Interaction of the *Bacillus subtilis* RNase P with the 30S ribosomal subunit

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ABSTRACT

Ribonuclease P (RNase P) is a ribozyme required for the 5' maturation of all tRNA. RNase P and the ribosome are the only known ribozymes conserved in all organisms. We set out to determine whether this ribonucleoprotein enzyme interacts with other cellular components, which may imply other functions for this conserved ribozyme. Incubation of the *Bacillus subtilis* RNase P holoenzyme with fractionated *B. subtilis* cellular extracts and purified ribosomal subunits results in the formation of a gel-shifted complex with the 30S ribosomal subunit at a binding affinity of ~40 nM in 0.1 M NH₄Cl and 10 mM MgCl₂. The complex does not form with the RNase P RNA alone and is disrupted by a mRNA mimic polyuridine, but is stable in the presence of high concentrations of mature tRNA. Endogenous RNase P can also be detected in the 30S ribosomal fraction. Cleavage of a pre-tRNA substrate by the RNase P holoenzyme remains the same in the presence of the 30S ribosome, but the cleavage of an artificial non-tRNA substrate is inhibited eightfold. Hydroxyl radical protection and chemical modification identify several protected residues located in a highly conserved region in the RNase P RNA. A single mutation within this region significantly reduces binding, providing strong support on the specificity of the RNase P-30S ribosome complex. Our results also suggest that the dimeric form of the RNase P is primarily involved in 30S ribosome binding. We discuss several models on a potential function of the RNase P-30S ribosome complex.

Keywords: RNase P; ribosome; 30S subunit; ribozyme

INTRODUCTION

Ribonuclease P is a universally conserved endonuclease required for the maturation of the 5' end of all tRNA (Frank and Pace 1998; Altman and Kirsebom 1999). The bacterial RNase P holoenzyme is composed of an RNA subunit of 330–420 nucleotides (nt) and a protein subunit of ~120 amino acids. The RNA subunit alone is a ribozyme that catalyzes the same endonucleolytic cleavage of a tRNA precursor (Guerrier-Takada et al. 1983). Under physiological conditions, the protein subunit is required to increase the catalytic efficiency of this enzyme by ~10,000-fold (Reich et al. 1988; Kurz et al. 1998). Besides the ribosome, RNase P is the only other ribozyme identified to date that is present in all organisms.

RNase P also cleaves other RNA substrates in addition to the precursors of tRNA. These substrates include the pre-

cursors of the tmRNA that is required for the release of stalled ribosomes (Keiler et al. 1996), the *Escherichia coli* 4.5S RNA that is required for protein translocation (Peck-Miller and Altman 1991), and a small *E. coli* phage RNA that is involved in translational regulation (Hartmann et al. 1995). These substrates contain terminal structures similar to the structure of the coaxially stacked acceptor and the T stem-loop of a tRNA. Like tRNA, this structure serves as the recognition site for RNase P. Like tRNA, these RNAs function without being translated into proteins.

An *E. coli* substrate that does not have an apparently similar structure to a tRNA is the polycistronic mRNA of the histidine operon (Alifano et al. 1994). Acting on the processing products by RNase E, RNase P cleaves at a single site to produce a ~3900-nt-long mRNA. The processed mRNA is significantly more stable than the full-length transcript. Processing by RNase P requires the presence of initiating ribosomes at the intercistronic region located immediately downstream of the cleavage site. The ribosome requirement for this processing reaction may be interpreted in two ways: (1) translation is needed to allow the formation of a specific mRNA structure for the recognition by RNase P, or (2) physical association of RNase P with the ribosome is necessary for the cleavage at this site. The latter

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Abbreviations: DMS, dimethylsulfate; DTT, dithiothreitol; P RNA, the RNA subunit of *B. subtilis* RNase P; P protein, the protein subunit of *B. subtilis* RNase P.

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interpretation implies that RNase P may physically interact with other cellular components to broaden its range of substrates.

Physical association of *E. coli* RNase P with the ribosomal fraction was shown more than three decades ago (Robertson et al. 1972). The *E. coli* RNase P could be isolated from the ribosomal pellet prepared at 0.06 M NH_4Cl by a 0.2 M NH_4Cl wash. In the presence of the ribosome, the *E. coli* RNase P was active in cleaving a pre-tRNA substrate. Although it was not determined with which ribosomal subunit RNase P associates, this result indicates that RNase P is indeed capable of association with cellular components other than its substrates.

Several in vitro results indirectly suggest that RNase P may interact with other cellular components. The RNase P holoenzyme can cleave RNA substrates that are made of a single hairpin-loop plus a few single-stranded residues, or even single-stranded RNAs at the time scale of seconds to minutes (Loria and Pan 2000; Hansen et al. 2001). This high nonspecific activity of the RNase P holoenzyme suggests that an in vivo inhibitor may exist to prevent RNase P from indiscriminately cleaving non-tRNA substrates. The *Bacillus subtilis* RNase P holoenzyme can exist in a monomeric form composed of one RNase P RNA (P RNA) and one RNase P protein (P protein) subunit and a dimeric form composed of two P RNA and two protein subunits (Fang et al. 2001). Both the holoenzyme monomer and the holoenzyme dimer are equally active in cleaving a pre-tRNA substrate. A crude estimate of intracellular concentration of the RNase P holoenzyme suggests that both monomer and dimer can exist in significant fractions in vivo. It is possible that one of these two oligomers performs another, yet to be determined function.

This work demonstrates that the *B. subtilis* RNase P holoenzyme forms a specific complex with the 30S ribosomal subunit. Formation of the RNase P–30S complex requires the RNase P protein and involves a conserved region near the active site of the RNase P RNA. A single mutation within this conserved region significantly reduces binding. The cleavage efficiency of a pre-tRNA substrate by RNase P remains the same, but the cleavage efficiency of an artificial non-tRNA substrate is markedly reduced in the presence of the 30S ribosome. We also show that RNase P binds to the ribosome primarily as a dimer.

RESULTS

RNase P binding to the 30S ribosome

Native gel analysis was carried out to determine whether RNase P forms a complex with components in cellular extract fractions. P RNA alone on a native gel migrates primarily as a monomer. Addition of the P protein shifts the P RNA to a slower migrating band plus a smear of bands in between (Fig. 1A). This result is consistent with the holo-

enzyme existing in a dimer–monomer equilibrium under this condition (Barrera et al. 2002). Because the P protein has only one-tenth the mass of the P RNA, the slower migrating major band is likely the RNase P holoenzyme dimer, whereas the holoenzyme monomer migrates close to the free P RNA. A severely retarded band was observed when the RNase P holoenzyme was mixed with *B. subtilis* cellular extracts (Fig. 1A). This shifted band is only present using the S30 fraction that contains ribosomes and other soluble proteins, but is absent using the S100 fraction that contains only soluble proteins. No gel shift using either S30 fraction (Fig. 1A) or the S100 fraction (data not shown) was observed with P RNA alone. The shift of the RNase P holoenzyme is evident using just 70S ribosomal fraction that is primarily made of ribosomes.

The 70S ribosomal fraction was fractionated further to determine which component binds to RNase P. First, the ribosomal subunits were dissociated at low Mg^{2+} concentrations followed by a sucrose gradient to separate 30S from 50S subunits (Fig. 1B). Second, after pooling the 30S and 50S fractions separately, ultracentrifugation was carried out to further separate the ribosomal subunits from minor impurities carried over from the sucrose gradient. When the gel shift assay was performed using purified ribosomal subunits, only the 30S subunit produced the same shift as the 70S ribosomal fraction (Fig. 1C). No shift of the RNase P was observed with the 50S subunit. Combining the purified 30S and 50S ribosomes did not generate further shift of the 30S-shifted band.

Binding of the RNase P holoenzyme to the 30S ribosomal subunit was tested through competition with a pre-tRNA substrate, a mature tRNA, and the Poly-U RNA (Fig. 1D). RNase P binding to the 30S ribosome is not affected significantly by the present of the yeast tRNA^{Phe} product at a concentration approximately fivefold higher than the binding constant of the RNase P–tRNA product. Poly-U, on the other hand, completely disrupts RNase P binding to the 30S ribosome under this condition. Disruption of RNase P binding by poly-U has two possible explanations: (1) Poly-U binding to the 30S ribosome results in a conformational change of the ribosome (Grozdanovic and Hradec 1975), or (2) the single-stranded poly-U binds directly to the RNase P holoenzyme. In the presence of 0.07 mg/mL poly-U, RNase P cleavage of a pre-tRNA^{Phe} substrate was inhibited by approximately fivefold under k_{cat}/K_m conditions (data not shown).

The binding affinity of the RNase P–30S ribosome complex was measured by the gel shift assay (Fig. 1E,F). At 0.1 M NH_4Cl and 10 mM MgCl_2 , the binding constant is 39 ± 9 nM in the absence of tRNA competitor. The addition of a mixture of *E. coli* tRNA at 4.4 μM (0.1 mg/mL) reduces the binding constant by 10-fold ($K_d \sim 420 \pm 120$ nM). Because the mature tRNA also binds to the RNase P holoenzyme alone under this condition, it is likely that the reduction in the binding affinity is primarily due to the formation of

FIGURE 1. (Legend on facing page)

FIGURE 2. Detection of endogenous RNase P that copurifies with the ribosomal fractions by primer extension. Two product bands are detected in the 30S fraction. The shorter product probably is derived from removal of one or two 5'-terminal nucleotides in the P RNA during 30S purification. The single-stranded nt 1–4 in the P RNA are dispensable for all known functions of RNase P. The minor products in the 50S fraction are either derived from the 30S impurity in the pooled 50S fraction (see Fig. 1B) or are unidentified reverse transcription products.

RNase P-tRNA complex. RNase P preferentially binding to some tRNA species in the *E. coli* tRNA mixture may explain the discrepancy of the *E. coli* tRNA and the yeast tRNA^{Phe} results.

The specificity of the RNase P-30S complex is further demonstrated by using a P RNA fragment in the gel shift assay (Fig. 1E,F). This fragment contains residues 1–239 of the P RNA and lacks a portion of the active site of this ribozyme. No gel-shifted complex was observed when this P RNA fragment plus P protein was incubated with the 30S ribosome.

Does the endogenous RNase P associate with the ribosomal subunits? We first used primer extension to determine whether the purified 30S and 50S ribosome fractions contain endogenous RNase P (Fig. 2). RNase P can be easily detected in the 30S ribosome fraction, whereas a significantly lower amount of RNase P is present in the 50S ribosome fraction. Using an added P RNA control in the primer extension, we estimate that ~1% of the purified 30S ribosome contains endogenous RNase P. This amount accounts for ~30% of the total P RNA present in the total RNA mixture, as determined by primer extension (data not shown). Approximately 6000 ribosomes per cell were isolated in our preparation. Hence, the total number of RNase P molecules in a rapidly growing *B. subtilis* cell is on the order of 200. This estimate is higher than that obtained previously based on the amount of purified RNase P from S100 fractions (Reich et al. 1986).

RNase P associates with the 30S ribosome as dimers (see below). Unexpectedly, the sucrose density gradient interferes with RNase P dimerization; therefore, at least half of the endogenous RNase P associated with the 30S ribosome prior to the sucrose density gradient became dissociated after the sucrose density gradient (data not shown).

A substantial amount of the endogenous *B. subtilis* RNase P copurifies with the 30S ribosome starting with a cell lysate under the ionic condition of 10 mM MgCl₂ and 0.1 M NH₄Cl. A previous *B. subtilis* RNase P purification was carried

out starting with a cell lysate in the presence of 10 mM MgCl₂ and 0.2 M NH₄Cl (Reich et al. 1986). Under this higher ionic condition, more RNase P was present in the S100 fraction. These results can be reconciled by the possibility that RNase P binding to the ribosome is very sensitive to the monovalent salt concentration (Barrera et al. 2002). A change of NH₄Cl concentration from 0.1 M to 0.2 M can result in a significant loss in the affinity of RNA-protein interactions, for example, an ~120-fold decrease in the dimerization of the RNase P holoenzyme (Barrera et al. 2002). A very similar result was obtained for *E. coli* RNase P that copellets with the 70S ribosomal fraction at 10 mM MgCl₂ and 0.06 M NH₄Cl, but appears in the supernatant after a 0.2 M NH₄Cl wash of the ribosome pellet (Robertson et al. 1972).

Activity of the RNase P-30S ribosome complex

Two substrates were used to test the effect of 30S ribosome binding on the catalytic activity of the RNase P holoenzyme. The pre-tRNA^{Phe} substrate contains a 14-nt 5' leader linked to tRNA^{Phe}. The hairpin-loop substrate contains a 7-nt 5' leader linked to a 6-bp helix capped with an UUCG tetraloop and 3'ACCA (5' a₄uauGCGGAUUCGAUUCGC UCCA; the cleavage site is between the underlined nucleotides). As reported previously, this hairpin-loop substrate is cleaved by the RNase P holoenzyme at 1 sec⁻¹ at a binding affinity of ~1 μM (Loria and Pan 2000).

FIGURE 1. (A) Native gel analysis for RNase P binding with various fractions of *B. subtilis* extract. The holoenzyme dimer and monomer are well separated, but the dissociation of the dimer during gel electrophoresis generates a smear between the dimer and the monomer. A small fraction of the P RNA (<5%) also dimerizes in the absence of the P protein; such a dimerization of P RNA alone is due to the formation of an intermolecular P1 helix (X. Fang and T. Pan, unpubl. results). A severely retarded band is present with the S30 fraction, and incubation with the S100 fraction only generates a minor well shift. (B) Sucrose gradient of the 70S ribosomal fraction (dissolved S100 pellet) showing the separation of the pooled 30S and 50S ribosomal fractions. Two thick lines indicate the pooled fractions. (C) Gel shift of the RNase P holoenzyme by the purified ribosomal fractions. (D) Competition assay in the presence of 0.2 μg/μL Poly-U RNA, 5 μM yeast tRNA^{Phe}, and 5 μM pre-tRNA^{Phe} substrate ("tRNA^{Phe}"). Because 30S binding does not affect the catalytic activity of the RNase P holoenzyme on this pre-tRNA substrate, it was certain that all pre-tRNA substrates had been cleaved before the sample was loaded onto the native gel. (E) Percent gel-shifted complex as a function of 30S ribosome concentration using the RNase P holoenzyme (filled circles) and a P RNA fragment containing residues 1–239 plus stoichiometric amount of P protein (open circles). (F) Percent gel-shifted complex as a function of 30S ribosome concentration in the presence of 0.1 mg/mL *E. coli* tRNA (type XXI from Sigma-Aldrich).

TABLE 1. Catalytic activity of the RNase P holoenzyme with and without 30S ribosome.

Enzyme	pre-tRNA ^{Phe}		Hairpin-loop	
	k_{obs} (sec ⁻¹) ^a	$k_{\text{obs}}/[E]$ (μM^{-1} sec ⁻¹)	k_{obs} (sec ⁻¹) ^a	$k_{\text{obs}}/[E]$ (μM^{-1} sec ⁻¹)
RNase P – 30S ^b	0.11 ± 0.01	5.5	0.010 ± 0.001	0.5
RNase P + 30S	0.17 ± 0.02	6.1 ^c	0.0016 ± 0.0002	0.06 ^c
30S alone	0.022 ± 0.002	5.5 ^c	— ^d	—

^aSingle turnover conditions: 50 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, 0.1 M NH₄Cl, 37°C.^b–30S: 20 nM RNase P; +30S: 20 nM RNase P + 0.8 μM 30S ribosome; 30S alone: 0.4 μM 30S ribosome.^cAssuming that 1% of the 30S ribosome contains RNase P.^dNot determined.

The presence of a 40-fold excess of 30S ribosome over RNase P has little effect on the cleavage of the pre-tRNA substrate (Table 1). Because the total amount of the 30S ribosome in the reaction is 20-fold higher than the binding affinity of the RNase P-30S ribosome complex, >90% of the RNase P in the reaction should initially associate with the 30S ribosome. The same cleavage activity was obtained for the endogenous RNase P in the 30S ribosomal fraction. In contrast, the presence of the 30S ribosome significantly inhibits the cleavage of the hairpin-loop substrate (Table 1).

It is unclear whether the RNase P holoenzyme remains associated with the 30S ribosome upon the formation of an active ES complex. Our previous work indicates that the RNase P/pre-tRNA^{Phe} complex is a monomer, even when the RNase P itself is fully a dimer in the absence of the substrate (Barrera et al. 2002). Because only RNase P dimer appears to bind to the 30S ribosome (see below), it is therefore possible that the RNase P/pre-tRNA^{Phe} complex and the RNase P/30S complex are mutually exclusive. Hence, the mechanistic reason for the enhancement in substrate specificity may be due to the ability of a pre-tRNA substrate to dissociate RNase P from the 30S ribosome upon complex formation, whereas the hairpin-loop substrate is unable to do so. Alternatively, the excess 30S ribosome present in the reaction may bind the hairpin-loop substrate directly to prevent RNase P binding to this substrate.

Structural mapping of the RNase P–30S complex

Hydroxyl radical protection was carried out to determine the ribose-phosphate positions in the RNase P RNA that become protected in the RNase P-30S ribosome complex (Fig. 3A,C). A single protected region in the J18/2 region of the P RNA was identified. The J18/2 region and residues A314 and G315 in particular are highly conserved in all RNase P RNA and they are located near the active site of this ribozyme (Chen et al. 1998; Frank et al. 2000). Even though this method alone cannot distinguish direct contacts or conformational changes of the P RNA upon 30S ribosome binding, the protection of such a highly conserved

region in the P RNA raises the possibility that ribosome binding to RNase P may also occur in other organisms.

Chemical modification by DMS and kethoxal were performed to determine the nucleotide bases in the P RNA that become protected in the RNase P-30S complex (Fig. 3B,C). Protection was observed for 3 nt at the base of the P18 helix upon 30S ribosome binding. Although these nucleotides are located at the end of an RNA helix, they are nevertheless modified by DMS in the RNase P holoenzyme in the absence of the 30S subunit. The protection of these nucleotides

probably is derived from stabilization of the P18 helix upon ribosome binding. The protected residues in the P18 helix are immediately adjacent to the protected residues against hydroxyl radical attack. A weak protection for the conserved A314 can be seen, whereas residues A316 and A318 appear to be unprotected. Taking the results from hydroxyl radical protection and chemical modifications together, 30S ribosome binding appears to involve the conserved J18/2 region in the RNase P RNA.

Hydroxyl radical protection can also be used to determine whether the ribosome bound RNase P holoenzyme is a dimer or a monomer (Fig. 4A). Previous comparison of the protection pattern shows significant loss of protection in four regions in the P RNA when the RNase P is converted to a monomer from a dimer (Barrera et al. 2002). The extent of protection of these four regions can therefore be used as signatures for the RNase P dimer. The protected pattern of the holoenzyme under our condition is that of a dimer. Upon 30S ribosome binding, protections of these four regions remain essentially unchanged. This result suggests that at least a majority of the RNase P bound to the 30S ribosome is in its dimeric form.

By varying the amount of the 30S ribosome, an estimate of the binding affinity can be obtained by the hydroxyl radical protection method (Fig. 4B). The affinity determined this way is 20 ± 5 nM, which is within twofold of the K_d value determined by nondenaturing gel electrophoresis.

RNase P mutations and the effect on binding to 30S ribosome

The J18/2 region that is protected against hydroxyl radical attack upon 30S ribosome binding includes two residues, A314 and G315, that are universally conserved in prokaryotic and eukaryotic RNase P RNAs. To determine whether mutation of these residues affects RNase P binding to the 30S ribosome, A314 was mutated to C or G and G315 was mutated to A, C, or U. Both A314 mutants have approximately twofold higher K_m and k_{cat} , resulting in similar $k_{\text{cat}}/$

FIGURE 3. (A) Hydroxyl radical protection of the RNase P–30S ribosome complex. A thick line on the *right* indicates the protected region. (B) DMS and kethoxal modification of the RNase P–30S ribosome complex. (*Left*) Reverse transcriptase sequencing using the primer complementary to residues 367–351 of the P RNA; (*middle*) primer extension with the 367–351 complementary primer; (*right*) primer extension with the 402–381 complementary primer. A thick line on the *right* indicates the protected residues. (C) Summary of the protection and chemical modification results. Residues protected against hydroxyl radical attack are enclosed by an oval. Residues protected against DMS modification are shaded. The four regions involved in holoenzyme dimerization are shown as I–IV. Regions I, II, III, and IV include residues 107–116, 157–161, 198–223, and 264–273, respectively. Residues not examined for hydroxyl radical protection due to gel resolution are shown in lowercase.

K_m values compared to the wild-type holoenzyme (Table 2). In contrast, all three G315 mutants are catalytically impaired, they have significantly lower k_{cat} , and two of the three mutants also have high K_m values.

Native gel analysis shows that both A314 mutants bind similarly well to the 30S ribosome (Fig. 5; data not shown).

All three G315 mutants, on the other hand, show significantly reduced binding to the 30S ribosome (Fig. 5). The relative extent of the holoenzyme dimer is also markedly decreased for the G315 mutants, as suggested by the fraction of the dimer in the absence of the ribosome. It is therefore possible that the effect of the G315 mutation on

FIGURE 4. (A) The extent of the protection of the four regions involved in the formation of the RNase P holoenzyme dimer in the RNase P–30S ribosome complex. For each position, the amount of radioactivity in the absence of 30S subunit divided by the amount of radioactivity in the presence of 30S subunit is shown as closed squares. The amount of radioactivity of the RNase P monomer divided by the amount of radioactivity of the RNase P dimer is shown as open circles (data taken from Barrera et al. 2002). (B) The inverse of the extent of protection versus 30S ribosome concentration can be fit to obtain an estimated binding affinity.

30S ribosome binding is a consequence of weakened dimerization of these mutant holoenzymes.

A quadruple mutant in the conserved P4 helix was also tested to determine whether other mutations around the active site also affect 30S ribosome binding (Fig. 5; Table 2). The P4 mutant has four nucleotide changes, C51G, C52G, G378C, and G379C that convert two conserved C–G base pairs to G–C. The P4 mutant has comparable k_{cat} and K_m values in multiple turnover reactions, but has an eightfold lower k_c at saturating enzyme concentration. This mutant binds to the 30S ribosome similarly well, and its extent of dimerization is also comparable to the wild-type holoenzyme. This result supports the notion that dimerization of the holoenzyme is an important factor in 30S ribosome binding. Moderate change on catalytic activity, on the other hand, exerts only a minor effect in the formation of the RNase P–30S ribosome complex.

DISCUSSION

The structure of the RNase P–30S ribosome complex

This work demonstrates that the *B. subtilis* RNase P holoenzyme specifically interacts with the 30S ribosomal subunit. The binding affinity is ~40 nM in 0.1 M NH_4Cl and 10

mM MgCl_2 . Because the ribosome concentration in a rapidly growing bacterial cell is >10 μM , it is likely that this complex forms at some stages in the life cycle of *B. subtilis*.

Two lines of evidence suggest that binding to the 30S ribosome involves the dimeric form of the *B. subtilis* RNase P holoenzyme. First, the hydroxyl radical protection of residues in all four regions specific for the RNase P holoenzyme dimer remains unchanged upon 30S binding. Second, native gel analysis of the G315 mutants suggests that impaired dimerization may be a primary reason for their significantly reduced binding to 30S ribosome.

A conserved region in the P RNA is involved in 30S ribosome binding. This region is located near the active site of the ribozyme, but previous work indicates that it is not a part of the four regions that are candidates for direct involvement in the dimerization of the RNase P holoenzyme (Barrera et al. 2002). The function for the two universally conserved residues in this region, A314 and G315, has not been assigned previously. The strong defect of G315 mutants on ribosome binding clearly shows that the sequence identity of G315 is very important. Unfortunately, the reduction in the catalytic efficiency of all G315 mutants is sufficiently significant to make them unsuitable for the functional elucidation of the RNase P–30S complex in vivo. An ideal mutant for this purpose would have similar cata-

TABLE 2. Catalytic activity of the mutant RNase P holoenzymes

Enzyme	k_{cat} (sec ⁻¹) ^a	K_m (μM ⁻¹ sec ⁻¹) ^a	Relative k_{cat}/K_m	Relative k_c ^c
Wild type	0.27 ± 0.03	0.091 ± 0.044	1.0	1.0
A314C	0.66 ± 0.05	0.17 ± 0.04	1.3	1.4
A314G	0.51 ± 0.03	0.16 ± 0.03	1.1	— ^d
G315A	0.053 ± 0.008	0.40 ± 0.14	0.04	0.9
G315C	0.048 ± 0.004	0.11 ± 0.03	0.15	2.1
G315U	0.079 ± 0.014	0.63 ± 0.19	0.04	—
P4 mutant ^b	0.21 ± 0.02	0.12 ± 0.04	0.6	0.13

^aMultiple turnover conditions: 50 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, 0.1 M NH₄Cl, 37°C.

^bP4 mutant has four nucleotide changes: C51G, C52G, G378C, and G379C.

^cSingle turnover conditions: 2.4 μM RNase P, 50 mM MES at pH 5.8, 10 mM MgCl₂, 0.1 M NH₄Cl, 37°C.

^dNot determined.

lytic efficiency for pre-tRNA cleavage, but significantly reduced binding affinity to the 30S ribosome.

It is likely that the P protein is also involved in the RNase P-30S ribosome interactions. Within the RNase P holoenzyme, the P protein has a single-stranded RNA-binding site that is used for binding of the 5' leader of a pre-tRNA substrate (Niranjanakumari et al. 1998). This site can bind two to four single-stranded residues and binding of four residues is sufficient for achieving the maximal binding affinity (Crary et al. 1998). This binding site could be used to bind a small region in the ribosomal RNA that encompasses two to four residues. Conversely, some ribosomal proteins may also be involved in the interaction with the RNase P RNA.

Recent structural analysis of the RNase P holoenzyme suggests that P protein directly binds to the vicinity of the J18/2 region of the P RNA (Kurz and Fierke 2002; Tsai et al. 2003). The proximity of the P protein to this region of P RNA supports the idea that both P RNA and P protein are involved in interactions with the 30S ribosome.

Three functional models of the RNase P-30S ribosome complex

The first model hypothesizes that 30S ribosome acts as an inhibitor to prevent undesirable RNA cleavage by this highly active endonuclease. The presence of the 30S ribosome has little effect on the cleavage of a pre-tRNA by the RNase P, likely due to the mutual exclusivity of the RNase P-30S and the RNase P-pre-tRNA complexes. Hence, enhancement of the catalytic activity of pre-tRNA cleavage by RNase P upon 30S ribosome binding is not a function for this complex. However, the presence of the 30S ribosome does markedly reduce the

cleavage efficiency of an artificial, non-tRNA substrate. Regardless of the mechanistic reason for this inhibition, this result shows that the formation of this complex does have a net effect on the substrate specificity of the RNase P reaction. Hence, binding of RNase P to 30S ribosome could markedly reduce its chance to randomly cleave RNA in the cell.

The second model hypothesizes that the RNase P-30S ribosome complex acts as a regulator for cell growth. This model would imply that different amounts of RNase P-30S complex is present at different stages of cell growth. Indeed, the amount of endogenous *B. subtilis* RNase P that associates with the

30S ribosome isolated from the mid-log phase of growth is significantly higher than the amount isolated from the stationary phase of growth (data not shown). This result is counterintuitive at first because more tRNA precursors are produced at the mid-log phase of growth and therefore, RNase P should be kept away from binding to ribosome through the formation of the ES complex. On the other hand, this result may be explained by differential property or the amount of free 30S ribosome at these growth stages. Despite the higher transcriptional level of the tRNA genes at the mid-log phase, rapid processing of the tRNA precursors may result in a steady-state pool of ES complexes that is lower than the amount of the 30S ribosome capable of association with RNase P.

What could an RNase P-30S ribosome complex do? Our third model hypothesizes that RNase P acts as a ribosome-associated endonuclease to cleave specific, but non-tRNA substrates in vivo. Potential targets are mRNAs that contain specific structures involved in translational regulation. Such mRNAs can be bound by the 30S ribosome, but translation can only occur after they have been cleaved at specific sites. A particular example for this endonucleolytic reaction in *B. subtilis* may be the *ermC* mRNA that codes for a methyltransferase that methylates a specific residue of rRNA

FIGURE 5. Native gel analysis of mutant holoenzymes. The RNase P concentration was 0.2 μM in all cases.

(Drider et al. 2002). Translational regulation of the *ermC* mRNA involves an erythromycin-dependent cleavage at a specific site that alters the stability of this mRNA. Cleavage of this mRNA also depends on stalled ribosomes and an RNA structure within this mRNA. Even though RNase P holoenzyme can cleave artificial non-tRNA substrates in vitro, the cleavage efficiency for non-tRNA substrates in vivo may not be sufficient without the aid of a cofactor. Association of RNase P with the 30S ribosome could enhance the recognition of RNase P with specific mRNA substrates that are also bound by the ribosome.

Does the RNase P-30S ribosome association also occur in other bacteria? Bacterial RNase P RNA is made of two types, A and B, that have the same core structure, but several distinct secondary structure motifs (Brown 1999). The *B. subtilis* and *E. coli* RNase P RNA are representative of types B and A, respectively. The RNase P protein from these two bacteria can substitute for each other in the catalytic activity, suggesting that they interact similarly with the core structure of the P RNA (Guerrier-Takada et al. 1983). A previous work on *E. coli* suggests that an RNase P-30S ribosome complex also forms there (Robertson et al. 1972). It is likely that similar regions in the *E. coli* RNase P are involved in ribosome binding. Whether a dimeric form of the *E. coli* RNase P associates with the ribosome remains to be determined.

Perhaps one should not be surprised to find that two evolutionarily conserved ribozymes interact with each other for some mutual benefits. The specificity of RNase P interaction with the 30S, but not with the 50S ribosomal subunit raises intriguing possibilities for this ribozyme to perform other unknown functions. In vivo studies are needed to fully explore the functional spectrum of this highly conserved ribozyme.

MATERIALS AND METHODS

Preparation of cell lysates

An overnight culture of *B. subtilis* 168A1 cells was used to inoculate a liter of LB in 1:200 dilution. Cells were grown to A_{600} of 0.4–0.6 and harvested. The cell pellet was washed with 15 mL S30 buffer (10 mM Tris-acetate at pH 8.2, 14 mM Mg(OAc)₂, 60 mM KOAc, 1 mM DTT) plus 5 mM β -mercaptoethanol. The pellet suspended in the S30 buffer was passed through a French Press twice and collected on ice. The cell lysate was spun at 30,000g for 30 min at 4°C. The supernatant was collected and designated as the S30 fraction. A portion of the S30 fraction was centrifuged at 100,000g for 2.5 h at 4°C. The supernatant was collected and designated as the S100 fraction, and the pellet primarily contained ribosomes. The S100 pellet was dissolved in the S30 buffer and designated as the 70S fraction. All fractions were pooled separately and the aliquots were stored at –80°C.

Isolation of ribosomal subunits

The 30S and 50S ribosomal subunits were isolated through a 5–40% sucrose gradient containing 10 mM Tris-acetate (pH 8.2),

60 mM KOAc, 1 mM Mg(OAc)₂, and 1 mM DTT, prepared and chilled to 4°C prior to use. Approximately 10 A_{260} units of the dissolved S100 pellet were diluted in the same buffer and left overnight at 4°C. The 70S fraction was layered on the top of the sucrose gradient and centrifuged for 6 h at 27,000 rpm at 7°C. Fractions of 0.5–1.0 mL were collected manually and the absorbance at 260 nm was measured to determine the location of the 50S and 30S subunits. The 30S and 50S ribosomal subunit fractions were pooled and centrifuged again at 4°C for 2 h at 95,000 rpm. The pellets were dissolved in 20 mM Tris-OAc at pH 7.5, 10 mM MgCl₂, 0.1 M NH₄Cl, and 2 mM β -mercaptoethanol, and aliquots were stored at –80°C. The ribosome concentration was determined by UV absorbance (1 A_{260} unit = 69 pmoles for 30S and 34 pmoles for 50S subunit; Stern et al. 1988).

This isolation method ensures high purity of the 30S subunit through incubation and performing the sucrose density gradient at low Mg²⁺ concentrations (Spedding 1990).

Preparation of the RNase P holoenzyme and the RNase P-30S complex

The RNase P holoenzyme was obtained by in vitro reconstitution using in vitro transcribed P RNA as described previously (Loria et al. 1998). Briefly, the P RNA transcript in Tris-HCl at pH 7.5 alone was heated for 2 min at 85°C followed by incubation for 3 min at room temperature. MgCl₂ was added and the sample incubated for 3 min at 50°C. NH₄Cl and an equal-mole of the *B. subtilis* P protein were added and the sample was incubated for 5 min at 37°C. To obtain the RNase P-30S complex, the 30S subunit was mixed with the reconstituted holoenzyme and the sample was incubated for 30 min at 37°C. The final buffer concentration for the holoenzyme reconstitution and ribosome binding was 20 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, and 0.1 M NH₄Cl.

The templates for in vitro transcription of A314 and G315 mutants were generated by PCR and confirmed by sequencing. The template for the P4 mutant was obtained by PCR followed by cloning of the PCR product into a pUC18-based plasmid DNA.

Analysis of the RNase P-30S complexes

Nondenaturing gel electrophoresis

Mixtures containing 0.2 μ M 5' ³²P-labeled RNase P holoenzyme with various fractions (S30, S100, 70S, 50S ribosome, and 30S ribosome) were analyzed on 6% nondenaturing polyacrylamide gels run for 4 h at 12°C. The running buffer contained 50 mM Tris-acetate at pH 7.5 and 10 mM Mg(OAc)₂. The radiolabeled P RNA was detected by phosphorimaging.

To determine the apparent binding constant of the RNase P-30S ribosome complex, the amount of the gel-shifted complex is plotted against the varying concentration of the 30S ribosome. These values are fit to a binding equation, $y = a^*[\text{ribosome}]/(K_d^{app} + [\text{ribosome}])$, to obtain the apparent K_d . Under the conditions where the concentration of the RNase P dimer is similar to the low ribosome concentration, $K_d \approx K_d^{app} - [\text{RNase P dimer}]/2$.

Primer extension

Primer extension was used to detect endogenous RNase P RNA in the 30 S ribosome. The primer, 5' AACTACGGGCATCT, was

complementary to nt 85–71 of the *B. subtilis* P RNA. ^{32}P -labeled primer was annealed with the 30S or 50S ribosomal fraction by heating for 1 min at 95°C followed by incubation on ice for 4 min. Reverse transcription was carried out using AMV reverse transcriptase (Amersham) for 10 min at 50°C. The reaction was quenched upon the addition of 9 M Urea/25 mM EDTA and analyzed on polyacrylamide gels containing 7 M urea.

Hydroxyl radical protection

Hydroxyl radical protection was performed in 20 mM Tris-HCl at pH 7.5, 10 mM MgCl_2 , 0.1 M NH_4Cl , 0.2 μM RNase P, and 0–1.6 μM 30S ribosome. The preformed RNase P–30S complex was mixed with 1 mM ascorbic acid, 5 mM DTT, 1 mM Fe(II)/1.2 mM EDTA and incubated for 30 min at 37°C. The reaction was quenched upon the addition of 10 mM thiourea. Ribosomal proteins were removed by phenol extraction, and the RNA was concentrated by ethanol precipitation. The pellet was resuspended in 9 M urea/25 mM EDTA and the samples were analyzed by polyacrylamide gels containing 7 M urea.

The hydroxyl radical protection was primarily carried out using 5' ^{32}P -labeled P RNA. Due to the high autolytic cleavage rate of the holoenzyme under these conditions (Barrera et al. 2002), it was technically difficult to use 3' ^{32}P -labeled P RNA to obtain better resolution of the protected region (data not shown).

Chemical modification

DMS/kethoxal modifications were carried out in 20 mM Tris-OAc at pH 7.5, 10 mM MgCl_2 , 0.1 M NH_4Cl , 0.2 μM RNase P, and 0 or 0.8 μM 30S ribosome. Modification reaction was initiated upon the addition of DMS (1:4 diluted in H_2O) and 3.7 mg/mL Kethoxal (stock 74 mg/mL) and incubated for 10 min at 37 °C. DMS reaction was quenched upon the addition of 500 mM Tris-HCl at pH 7.5, 50 mM β -mercaptoethanol, and 50 mM EDTA. Kethoxal reaction was quenched using 25 mM K-boric acid at pH 7.0. Samples were stripped of proteins through three Phenol/chloroform extractions and two ethanol precipitations. The resulting RNA was analyzed by primer extension using two primers, 5'-GACGTGGTCTAACGTTCTGTAA and 5'-CCATCGTACTGCAA ACG, that are complementary to residues 401–381 and 367–351 of P RNA, respectively. Reverse transcription reactions were carried out for 30 min at 42°C using AMV reverse transcriptase. The reaction was stopped upon the addition of 9 M urea/25 mM EDTA at pH 8.0. Samples were boiled for 1 min prior to loading on 8% polyacrylamide gels containing 7 M urea.

Catalytic activity

The catalytic activity of the RNase P holoenzyme in the presence and absence of the 30S ribosome at 37°C was tested using a pre-tRNA^{Phe} substrate described previously (Barrera et al. 2002). Single turnover reactions contained 50 mM Tris-HCl at pH 7.5, 10 mM MgCl_2 , 0.1 M NH_4Cl , and <2 nM 5' ^{32}P -labeled substrate. Percent product was fit to a single exponential to obtain the observed reaction rate.

To compare catalytic efficiency of the mutant RNase P, both multiple and single turnover reactions were carried out in the absence of the 30S ribosome. Multiple turnover reactions used 5–50 nM RNase P holoenzyme and 0.05–1 μM substrate. Single turnover reactions used 2.4 μM RNase P and <2 nM substrate.

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REFERENCES

- Alifano, P., Rivellini, F., Piscitelli, C., Arraiano, C.M., Bruni, C.B., and Carlomagno, M.S. 1994. Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA. *Genes & Dev.* **8**: 3021–3031.
- Altman, S. and Kirsebom, L. 1999. Ribonuclease P. In *The RNA world*, 2nd ed. (eds. R.F. Gesteland et al.), pp. 351–380. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Barrera, A., Fang, X., Jacob, J., Casey, E., Thiyagarajan, P., Pan, T. 2002. Dimeric and monomeric *Bacillus subtilis* RNase P holoenzyme in the absence and presence of pre-tRNA substrates. *Biochemistry* **41**: 12986–12994.
- Brown, J.W. 1999. The Ribonuclease P Database. *Nucleic Acids Res.* **27**: 314.
- Chen, J.L., Nolan, J.M., Harris, M.E., and Pace, N.R. 1998. Comparative photocross-linking analysis of the tertiary structures of *Escherichia coli* and *Bacillus subtilis* RNase P RNAs. *EMBO J.* **17**: 1515–1525.
- Crary, S.M., Niranjanakumari, S., and Fierke, C.A. 1998. The protein component of *Bacillus subtilis* ribonuclease P increases catalytic efficiency by enhancing interactions with the 5' leader sequence of pre-tRNA^{Asp}. *Biochemistry* **37**: 9409–9416.
- Drider, D., DiChiara, J.M., Wei, J., Sharp, J.S., and Bechhofer, D.H. 2002. Endonuclease cleavage of messenger RNA in *Bacillus subtilis*. *Mol. Microbiol.* **43**: 1319–1329.
- Fang, X.W., Yang, X.J., Littrell, K., Niranjanakumari, S., Thiyagarajan, P., Fierke, C.A., Sosnick, T.R., and Pan, T. 2001. The *Bacillus subtilis* RNase P holoenzyme contains two RNase P RNA and two RNase P protein subunits. *RNA* **7**: 233–241.
- Frank, D.N. and Pace, N.R. 1998. Ribonuclease P: Unity and diversity in a tRNA processing ribozyme. *Annu. Rev. Biochem.* **67**: 153–180.
- Frank, D.N., Adamidi, C., Ehringer, M.A., Pitulle, C., and Pace, N.R. 2000. Phylogenetic-comparative analysis of the eukaryal ribonuclease P RNA. *RNA* **6**: 1895–1904.
- Grozdanovic, J. and Hradec, J. 1975. Ribosome-dependent conversion of polyA-containing heterogenous nuclear RNA into smaller RNA molecules. *Nucleic Acids Res.* **2**: 821–830.
- Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. 1983. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* **35**: 849–857.
- Hansen, A., Pfeiffer, T., Zuleeg, T., Limmer, S., Ciesiolka, J., Feltens, R., and Hartmann, R.K. 2001. Exploring the minimal substrate requirements for *trans*-cleavage by RNase P holoenzymes from *Escherichia coli* and *Bacillus subtilis*. *Mol. Microbiol.* **41**: 131–143.
- Hartmann, R.K., Heinrich, J., Schlegel, J., and Schuster, H. 1995. Precursor of C4 antisense RNA of bacteriophages P1 and P7 is a substrate for RNase P of *Escherichia coli*. *Proc. Natl. Acad. Sci.* **92**: 5822–5826.
- Keiler, K.C., Waller, P.R., and Sauer, R.T. 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271**: 990–993.

- Kurz, J.C. and Fierke, C.A. 2002. The affinity of magnesium binding sites in the *Bacillus subtilis* RNase P $\times$ pre-tRNA complex is enhanced by the protein subunit. *Biochemistry* **41**: 9545–9558.
- Kurz, J.C., Niranjanakumari, S., and Fierke, C.A. 1998. Protein component of *Bacillus subtilis* RNase P specifically enhances the affinity for precursor-tRNA^{Asp}. *Biochemistry* **37**: 2393–2400.
- Loria, A. and Pan, T. 2000. The 3' substrate determinants for the catalytic efficiency of the *Bacillus subtilis* RNase P holoenzyme suggest autolytic processing of the RNase P RNA in vivo. *RNA* **6**: 1413–1422.
- Loria, A., Niranjanakumari, S., Fierke, C.A., and Pan, T. 1998. Recognition of a pre-tRNA substrate by the *Bacillus subtilis* RNase P holoenzyme. *Biochemistry* **37**: 15466–15473.
- Niranjanakumari, S., Stams, T., Crary, S.M., Christianson, D.W., and Fierke, C.A. 1998. Protein component of the ribozyme ribonuclease P alters substrate recognition by directly contacting precursor tRNA. *Proc. Natl. Acad. Sci.* **95**: 15212–15217.
- Peck-Miller, K.A. and Altman, S. 1991. Kinetics of the processing of the precursor to 4.5 S RNA, a naturally occurring substrate for RNase P from *Escherichia coli*. *J. Mol. Biol.* **221**: 1–5.
- Reich, C., Gardiner, K.J., Olsen, G.J., Pace, B., Marsh, T.L., and Pace, N.R. 1986. The RNA component of the *Bacillus subtilis* RNase P. Sequence, activity, and partial secondary structure. *J. Biol. Chem.* **261**: 7888–7893.
- Reich, C., Olsen, G.J., Pace, B., and Pace, N.R. 1988. Role of the protein moiety of ribonuclease P, a ribonucleoprotein enzyme. *Science* **239**: 178–181.
- Robertson, H.D., Altman, S., and Smith, J.D. 1972. Purification and properties of a specific *Escherichia coli* ribonuclease which cleaves a tyrosine transfer ribonucleic acid precursor. *J. Biol. Chem.* **247**: 5243–5251.
- Spedding, G. 1990. Isolation and analysis of ribosomes from prokaryotes, eukaryotes, and organelles. In *Ribosomes and Protein Synthesis, a Practical Approach* (ed. G. Spedding), pp. 1–29. Oxford University Press, Oxford, UK.
- Stern, S., Moazed, D., and Noller, H.F. 1988. Structural analysis of RNA using chemical and enzymatic probing monitored by primer extension. *Methods Enzymol.* **164**: 481–489.
- Tsai, H.Y., Masquida, B., Biswas, R., Westhof, E., and Gopalan, V. 2003. Molecular modeling of the three-dimensional structure of the bacterial RNase P holoenzyme. *J. Mol. Biol.* **325**: 661–675.

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