Higher Order Folding and Domain Analysis of the Ribozyme from *Bacillus subtilis* Ribonuclease P†

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ABSTRACT: Folding of the ribozyme from *Bacillus subtilis* ribonuclease P (denoted P RNA) has been examined by Fe(II)-EDTA protection and bimolecular association. Fe(II)-EDTA results show that, in the presence of Mg^{2+}, P RNA is folded into a core structure which includes most of the phylogenetically conserved nucleotides. Folding is cooperative and is complete at 5−6 mM Mg^{2+} with the $[Mg^{2+}]_{1/2}$ at 2−3 mM. The Hill constant indicates that this folding transition requires binding of at least three additional Mg^{2+} ions. Two RNA molecules consisting of nucleotides 62−239 [p(62−239)] and 240−401 + 1−61 [p(240−61)] of the *B. subtilis* P RNA have been constructed. These RNAs can in principle form the catalytically active structure primarily, if not solely, through tertiary interactions. Although either molecule by itself is inactive, the bimolecular complex is as active as the circularly permuted P RNAs from which it is derived. The binding constant of the complex can be as low as 0.1 μM and is strongly dependent on Mg^{2+} and K^+ concentrations. Association of these molecules also induces a Mg^{2+} dependent cleavage at nucleotide 103 in p(62−239). p(62−239) gives a Fe(II)-EDTA protection pattern very similar to the wild-type P RNA at identical Mg^{2+} concentrations. However, Fe(II)-EDTA protection in p(240−61) is completely lost, even though it contains many nucleotides that are conserved among all P RNAs. These results suggest that, like other RNAs, P RNA contains domains that can fold in the absence of the rest of the molecule. The implications of these results are discussed in the context of the P RNA structure and catalysis.

One of the largest ribozymes known to date is the catalytic RNA component from eubacterial RNase P (P RNA).[1] The P RNAs range from 330 to 420 nucleotides and catalyze a site-specific cleavage reaction to generate the mature 5′ end of tRNAs (Pace & Smith, 1990; Altman et al., 1993b). Such specificity and activity implies a complex three-dimensional structure of the P RNA (Haas et al., 1991; Westhof & Altman, 1994; Harris et al., 1994). The secondary structure of P RNA has been derived from phylogenetic analysis (Haas et al., 1994). Approximately 55% of all nucleotides of the *Bacillus subtilis* P RNA are involved in forming the secondary structure which consists of a total of 18 helices and hairpins. In addition to these secondary structural elements, numerous tertiary interactions form to fold the P RNA into a higher order three-dimensional structure. For the *Escherichia coli* P RNA, two structural models have recently been presented based on phylogenetic and biochemical data (Westhof & Altman, 1994; Harris et al., 1994).

Like proteins, the complex structures of RNA may also be composed of smaller domains that can fold independently. These domains associate through tertiary interactions to form the structure capable of catalysis. An independently folded RNA domain has been found in the P4−P6 segment of the *Tetrahymena* group I ribozyme (Murphy & Cech, 1993; Wang et al., 1994). Using primarily the solvent-based Fe(II)-EDTA assay, folding of the P4−P6 domain is shown to be identical either by itself or embedded in the intact ribozyme. Fe(II)-EDTA is a free radical reaction that attacks the ribose backbone of RNA, causing strand scission. In a folded RNA structure, some ribose moieties are less accessible to solvent and therefore protected against hydroxyl radical attack (Latham & Cech, 1989; Celander & Cech, 1991). By analyzing the protection pattern of a folded RNA, regions of nucleotides that are included in the core of the folded structure can be located.

This paper describes the core of *B. subtilis* P RNA revealed by Fe(II)-EDTA cleavage. In addition, evidence is provided for one independently folded domain in P RNA. Two RNA molecules are constructed from *B. subtilis* P RNA which combine primarily, if not solely through tertiary interactions. Either molecule by itself is completely inactive. When incubated together, the catalytic activity is comparable to that of the circularly permuted P RNAs from which it is derived. Fe(II)-EDTA protection shows that one of the two RNAs can fold into a structure similar to the wild-type P RNA. This independently folded domain includes numerous nucleotides that are conserved among all eubacterial P RNAs, but does not contain other conserved nucleotides that are considered to form the active site.

MATERIALS AND METHODS

Cloning of p(62−239) and p(240−61). cDNAs were obtained by reverse transcription of the circular P RNA

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[1] Abbreviations: ATP, adenosine triphosphate; CTP, cytidine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; K_d, binding constant; MOPS, 3-[N-Morpholino]propanesulfonic acid; pCp, 5′,3′-cytidine bisphosphate; P RNA, the catalytic RNA component of RNase P; p(62−239), the RNA construct consisting of residues 62−239 of the *B. subtilis* P RNA; p(240−61), the circularly permuted RNA construct consisting of residues 240−401 + 1−61 of the *B. subtilis* P RNA.

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followed by PCR amplification using appropriate primers (Pan & Zhong, 1994). For the RNA containing nucleotides 62−239 of *B. subtilis* P RNA, a 5′ primer 5′-TAATACGACTCACTATAACGGTGCTGAGATGCCCGTAGT and a 3′ primer 5′-TCGAGGGGTTTACCGCGTT were used. The 5′ primer contained the T7 RNA polymerase promoter, and the 3′ primer generated a *Xho*I site when the cDNA was cloned into a *Sma*I site. For the RNA containing nucleotides 240−401 and 1−61, a 5′ primer 5′-TAATACGACTCACTATAGCGAGAAACCCAAATTTTGG and a 3′ primer 5′-GGATGAATGGTCTCGCGAGCATGGACTTTCC were used. The 3′ primer contained a *Fok*I site which served as the restriction site for run-off transcription to produce the precise 3′ end.

Fe(II)-EDTA Protection. The RNAs were obtained from *in vitro* transcription by T7 RNA polymerase using linearized plasmid DNAs as templates (Milligan et al., 1987). The RNA transcripts were 5′ ^{32}P-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, or 3′ ^{32}P-labeled using [5′-^{32}P]-pCp and T4 RNA ligase (England & Uhlenbeck, 1980). The labeled RNAs were purified on denaturing gels and stored in water at −20 °C. Fe(II)-EDTA protection was carried out as described for the *Tetrahymena* ribozyme (Celander & Cech, 1991; Murphy & Cech, 1993) with minor modifications. All reagents except the buffer and the ultrapure $MgCl_2$ (Aldrich Chemicals) were prepared just prior to use. The RNA was renatured in 20 mM Tris-HCl or 20 mM MOPS, pH 7.5, by heating at 85 °C for 2 min, followed by incubation for 3 min at ambient temperature. $MgCl_2$ was added to appropriate concentrations and the mixture further incubated for 10 min at 50 °C. In some cases, 0.1 M KCl was added at this stage, although no changes in the protection pattern were observed. Ascorbic acid and DTT were then added to 1 and 5 mM, respectively, and the reaction started upon addition of a 10× mixture containing 10 mM Fe(II)$(NH_4)_2$-$(SO_4)_2$/20 mM EDTA, pH 8.0. The reaction proceeded at 37 °C for 60 min and was quenched upon addition of 10 mM thiourea. The reaction mixture was then fractionated using 6% or 15% polyacrylamide gels containing 7 M urea. The gels were dried and the cleavage products quantitated using a Phosphorimager (Fuji Medical).

Kinetic Assays. Kinetic analysis was performed using a pre-tRNA^{Phe} substrate (Pan & Zhong, 1994) containing 14 nucleotides 5′ to the cleavage site. The 5′ ^{32}P-labeled substrate was renatured in 50 mM Tris-HCl, pH 8.1, by heating at 85 °C for 2 min, followed by 3 min at ambient temperature. $MgCl_2$ and KCl were then added to designated concentrations, and the mixture was preincubated for 5 min at 37 °C. Ribozymes were renatured likewise except only $MgCl_2$ was added after heating, and the mixture was preincubated at 50 °C for 10 min. The reaction was started by mixing the ribozyme and the substrate. Aliquots were withdrawn at timed intervals, and the reaction was quenched by mixing with two volumes of 9 M urea/25 mM EDTA. Unreacted substrate and product were separated by denaturing polyacrylamide gel electrophoresis and quantitated using a Phosphorimager. Binding constants were calculated from the concentration dependence of reaction rates using the best-fit parameters obtained from the program Kaleidagraph (Abelbeck Software).

Cleavage at Nucleotide 103. Less than <0.01 μM of α-^{32}P-labeled p(62−239) was premixed with p(240−61) at designated concentrations in 50 mM Tris-HCl, pH 8.1. The mixture was then heated at 85 °C for 2 min, followed by 3 min at ambient temperature. The reaction was initiated by addition of KCl and $MgCl_2$ to appropriate concentrations and the reaction mixture incubated at 37 °C. Progress of the cleavage reaction was analyzed as described under kinetic assays.

RESULTS

Fe(II)-EDTA Protection of the Wild-Type P RNA. To ensure that the P RNA was conformationally homogenous, P RNAs purified from denaturing gels were renatured using different heat−cool protocols (Walstrum & Uhlenbeck, 1990; Herschlag & Cech, 1990) and examined by nondenaturing gel electrophoresis. When the purified P RNA was directly loaded on a nondenaturing gel, multiple bands were observed. Using the heat−cool conditions described in Materials and Methods, a single band corresponding to the fastest-migrating species in the untreated P RNA sample was present (data not shown). Since the P RNA renatured under this condition is also the most active in cleaving the pre-tRNA^{Phe} substrate, it is assumed that this fast-migrating conformer has the "native" structure of the P RNA.

Fe(II)-EDTA protection experiments were performed to locate ribose moieties that become protected upon folding. The results are shown in Figures 1 and 2. Using a phosphorimager, most radioactive bands can be quantitated. The result is presented as follows: first, the amount of radioactivity in each band without Mg^{2+} is divided by the amount of radioactivity with 10 mM Mg^{2+}. This ratio is then normalized to the total amount of radioactivity in each lane. By this measure, the higher the normalized ratio, the more protected the corresponding ribose moiety is in the folded structure. This ratio is arbitrarily defined as a "protection factor" (Figure 2). In this study, ribose moieties with a "protection factor" of larger than 1.5 are considered to be protected against hydroxyl radical attack. A significant fraction of all ribose moieties is protected in the presence of Mg^{2+}, indicating that, like group I introns (Latham & Cech, 1989; Celander & Cech, 1991), P RNA also folds into a compact structure with a "core" relatively inaccessible to solvent. Most nucleotides in J3/4, P4, J18/2, J19/4, and J15/15.1 that are highly conserved among all P RNAs are fully protected, indicating that they are located in the folded core of this catalytic RNA. It is striking that loops L8 and L15.1 also show strong protection in the presence of Mg^{2+}, although they are absent in many other P RNAs (Haas et al., 1991, 1994). In addition to these loops, the core contains the helical stems of P3, P5, and P5.1.

The only GNRA tetraloop in the *B. subtilis* P RNA, L12, also shows strong protection, suggesting that this loop interacts with another part of the RNA, as observed in other ribozymes (Michel & Westhof, 1990; Jaeger et al., 1994; Murphy & Cech, 1994). Loop L15, which has been shown to interact with the CCA nucleotides in the pre-tRNA substrate (LaGrandeur et al., 1994), appears to be rather exposed.

Folding of the *B. subtilis* P RNA is cooperative with respect to the Mg^{2+} concentration (Figure 3). Folding seems to be complete with at most 6 mM Mg^{2+}, since no changes in protection patterns were observed with Mg^{2+} concentrations up to 50 mM (not shown). A Hill plot gives a Hill coefficient of 3.2 (Figure 3B), suggesting that folding

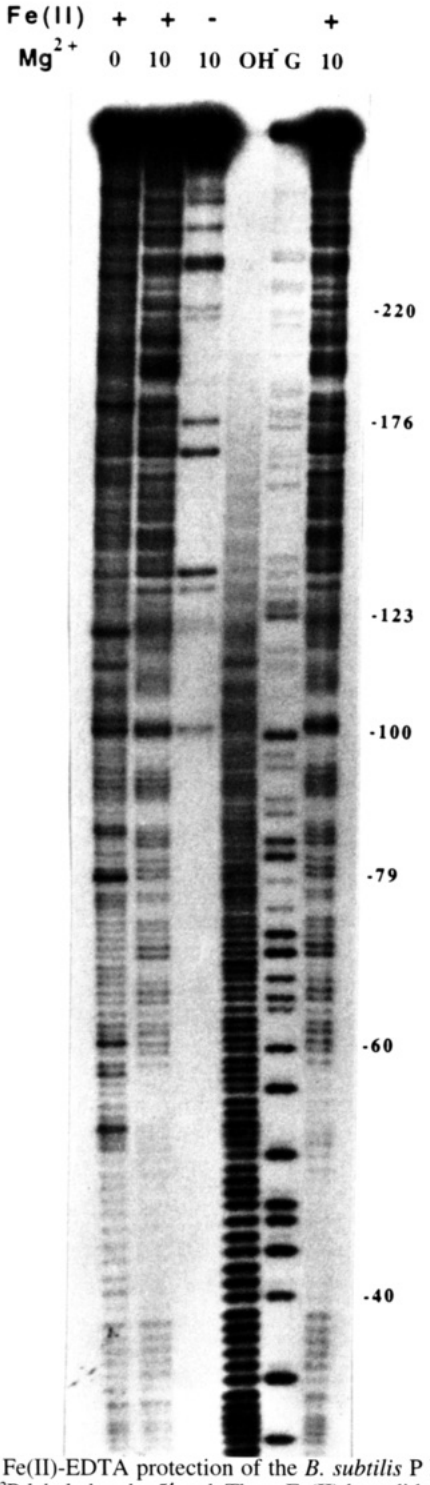

FIGURE 1: Fe(II)-EDTA protection of the *B. subtilis* P RNA. The RNA was ^{32}P-labeled at the 5′ end. The −Fe(II) lane did not contain ascorbic acid, DTT, and Fe(II)-EDTA. OH^- and G: partial alkaline hydrolysis and nuclease T1 digestion, respectively. The ribose positions are indicated by residue numbers to the right.

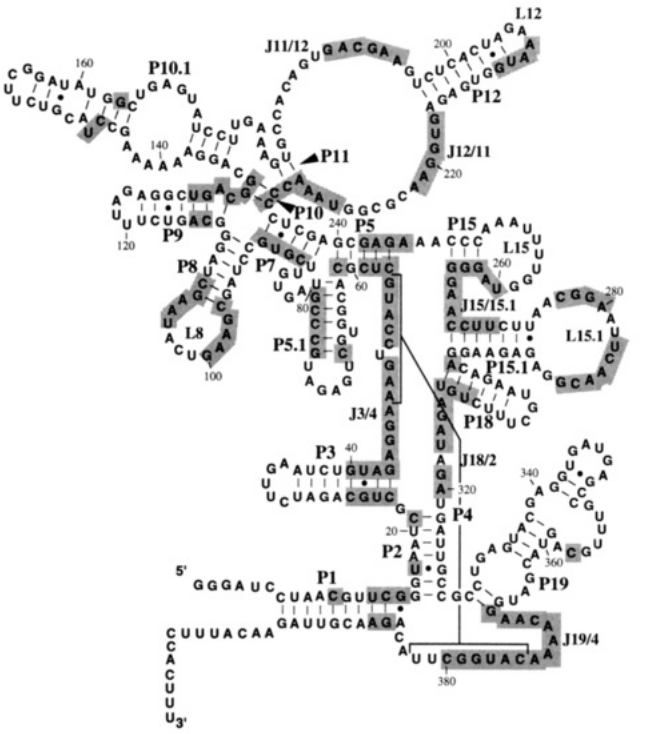

FIGURE 2: Summary of Fe(II)-EDTA protection results (the P RNA figure provided by James W. Brown and the Ribonuclease P Sequence Database). The nomenclature of the helical structures is according to N. Pace and co-workers (Haas et al., 1994). The radioactive bands were quantitated as described in text. Residues with an average protection factor of >1.5 from duplicate experiments are shaded. Due to heterogeneity of the 3′ ^{32}P-labeled RNAs, the shaded areas for residues 215-401 may be shifted by one nucleotide at either end.

requires binding of at least three additional Mg^{2+} ions. From the Hill plot, ~3 mM Mg^{2+} is needed to fold 50% of the RNA. This Mg^{2+} concentration is an overestimate, since EDTA is present in ~1 mM excess over Fe(II) in the reaction mixture. Therefore, the $[Mg^{2+}]_{1/2}$ for folding of *B. subtilis* P RNA should be in the range of 2−3 mM.

Design and Kinetic Analysis of Putative P RNA Domains. Dividing a biopolymer into folding domains provides an effective means to probe its structure and function. Studies on group I intron ribozymes (Murphy & Cech, 1993; Wang et al., 1994) demonstrate that, like proteins, RNAs also have domains that can fold into higher order structures without the rest of the molecule. By appropriate design of bimolecular RNA constructs that associate to form the tertiary structure of P RNA, it might also be possible to detect folding domains of P RNA by Fe(II)-EDTA protection. Although bimolecular constructs were made with the *E. coli* P RNA (Guerrier-Takada & Altman, 1992), they were designed by disrupting helical stems so that they could associate through Watson−Crick base pairs. It was unclear whether any of these RNA molecules alone was able to form any tertiary structure, due to the elimination of at least one helical element. RNA constructs designed here have presumably no disruption of helical elements based on the phylogenetically derived secondary structure. These molecules can therefore associate primarily, if not solely, through tertiary interactions. The design begins with the circular P RNA, followed by breaking the phosphodiester bond at two positions. These two positions serve as the new termini of the two smaller RNAs. Careful choice of these positions should cause minimal or no effect on the catalytic activity of the P RNA.

Circularly permuted P RNAs provide a guide in choosing the backbone positions as the termini of these two molecules. Previously, we have examined the effect of a single break in *B. subtilis* P RNA and shown that many of the phosphodiester bonds can be broken with little or no effect on substrate binding (Pan & Zhong, 1994). Two circularly permuted P RNAs with termini at nucleotides 62 and 240 were found to bind substrate equally well compared to the

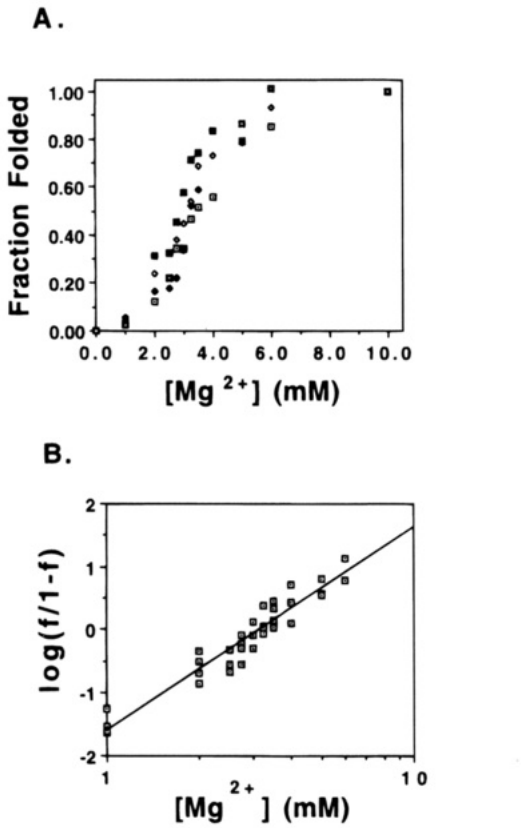

FIGURE 3: (A) Fraction of P RNA folded as a function of Mg^{2+} concentration. Protection of residues 45−47, 55−57, 75−77, and 97−99 is normalized to no Mg^{2+} sample (0% folded) and 10 mM Mg^{2+} sample (100% folded). (B) Hill plot of the data points in panel A. The slope of the plot is 3.2.

wild-type P RNA. These two positions are therefore chosen as the termini for the bimolecular constructs. In this design, one RNA has its 5′ end at nucleotide 62 and 3′ end at 239 [denoted p(62−239)], while the other RNA has its 5′ end at nucleotide 240 and 3′ end at 61 [p(240−61)]. When 1.2 μM p(62−239) or p(240−61) was incubated with trace amount of substrate, neither RNA alone was active. However, when both molecules were combined, the activity was restored to an extent comparable to the wild-type P RNA under identical conditions (Figure 4).

Binding constants for p(62−239) plus p(240−61) were determined by single-turnover kinetics. In this experiment, the concentration of p(62−239) was kept constant, but several times above the amount of a ^{32}P-labeled pre-tRNA^{Phe} substrate, while the concentration of p(240−61) was varied. The observed cleavage rates as a function of p(240−61) concentration corresponded to a binding curve from which the binding constants (K_d) can be deduced (Table 1). Two variables were considered in this study: the presence of triphosphates at the 5′ termini and the effect of Mg^{2+} concentrations on K_d. The RNA transcripts contained triphosphates at their 5′ ends. In the case of circularly permuted P RNAs, the presence of a 5′ triphosphate caused an increase in K_M of 4−5-fold, while reducing the k_{cat}/K_M by 2−3-fold (Pan & Zhong, 1994). The triphosphates of these RNAs were removed by alkaline phosphatase to produce RNAs containing 5′-hydroxyl groups. For this bimolecular complex, the presence of triphosphates caused only about 2-fold increase in K_d (Table 1), suggesting that the extra charge at the 5′ ends does not significantly destabilize the interaction of p(62−239) with p(240−61).

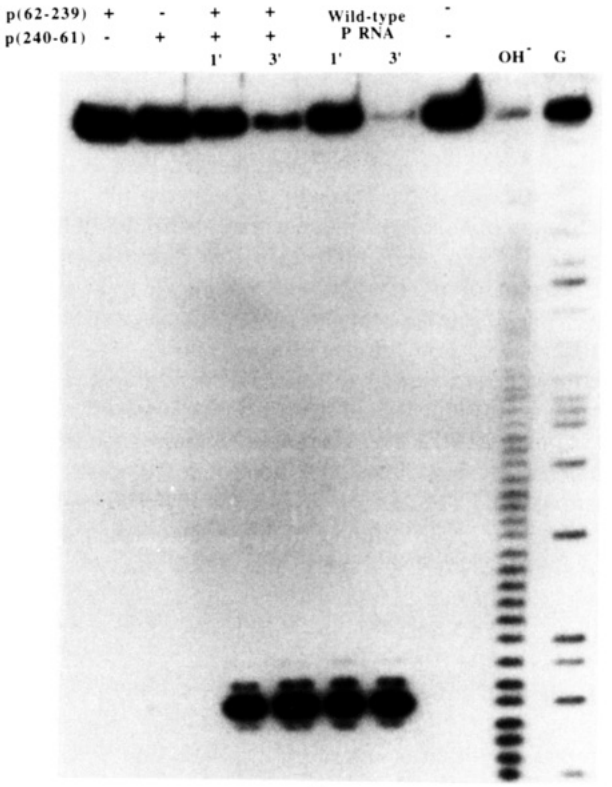

FIGURE 4: Cleavage of pre-tRNA^{Phe} substrate by the bimolecular complex, p(62−239) + p(240−61), under single-turnover conditions in 50 mM $MgCl_2$ and 1 M KCl, at 37 °C. All RNAs contain 5′-hydroxyl groups. From left to right: 1.25 μM p(62−239), 3 min; 1.25μM p(240−61), 3 min; 0.05 μM p(62−239) + 1.25 μM p(240−61), 1 and 3 min; 0.05 μM wild-type P RNA, 1 and 3 min; substrate alone.

Table 1: Binding Constants of p(62−239) + p(240−61)

cleavage of pre-tRNA^{Phe}	K_d (μM)[a]	v_{max} (min^{-1})	v_{max}/[p(62−239)] (10^7M^{-1}min^{-1})
50 mM Mg^{2+} [d]			
5′-OH	0.12	1.4[b]	2.8[c]
5′-ppp	0.26	0.8[b]	1.5
25 mM Mg^{2+}			
5′-OH	∼2−3	∼0.5[c]	∼0.2
5′-ppp	∼2−3	∼0.3[c]	∼0.1
cleavage at nucleotide 103 (50 mM Mg^{2+}, 5′-OH)	0.21	0.0067	

[a] Average values from duplicate experiments. [b] At 0.05 μM p(62−239). [c] At 0.25 μM p(62−239). [d] All contain 1 M KCl. [e] k_{cat}/K_M (10^7 M^{-1} min^{-1}) of P RNAs at 50 mM $MgCl_2$ and 1 M KCl: wild-type: 7.7; CP62$^{5'OH}$: 4.6; CP240$^{5'OH}$: 1.7.

Since K_M for *B. subtilis* P RNA is strongly dependent upon Mg^{2+} concentration (Reich et al., 1988; Pan & Zhong, 1994), binding constants were also measured in the presence of 25 and 50 mM Mg^{2+}. The 2-fold decrease in Mg^{2+} concentration resulted in an increase in K_d by almost 10-fold (Table 1), suggesting that Mg^{2+} is a crucial factor for the affinity of these two RNAs. Overall, the binding constants were in the range of 0.1−3 μM. Cleavage rates at saturating concentrations of p(240−61) (v_{max}) can also be estimated from the binding curves (Table 1). In this case, v_{max} represents a first-order rate constant, (k_{cat}/K_M)[p(62−239)]. By this measure, the apparent k_{cat}/K_M for the bimolecular complex is comparable to their parent molecules which are circularly permuted P RNAs.

Specific Cleavage at Nucleotide 103. An inadvertent site-specific cleavage at nucleotide 103 in p(62−239) was found

upon association of p(62−239) with p(240−61) in the absence of pre-tRNA substrate. Cleavage at nucleotide 103 occurred only in the presence of p(240−61) and Mg^{2+} (Figure 5A). It is unclear at the moment whether this reaction is catalyzed by the same active site residues involved in the standard RNase P reaction. Nevertheless, concentration dependence of this cleavage rate is useful in determining the affinity of bimolecular association. When the concentration of the "substrate", p(62−239), was kept constant while the amount of p(240−61) was varied, cleavage rates at nucleotide 103 as a function of p(240−61) concentration also corresponded to a simple binding curve. The binding constants determined by this method closely matched those determined by cleavage of the pre-$tRNA^{Phe}$ substrate (Table 1). Consistent with the remarkable Mg^{2+} dependence of the binding constants, cleavage at nucleotide 103 also showed a strong Mg^{2+} dependence (Figure 5B). At 50 mM Mg^{2+}, the bimolecular association also depended on the concentration of potassium ions (Figure 5C). Thus, like the substrate binding by the wild-type P RNA, ionic strength of the solution plays a significant role in forming the complex of p(62−239) and p(240−61).

Fe(II)-EDTA Protection of p(62−239) and p(240−61). Results for p(62−239) are shown in Figures 6 and 7. A substantial portion of the ribose moieties was protected in this molecule. Mg^{2+} dependence of these protections was also similar to that of the wild-type P RNA (Figure 6B). While fewer ribose moieties were protected overall, the unprotected regions in p(62−239) compared to the wild-type P RNA were mostly located in L8 and P5.1. Protection became more prevalent for ribose moieties in the nonconserved stem-loop P10.1. A plausible interpretation of this result is that this molecule functions as an independently folded domain, since most residues protected in this domain are identical to their counterpart in the wild-type P RNA. The total loss in protection for L8 and P5.1 indicates that these regions interact with p(240−61), contributing to the high affinity between these RNAs. In contrast to p(62−239), the only protected region in p(240−61) was located in the nonconserved stem-loop P19 (Figure 7B). Even at 50 mM Mg^{2+}, no protection was seen for almost all other residues in p(240−61), including the highly conserved nucleotides of J3/4, P4, J18/2, J19/4, and J15/15.1. Thus, it appears that folding of these regions are dependent upon interactions with residues in L8 and P5.1.

DISCUSSION

Fe(II)-EDTA protection of the catalytic RNA from *B. subtilis* RNase P has been described. The results confirm once again that catalytic RNAs contain core structures that are relatively solvent excluded. In the case of *B. subtilis* P RNA, the core includes J3/4, P4, J18/2, J19/4, and J15/15.1 that are phylogenetically conserved among all eubacterial (Haas et al., 1994) as well as eukaryotic P RNAs (Darr et al., 1992; Tranguch & Engelke, 1993; Altman et al., 1993a). Since some of these nucleotides are likely to form part of the active site (Westhof & Altman, 1994; Harris et al., 1994), the active site in P RNA seems to be less accessible to solvent in the absence of substrate. Such a folded structure is not unlike that of a group I intron ribozyme in which many phylogenetically conserved residues are also included in the core (Cech, 1990; Michel & Westhof, 1990). In addition, the active site in the group I ribozyme is also less accessible to solvent in the absence of substrate and the guanosine cofactor.

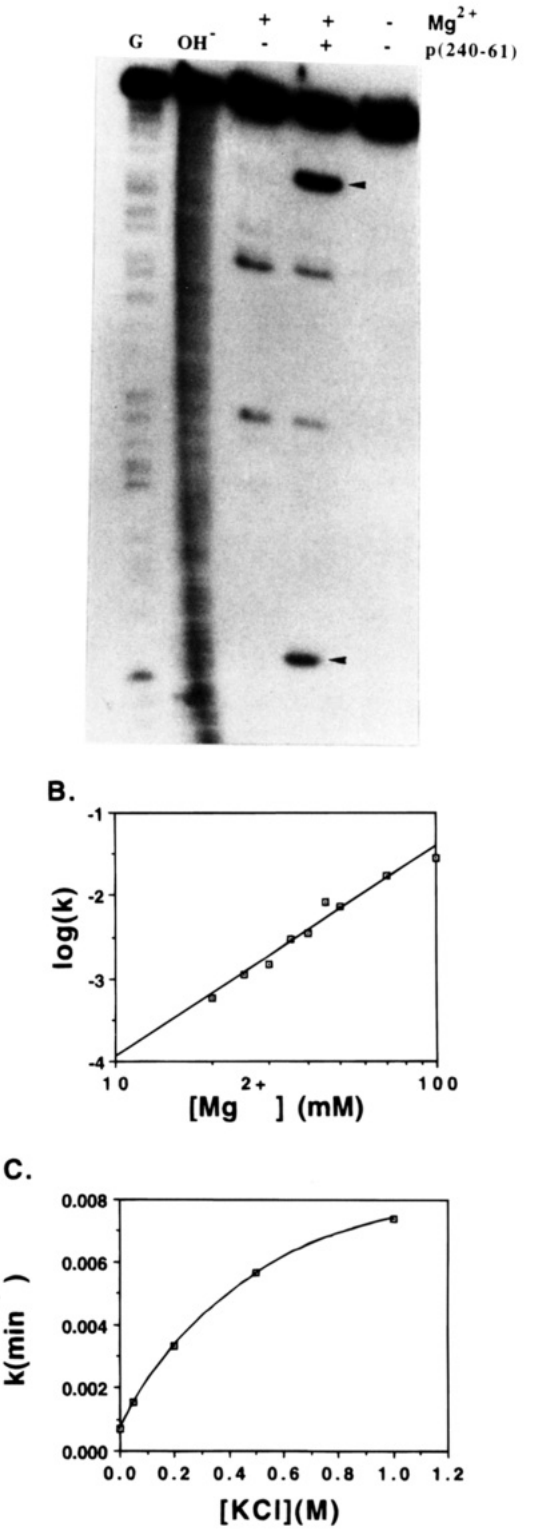

FIGURE 5: (A) Cleavage at nucleotide 103 in 1 M KCl, ±50 mM $MgCl_2$, at 37 °C for 60 min. p(62−239) was labeled with [α-^{32}P]-CTP, and the two cleavage products are marked by arrows. From left to right: G and OH^-: partial nuclease T1 digestion and alkaline hydrolysis of 5′ ^{32}P-labeled p(62−239); <0.1 μM $p(62-239)^{5'OH}$; <0.1 μM $p(62-239)^{5'OH}$ + 1 μM $p(240-61)^{5'OH}$; <0.1 μM $p(62-239)^{5'OH}$ + 1 μM $p(240-61)^{5'OH}$, no Mg^{2+}. (B) Cleavage rates at nucleotide 103 as a function of Mg^{2+} concentration. The slope of the plot is 2.5. (C) Cleavage rates at nucleotide 103 as a function of K^+ concentration.

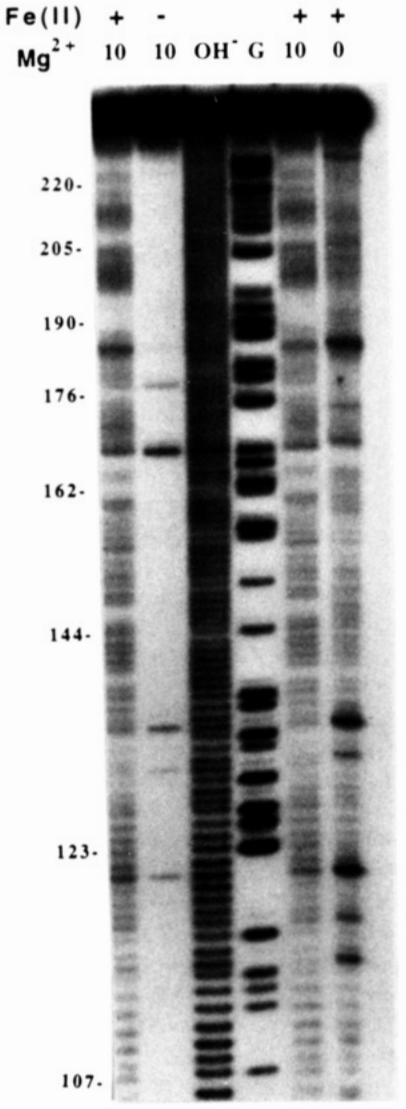

FIGURE 6: (A) Fe(II)-EDTA protection of p(62−239). The legends are the same as described for Figure 1. (B) Fraction of p(62−239) folded as a function of Mg^{2+} concentration. Protection of residues 165 and 193 are normalized to no Mg^{2+} sample (0% folded) and 10 mM Mg^{2+} sample (100% folded).

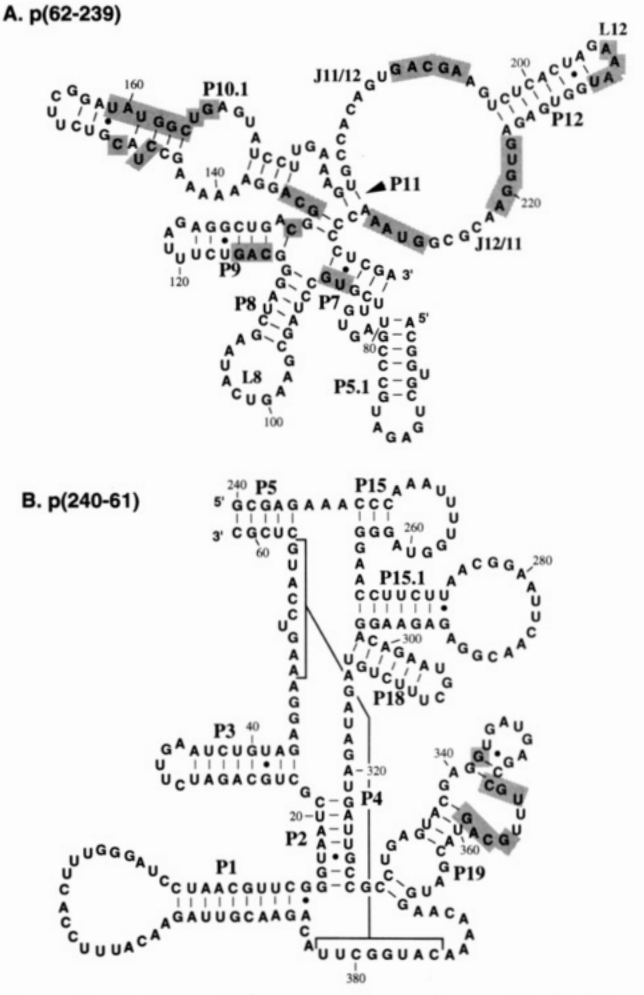

FIGURE 7: Summary of Fe(II)-EDTA protection results of p(62−239 and p(240−61). Residues with a protection factor of >1.5 are shaded. Due to the resolution of gel electrophoresis, the shaded areas for residues 215−239 may be shifted by one nucleotide at either end.

It is interesting to note that L8, L15.1, and part of P5.1 in *B. subtilis* P RNA are highly protected. In the *E. coli* P RNA, P-L5.1 and P-L15.1 are substituted by three additional helices of P6, P16, and P17, while the decanucleotide loop L8 in *B. subtilis* P RNA is reduced to a pentanucleotide loop in *E. coli* P RNA. Pace and co-workers have postulated (Haas et al., 1991) that this region, or subdomain, is similar in the tertiary structure in both P RNAs, despite the large differences in their secondary structures. The Fe(II)-EDTA protection pattern clearly suggests the presence of a large number of interactions, in support of a complex folded structure in this region.

Another structural unit in the *B. subtilis* P RNA is the region encompassing P7, part of P8 and P9−P12. This unit contains a large number of conserved nucleotides in J11/12 and J12/11. Fe(II)-EDTA protection of p(62−239) shows that many residues in this domain are protected against hydroxyl radical attack and the pattern of protection closely matches that of the same nucleotides in the wild-type P RNA. One interpretation of these results is that this region represents an independently folded domain and the folding is identical with or without the remaining portion of the P RNA.

From these results a domain structure begins to emerge for the *B. subtilis* P RNA (Figure 8). Domain I contains P7, part of P8, P9−P12, J11/12, and J12/11, which is independently folded. Domain II includes the helical elements of P5, P-L5.1, J5.1/7, L8, J5/15, P-L15, J15/15.1, and P-L15.1. Domain III contains the phylogenetically conserved P1−P4, P18, J3/4, J18/2, and J19/4. The division of domains II and III partly refers to the observation that eukaryotic P RNAs appear to contain domain III, but lack domain II. In the *B. subtilis* P RNA, tertiary folding of domain III cannot occur without domain II. It remains to be seen whether

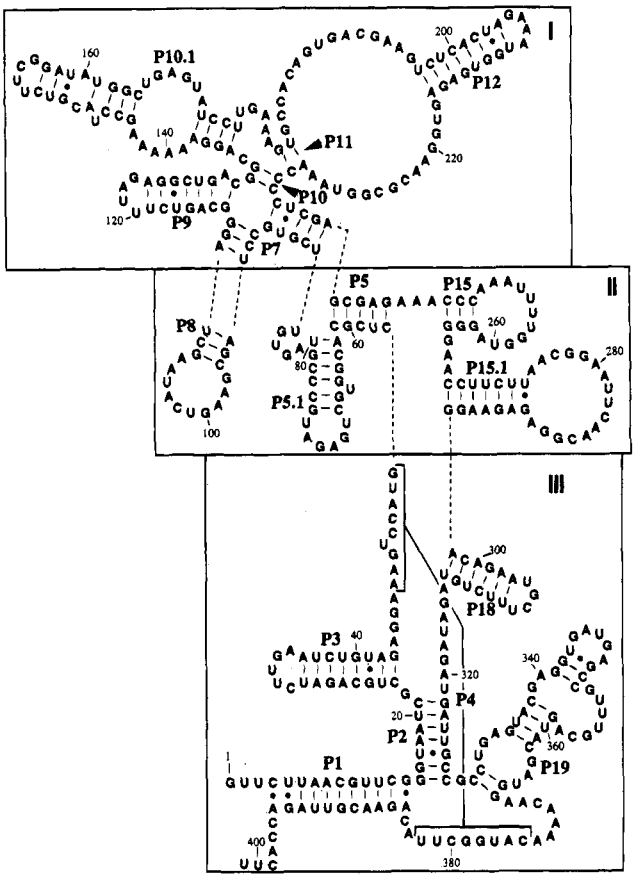

FIGURE 8: Putative domains of *B. subtilis* P RNA. Domain I can fold independently, while folding of domain III is dependent on the presence of domain II and/or domain I.

concomitant folding of domains II and III can be achieved in the absence of domain I. It is instructive to compare the domain compositions in *B. subtilis* P RNA with those of *E. coli* P RNA. *E. coli* P RNA lacks P10.1 but has two additional stem-loops P12 and P13 in domain I with P14 being the likely equivalent of P12 in *B. subtilis* P RNA. *E. coli* P RNA also lacks P19 but has a longer stem P3a in domain III. The most dramatic difference is the domain II with substitutions of P-L5.1 and P-L15.1 with P6, P16, and P17 in the *E. coli* P RNA.

The Fe(II)-EDTA results can also be compared to the models of *E. coli* P RNA (Westhof & Altman, 1994; Harris et al., 1994), keeping in mind that Fe(II)-EDTA protection patterns can only be consistent with, but cannot prove, particular interactions. Although several stem-loops in *E. coli* P RNA are missing in *B. subtilis* P RNA and vice versa, comparisons can still be made assuming that the equivalent secondary structural elements have identical orientations in the tertiary structure. According to the Westhof and Altman model, L12 in *B. subtilis* P RNA could interact with the helix of P7. Indeed, Fe(II)-EDTA assays of the wild-type as well as p(62−239) show simultaneous protection of nucleotides in L12 and P7 (Figures 2 and 7). The model by Pace and co-workers (Harris et al., 1994) infers that the missing P6, P16, and P17 in *E. coli* P RNA might be replaced by L15.1 and P5.1. Indeed, Fe(II)-EDTA assay of the wild-type P RNA shows simultaneous protection of L15.1 and P5.1 (Figure 2). The protection of both elements is completely lost when they are separated in the context of the bimolecular construct, p(62−239) plus p(240−61) (Figure 7). In the model by Pace and co-workers, P15.1 might also be positioned so as to interact with L8, consistent with the protection pattern of these elements in the *B. subtilis* P RNA (Figures 2 and 7).

The loss of Fe(II)-EDTA protection of domains II and III and the micromolar binding constant between p(62−239) and p(240−61) indicate that intra- and interdomain tertiary interactions must be quite large. On the other hand, these interactions appear to be dynamic, since no additional protection of one molecule was seen in the presence of the other at saturating concentrations (data not shown). Similar results were observed for the *Tetrahymena* group I intron in which addition of a subdomain, P5abc, in *trans* restored the catalytic activity, but not the protection against hydroxyl radicals (Van der Horst et al., 1991; Mohr et al., 1994).

The ionic dependence of P RNA folding demonstrates once again the importance of Mg^{2+} in promoting RNA structure. Two observations are of particular interest. (1) Folding of P RNA is highly cooperative with a Hill coefficient of 3.2, suggesting binding of at least three additional Mg^{2+} ions in the completely folded structure. A previous study showed that substrate cleavage by the *E. coli* P RNA also depends on the cooperative binding of at least three additional Mg^{2+} ions (Smith & Pace, 1993). Thus, Mg^{2+} requirements for *B. subtilis* and *E. coli* P RNAs appear to be similar. Another study (Kazakov & Altman, 1991) has shown that Mg^{2+} can induce specific cleavage at five sites in the folded *E. coli* P RNA. It remains to be seen whether any of these sites correlates to the Mg^{2+} ions bound during the cooperative folding process. Mg^{2+} concentration at which 50% of the molecules are folded is between 2 and 3 mM, which compares favorably to the folding of the *Tetrahymena* ribozyme with $[Mg^{2+}]_{1/2}$ of ~1 mM (Celander & Cech, 1991; Murphy & Cech, 1993). (2) Although the P RNA is completely folded at 5−6 mM Mg^{2+}, substrate binding still shows a highly cooperative dependence on Mg^{2+} up to at least 50 mM (Reich et al., 1988; Pan & Zhong, 1994). Fe(II)-EDTA protection indicates that folding of the pre-$tRNA^{Phe}$ substrate is complete at 2−3 mM Mg^{2+} (T.P., unpublished results). Thus, the requirement of high Mg^{2+} concentration for the intermolecular ribozyme−substrate interaction cannot be explained by folding of individual RNA components. An intriguing parallel can be found in the bimolecular association of p(62−239) and p(240−61) (Table 1). The obvious property common in both systems is that these RNAs associate through tertiary interactions, rather than through Watson−Crick base pairing. It remains to be seen whether this observation is general among other tertiary RNA−RNA interactions. One solution to circumvent the high Mg^{2+} requirement is through binding of proteins, as observed in the RNase P holoenzymes (Guerrier-Takada et al., 1983; Reich et al., 1988) and several group I intron ribozymes (Akins & Lambowitz, 1987; Gampel & Cech, 1991; Mohr et al., 1994).

Finally, folding of *B. subtilis* P RNA domains may shed light on why the eukaryotic P RNAs are inactive in catalysis in the absence of proteins. Inspection of eukaryotic P RNA sequences reveals that they contain most structural elements of domains I and III, including many invariant nucleotides in eubacterial P RNAs. However, they all lack substantial portions of domain II. Since domain III in *B. subtilis* P RNA is incapable of tertiary folding in the absence of II and/or I, the same may be true for eukaryotic P RNAs. It will be

interesting to see whether the catalytic activity of eukaryotic P RNAs can be restored by addition of a RNA molecule resembling that of the domain II of eubacterial P RNAs.

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