MgCl_2$, 1M NH_4Cl), destabilization of 1-tRNA bound to P RNA decreases; the affinity of 1-tRNA is only 1.8 kcal/mol less favorable than that of tRNA (Table 1; Figure 3).

The decreased affinity of 1-tRNA is perhaps indicative of an unfavorable interaction in the ground state that can be

relieved in the transition state, thus decreasing the free-energy difference between these two states and allowing a consequent increase in the reaction rate constant (*53*, *54*). Either high concentrations of mono- and divalent cations (*45*) or the P protein can partially relieve the ground-state destabilization of the P RNA−1-tRNA complex, suggesting that at least part of the destabilization is caused by either an electrostatic repulsion between the two negatively charged RNAs or a decreased affinity for an essential magnesium ion.

In 1-tRNA the group adjacent to the cleavage site is a 5′ monophosphate group with a net charge of −2 at pH 6 (*55*). This group is significantly different than the usual phosphodiester bond that would be found in this position in any substrate with a longer leader sequence, so it is possible that unfavorable electrostatic interactions may be elevated near the cleavage site in this substrate. However, since binding of 1-tRNA by P RNA is still destabilized by ≥1.7 kcal/mol in the presence of either excess cations or the RNase P protein component, this repulsion can only be partially modulated by positive charge, suggesting that the destabilization of 1-tRNA is due to other factors as well.

Magnesium ions play multiple roles in the cleavage of pre-tRNA catalyzed by RNase P RNA. In particular, at least two magnesium ions enhance the affinity of pre-tRNA^{Asp} and a third magnesium ion stabilizes the transition state for cleavage (*41*, *45*, *52*). Therefore, the observed destabilization of 1-tRNA^{Asp} could be due to the decreased affinity of one or more of these crucial magnesium ions. Alternatively, destabilization of a ribozyme−substrate complex due to the proximity of a ground-state magnesium ion to a phosphodiester bond has precedent in the *Tetrahymena* ribozyme (*56*). Narlikar and colleagues argue that the oxygen in the reactive phosphate gains a partial positive charge through donation of electrons to the phosphodiester bond, resulting in an unfavorable interaction with the positively charged magnesium. This destabilization is relieved in the transition state as the phosphodiester bond breaks, and the previously destabilizing oxygen atom develops a negative charge, thus stabilizing its interaction with the magnesium ion.

Substrate Stabilization in 2-tRNA. The addition of a second nucleotide to the wild-type 5′ leader sequence of pre-tRNA enhances the affinity of both P RNA and holoenzyme for substrate (Table 1; Figure 2). To compensate for the destabilization observed in the binding of 1-tRNA, stabilizing interactions of up to 3 kcal/mol occur between 2-tRNA and RNase P. Previous data indicate that the first 2−4 nucleotides of the 5′ leader of pre-tRNA interact with nucleotides in the sequences comprising J18/2 and J6/7 in RNase P RNAs from a diverse subset of organisms (*16*, *37*, *57*). On the basis of footprinting analysis of *E. coli* RNase P RNA in the presence and absence of a substrate, LaGrandeur and colleagues (*16*) have proposed either direct contacts between RNase P RNA and the 5′ leader of pre-tRNA or a local conformational rearrangement in the enzyme upon the binding of pre-tRNA. Our data confirm that RNase P RNA interacts with the leader sequence since substrates with leaders longer than 1 nucleotide are bound >1.3 kcal/mol tighter than 1-tRNA; however, favorable interactions are localized to the second nucleotide. Additionally, P RNA still binds pre-tRNA weaker than tRNA by >0.9 kcal/mol. In contrast, RNase P holoenzyme binds substrates with leaders of 2, 3, and 4 nucleotides roughly 3 and 1.5 kcal/mol tighter than 1-tRNA and tRNA, respectively. Thus, although the potential for a stabilizing interaction between the enzyme and the substrate leader exists for P RNA, it is not fully observed in the absence of the protein component. This stabilization may be due to alterations in the conformation of the P RNA upon addition of the RNase P protein component, as visualized by iron−EDTA data (*58*).

Protein Subunit Enhances the Affinity of RNase P for 5-tRNA. Substrates with leaders greater than 4 nucleotides are bound by the holoenzyme as tightly as wild-type pre-tRNA^{Asp}, with an affinity that is around 2.2 kcal/mol higher than that of 4-tRNA. This enhanced affinity is not observed for substrates binding to P RNA alone at either high or low salt, indicating that this effect is not due solely to the masking of electrostatic repulsions or enhancement of magnesium affinity by a longer leader. Therefore, the protein component neither simply compensates for unfavorable electrostatic interactions that occur upon binding of substrate RNA to enzyme RNA nor merely stabilizes the global fold of P RNA. The protein specifically enhances the affinity of the pre-tRNA substrate either by directly contacting the leader sequence or by altering the conformation of the RNase P RNA to enhance favorable RNA−RNA contacts.

Substrate Affinity is Increased by Changes in Both the Substrate Association and Dissociation Rate Constants. The enhanced affinity of holoenzyme for 5-tRNA is partially observed as an increase in the substrate association rate constant to a value close to the diffusion-controlled rate constant (*54*, *59*). This rate constant increases 24-fold as the 5′ leader is lengthened from 4 to 5 nucleotides, but this difference is not sufficient to explain entirely the 40-fold difference in substrate binding affinity. Thus, the change in the binding affinity of substrates with various leader lengths does not precisely correlate with alterations in the substrate association rate constant and, therefore, must result from changes in both the substrate association and dissociation rate constants.

An increase in the dissociation rate constant could easily be due to loss of favorable contacts between the leader sequence in our truncated pre-tRNA^{Asp} substrates and RNase P. However, the decreases in the association constant are not due simply to a decrease in the diffusion-controlled association rate constant. Although our data fit well to a minimal mechanism of two consecutive first-order reactions and no evidence for the buildup of an additional intermediate has been observed, we cannot rule out a more complex mechanism in which rapid and reversible binding is either followed or proceeded by a conformational change. Since the association rate constant is dependent on the length of the 5′ leader sequence, we can reasonably narrow these choices to conformational changes in either the free substrate or the enzyme−substrate complex, and this conformational change decreases the observed association rate constant for substrate binding. Possible conformational changes include the breaking or forming of intermolecular substrate base pairs prior to binding and the rearrangement of the substrate in the active site after binding. A rearrangement of the enzyme−substrate complex seems especially likely if the bases in the −2 through −4 positions normally aid in aligning the substrate properly for cleavage.

Conclusions. These data suggest that the protein component of RNase P interacts with the 5′ leader of pre-tRNA substrates at nucleotides −2 and −5. P protein could be either making a direct contact with the 5′ leader to promote binding or positioning the P RNA such that it enhances interactions of P RNA with substrate. The recently elucidated crystal structure of the *B. subtilis* P protein reveals that the positions of the azidophenacyl-modified cysteines capable of forming cross-links with pre-tRNA^{Asp} are near a potential single-stranded RNA binding cleft (*60*) analogous to the single-stranded RNA binding pocket of the spliceosomal protein U1A (*61*). This cleft consists primarily of hydrophobic residues surrounded by positively charged amino acids. We thus speculate that the fifth nucleotide of the pre-tRNA^{Asp} 5′ leader may interact with this cleft, and be held in place by a combination of base stacking with the aromatic residues lining the bottom of the cleft and charge interactions between the phosphate backbone and positively charged amino acids surrounding the cleft in P protein.

The picture of catalysis of pre-tRNA^{Asp} by the RNase P holoenzyme that we describe here mirrors other RNA and protein enzymes. An examination of catalysis by the *Tetrahymena* ribozyme (*56*) confirmed that RNA enzymes employ at least some of the same techniques as protein enzymes to achieve high catalytic efficiency. In RNase P, as in the *Tetrahymena* ribozyme, binding interactions are used both to destabilize the bound substrate and to stabilize the transition state. The pre-tRNA^{Asp} substrate bound to RNase P holoenzyme is destabilized near the cleavage site to decrease the energy difference between the ground and transition states (assuming that the destabilization is relieved in the transition state). The substrate is held in this energetically unfavorable position by stabilizing interactions with other portions of the 5′ leader. These favorable interactions likely originate from both pre-tRNA^{Asp}−P RNA interactions (stabilizing the substrate at nucleotides −2, −3, and −4) and pre-tRNA^{Asp}−P protein contacts (stabilizing the substrate at nucleotide −5).

ACKNOWLEDGMENT

We thank Jeffrey Kurz and Jane Jackman for advice and thoughtful reading of this manuscript. We are also grateful to Anne Perrotta and Michael Been for helpful discussions. In addition, we thank Dr. Norman Pace for his gift of plasmids for *B. subtilis* RNase P RNA and pre-tRNA^{Asp}.

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BI980613C