Selection of Circularly Permuted Ribozymes from *Bacillus Subtilis* RNAse P by Substrate Binding†

Tao Pan* and Kun Zhong

Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637

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ABSTRACT: The effect of a single break in the phosphodiester backbone of *Bacillus subtilis* RNAse P RNA (P RNA) was examined using circular permutation analysis (CPA). This method reveals that many of the phosphodiester bonds in this catalytic RNA can be broken with little or no effect on substrate binding. Phosphate positions that show strong effects are located mostly in regions conserved among all RNAse P RNAs, or they are in regions known to interact directly with the pre-tRNA substrate. Two circularly permuted isomers of P RNA were constructed and analyzed in detail. The K_M for both circularly permuted isomers is nearly identical to that of the wild-type P RNA. Since the K_M of the P RNA is essentially the same as the binding constant to the substrate, this finding confirms the CPA results. The implications of backbone breakage are discussed with respect to folding and catalysis of the RNAse P RNA.

The ribose−phosphate moieties often play important roles in the folding and biological activity of RNA molecules (Quigley & Rich, 1976; Celander & Cech, 1991; Puglisi et al., 1992; Nolan et al., 1993). The role of particular backbone positions can be examined by site-specific modifications using 2′-deoxynucleotides (Perreault et al., 1990; Pyle & Cech, 1992) or phosphorothioates (Ruffner & Uhlenbeck, 1990; Christian & Yarus, 1992). One other approach to analyze the role of phosphodiester bonds in RNA folding and catalysis is circular permutation [reviewed in Pan and Uhlenbeck (1993)]. Circular permutation involves first covalently linking the normal 5′ and 3′ ends of the RNA, followed by breaking a single phosphodiester bond elsewhere to produce new 5′ and 3′ ends. In chemical terms, circular permutation is equivalent to converting a 5′,3′-phosphodiester linkage into a 2′,3′-cyclic phosphate and a 5′-hydroxyl group. Depending on the structural and functional requirement of the ribose phosphate that is broken, folding and activity of circular permuted (CP)[1] RNA isomers can vary extensively. Since circular permutation identifies the backbone positions that can become new termini with little or no effect on the functional aspects of the RNA, two such positions can be chosen as the termini for bimolecular RNA constructs that associate to reform the active molecule. Such constructs may be useful in locating RNA folding domains.

A technique, termed circular permutation analysis (CPA), has been developed in which the folding or activity of all CP RNA isomers can be analyzed in a single experiment (Pan et al., 1991; Gott et al., 1993). In CPA, a mixture of CP molecules, generated by partial alkaline hydrolysis of the circular RNA, is subjected to a procedure which permits separation of active from inactive CP isomers. The 5′ ends of the active CP isomers are then determined by either site-specific cleavage (Pan et al., 1991) or reverse transcription (Gott et al., 1993).

In this paper we apply CPA to the catalytic RNA from *Bacillus subtilis* RNAse P. RNAse P is a cellular enzyme responsible for producing the mature 5′ end of transfer RNAs through a hydrolytic cleavage reaction of the precursor tRNA (Altman et al., 1993; Pace & Smith, 1990). The RNA component (denoted P RNA) from the *B. subtilis* RNAse P is fully competent in binding and cleavage of precursor tRNAs at high ionic strength (Gardiner et al., 1985; Reich et al., 1988). To separate the CP isomers on the basis of their binding affinity to a pre-tRNA substrate, immobilized substrates are used that are linked to a solid support by a new procedure. The 5′ ends of the bound CP RNAs are then identified by reverse transcription. Binding affinities determined by CPA are confirmed in kinetic assays of two individual CP P RNAs. The effect of circular permutation of most phosphodiester bonds correlates well with phylogeny and biochemical data of the P RNA.

MATERIALS AND METHODS

Preparation of the Immobilized Pre-tRNA Substrate. The pre-tRNA substrate contains 14 nucleotides 5′ to the sequence of the yeast $tRNA^{Phe}$. Transcription by T7 RNA polymerase was carried out as previously described (Milligan et al., 1987) except that 2.5 mM N^6-(6-aminohexyl)adenosine 5′-monophosphate (Sigma, St. Louis, MO) over 0.5 mM ATP was included in the transcription reaction to generate RNA with a modified 5′-adenosine. The RNA transcript was precipitated directly from the transcription mixture by addition of 2.5 volumes of ethanol, redissolved in 65 mM $NaHCO_3$, pH 8.85, and 33 mg/mL of sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (NHS-SS-Biotin; Pierce, Rockford, IL). The reaction mixture was incubated for 1 h at 25 °C, and the biotinylated RNA products were purified on a 5% polyacrylamide gel containing 7 M urea. The purified

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* Corresponding author.

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[1] Abbreviations: ATP, adenosine triphosphate; CP, circularly permuted; CPA, circular permutation analysis; GMP, guanosine monophosphate; GTP, guanosine triphosphate; K_d, binding constant; N^6amA, N^6-(6-aminohexyl)adenosine 5′-monophosphate; P RNA, the catalytic RNA component of RNAse P.

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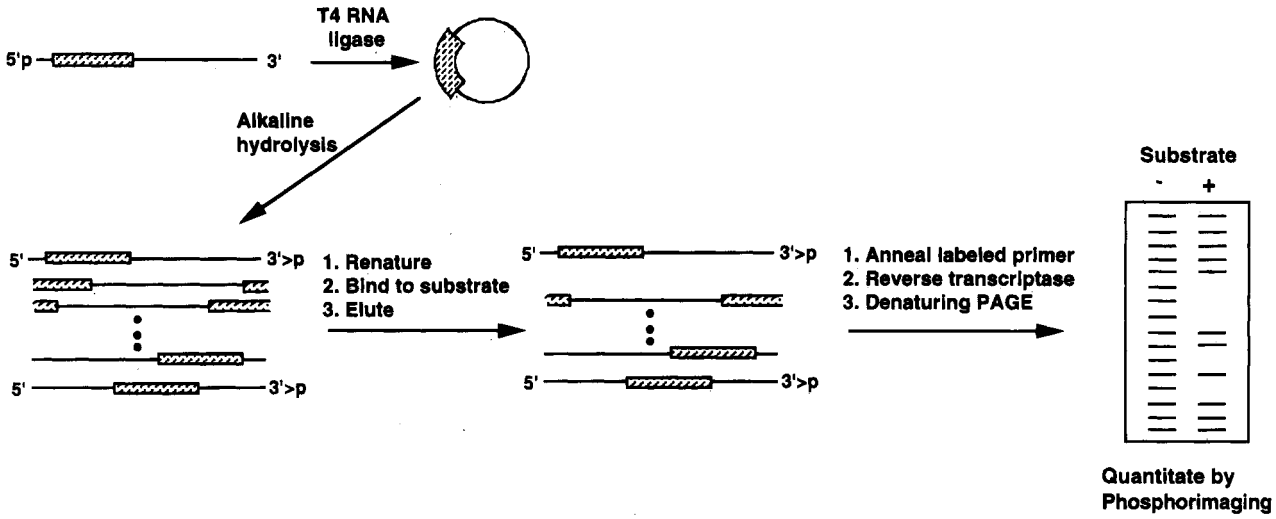

FIGURE 1: Experimental strategy for circular permutation analysis (CPA). The hatched boxes are graphic representations of a region in P RNA that becomes circularly permuted.

substrate was preheated in 50 mM Tris-HCl, pH 8.0, at 85 °C for 2 min, cooled to ambient temperature, and incubated with 10 mM $MgCl_2$ and 1 M KCl for 5 min. The biotinylated substrate (5 pmol) was then loaded on 10 μL of Streptavidin MagneSphere beads (Promega, Madison, WI), incubated at ambient temperature for 5 min, and washed with 20 μL of 50 mM Tris-HCl, pH 8.0, 1 M KCl, and 25 mM $CaCl_2$.

Circular Permutation Analysis. The *B. subtilis* P RNA was obtained by *in vitro* transcription of a plasmid containing the P RNA gene (Waugh & Pace, 1993). A 5-fold excess of 5′-GMP over GTP was included in the transcription to obtain RNAs with a 5′-monophosphate. Circularization was carried out by incubating 0.6 μM linear RNA with T4 RNA ligase at 16 °C overnight (England et al., 1980; Pan et al., 1991). Under these conditions, about 50% of the linear RNA was converted into the circular form. Alkaline hydrolysis was performed with ~0.7 μM purified circular RNA in 1 mM glycine and 0.4 mM $MgSO_4$, pH 9.54, by boiling for 1 min 30 s. The hydrolysis mixture was neutralized by addition of 120 mM Tris-HCl, pH 8.0. To renature the RNA, the mixture was heated at 85 °C for 2 min, cooled to room temperature, and incubated with 25 mM $CaCl_2$ and 1 M KCl at 50 °C for 10 min. CP RNAs (2.5 pmol) were then loaded in 10 μL on the streptavidin beads containing the immobilized pre-tRNA substrate and incubated for 15 min at 6 °C. The unbound RNA was then separated from the beads, and the bound RNA was eluted by addition of 10 μL of 45 mM Tris-HCl, pH 8.0, 0.9 M KCl, and 50 mM $MgCl_2$ and incubation at 6 °C for 20 min. The eluted RNA was then ethanol precipitated in 50 mM potassium acetate and 0.2 M KCl and stored in water.

To determine the 5′ ends of the circularly permuted P RNAs, three primers of the sequences 5′-GACGTGGTCTAACGTTCTGTAA, 5′-CCGTTAAGAAGGTTCCCCTACCAA, and 5′-GTAGGCTTTTTTCCTGCCGTCAG, complementary to nucleotides 381−401, 255−279, and 127−149, respectively, of the *B. subtilis* P RNA, were used. The eluted RNA was annealed with a molar excess of 5′-^{32}P-labeled primers by heating at 90 °C for 1.5 min followed by incubation on ice for 4 min. Reverse transcription was carried out at 50 °C and analyzed on 5% denaturing gels as described previously (Gott et al., 1993). The amount of reverse transcribed products was quantitated using a Phosphorimager (Molecular Dynamics).

Cloning of the Circularly Permuted P RNAs. cDNAs were obtained by reverse transcription of the circular P RNA followed by PCR amplification using appropriate primers. For the CP P RNA with the 5′ end at nucleotide 62, a 5′ primer 5′-TAATACGACTCACTATAACGGTGCTGAGATGCCCGTAGT and a 3′ primer 5′-GGATGAATGGTCTCGCGAGCATGGACTTTCC were used. The 5′ primer contained the T7 RNA polymerase promoter, and the 3′ primer contained a *FokI* site which served as the restriction site for run-off transcription to produce the precise 3′ end. For the CP P RNA with the 5′ end at nucleotide 240, a 5′ primer 5′-TAATACGACTCACTATAGCGAGAAACCCAAATTTTGG and a 3′ primer 5′-TCGAGGGGTTTACCGCGTT were used. The 3′ primer was designed such that when the double-stranded DNA was cloned into a *Sma*I restriction site, the plasmid DNA could be linearized with *Xho*I for run-off transcription.

Kinetic Assays. The K_M and k_{cat} of the cloned CP P RNAs were determined using the pre-tRNA^{Phe} substrate described above except the transcript begins with a 5′ G. The 5′-^{32}P-labeled pre-tRNA 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 25 mM and 1 M, respectively, and the mixture was preincubated for 5 min at 37 °C. The P RNAs were renatured likewise except no KCl was included and the mixture was preincubated at 50 °C for 10 min. The reaction was started by mixing the enzyme and the substrate. Aliquots were withdrawn at varying times and mixed with two volumes of 9 M urea/25 mM EDTA to quench the reaction. Unreacted substrates and products were separated by denaturing polyacrylamide gel electrophoresis and quantitated using a Phosphorimager.

RESULTS

Circular Permutation Analysis. The experimental strategy for CPA applied to P RNA is illustrated in Figure 1. The RNA was circularized using T4 RNA ligase and subjected to partial alkaline hydrolysis under denaturing conditions to produce all circularly permuted isomers. This mixture of CP isomers was then renatured and incubated with an

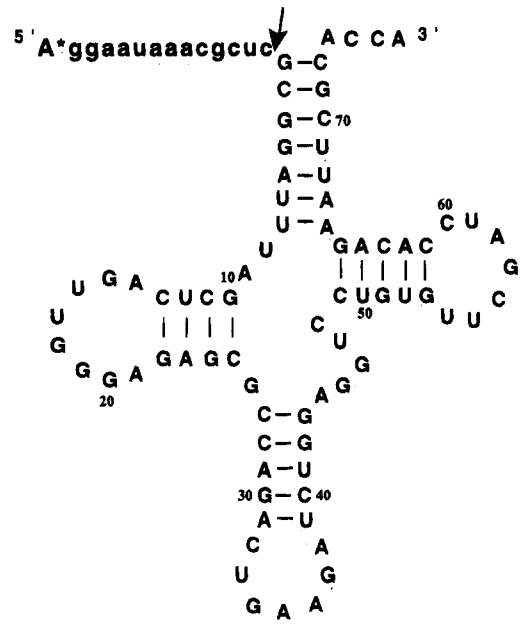

FIGURE 2: Sequence of the pre-tRNA^{Phe} substrate. The cleavage site is indicated by an arrow and the sequence 5′ to the cleavage site is shown in lower case. A* represents the modified adenosine [N^{6}amA. Final product: biotin-NH$(CH_2)_2$SS$(CH_2)_2$C(O)NH$(CH_2)_6$N^6-adenosine].

immobilized pre-tRNA substrate to separate the CP isomers with differential binding affinities. The binding condition could be controlled (see below) such that essentially no cleavage reaction occurred during the time of incubation. The bound RNAs were then eluted from the solid support and annealed to a 5′-^{32}P-labeled DNA primer for reverse transcription. The 5′ end of the CP isomers was identified by comparing the run-off bands to sequencing reactions using the circular RNA as the template. Reverse transcription was also done with the mixture of CP isomers prior to binding to the immobilized substrate to determine the amount of individual CP isomers present initially in the mixture.

The pre-tRNA^{Phe} substrate (Figure 2) is constructed to contain a region of 14 nucleotides 5′ to the cleavage site. Since very little interaction occurs between the P RNA and the nucleotides 5′ to the cleavage site (Smith & Pace, 1993), these nucleotides serve as a linker between the tRNA moiety and the site of immobilization. Since the RNA transcript begins with a 5′ adenosine, inclusion of a 5-fold molar excess of a modified adenosine (N^{6}amA; Figure 2) ensured that the majority of the RNA contained this residue at their 5′ end (data not shown). The modified adenosine contains a primary amino group which serves as the site of biotinylation. Although the biotinylating reagent would react readily with an aliphatic primary amino group, the reaction condition at high pH (8.8) also leads to hydrolysis of the succinimidyl group, resulting in rapid decomposition of the reagent. Thus, it was necessary to include a large molar excess (10−20-fold over N^{6}amA in the transcription mixture) of biotinylating reagent in the reaction to quantitatively biotinylate RNA transcripts containing N^{6}amA. The biotinylated pre-tRNA substrate could be separated on a denaturing gel from unreacted pre-tRNAs which presumably did not contain N^6-amA. For most experiments described below, a RNA sample was used in which more than 90% of the RNA contained biotin after purification. Kinetic analysis showed that k_{cat}/K_M of the biotinylated pre-tRNA^{Phe} was indistinguishable from the unmodified RNA (data not shown), suggesting that biotinylation of the RNA did not affect cleavage by P RNA.

To prevent substrate cleavage during binding, the divalent ion Mg^{2+} was substituted with Ca^{2+}. Ca^{2+} appears to be capable of sustaining substrate binding by *Escherichia coli* P RNA (Smith et al., 1992, 1993), while reducing the chemical step by about four orders of magnitude, so that substrate cleavage becomes negligible during the time of incubation (10 min; while the half-life for cleavage at saturation of substrate is >1 h). The apparent binding constant (K_d) of the wild-type P RNA to the immobilized substrate was ~1−2 μM at 50 mM Tris-HCl, pH 8.0, 1 M KCl, and 25 mM $CaCl_2$. This is only about 2−3-fold higher than the K_M of the substrate in solution at 25 mM $MgCl_2$ (Reich et al., 1988; also see below). Since substrate binding by *B. subtilis* P RNA is quite sensitive to the nature and concentration of divalent ions, this 2−3-fold increase in K_d is rather small, suggesting that P RNA binding to the immobilized substrate reflects its binding affinity in solution. Cleavage of the immobilized substrate was also carried out in 50 mM $MgCl_2$ with preformed enzyme−substrate complex. The catalytic efficiency (k_{cat}/K_M) was (4−5) × 10^6 M^{-1} min^{-1}, only about 5-fold less than that of the unmodified substrate in solution. On the basis of these criteria, the immobilized substrate appears to be competent as an authentic substrate of the *B. subtilis* P RNA.

The results of the CPA experiment are shown in Figures 3 and 4. When 2.5 pmol of CP P RNA mixture was applied to the solid support containing 5 pmol of substrate, about 20% of the CP RNAs were bound. This is similar to the amount of wild-type *B. subtilis* P RNA bound under identical conditions, suggesting that many CP isomers bound with similar affinity as the wild-type ribozyme. Using three reverse transcriptase primers, 377 of the 401 nucleotide positions (94%) could be analyzed (Figure 4). The effect of phosphodiester bond breakage on substrate binding could be assessed quantitatively by measuring the reverse transcription products of CP isomers. The result is presented as follows: first, the amount of the CP isomer before addition to solid support is divided by the amount after separation. This ratio is then normalized to that of the CP isomer with the 5′ end at nucleotide 1 (the wild-type P RNA, Figure 4). The normalized ratio will be referred to as the "depletion factor". By this measure, the higher the depletion factor, the more detrimental the effect of circular permutation of P RNA is on substrate binding.

Circularly permuted P RNAs at 236 of 377 analyzable backbone positions (63%) have depletion factors smaller than 1.5, suggesting that they bind substrate with similar affinity as the wild-type P RNA. As expected, many of these positions are located in phylogenetically nonconserved regions (P10.1, P19) or stem-loops with few conserved nucleotides (P1, P3, P9, P12). In general, CP P RNAs with breaks within the phylogenetically conserved regions have depletion factors larger than 1.5. This is consistent with these residues being involved in folding of the P RNA or in contacting the substrate directly. On the other hand, several breaks within the conserved regions result in CP P RNAs that are fully capable of substrate binding. These include ribose phosphates 21−23 and 52−53 and several nucleotides in the loops of 175−197 (J11/12) and 217−234 (J12/11). Thus, it is not always possible to predict the effect of circular permutation on substrate binding on the basis of the

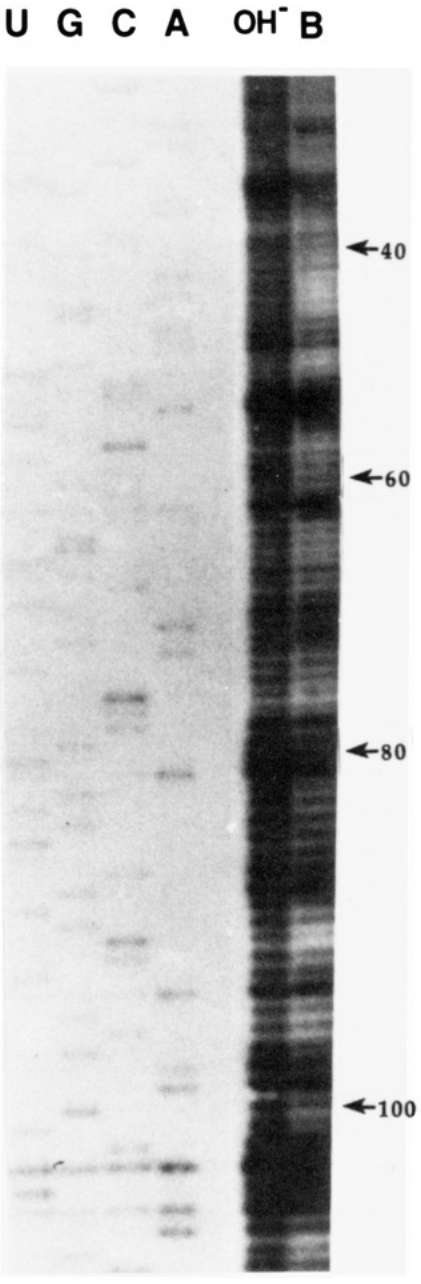

FIGURE 3: CPA of the *B. subtilis* P RNA. The 5′ ends of CP isomers are determined by run-off reverse transcription. (OH^-) The CP mixture prior to addition to the solid support. (B) The CP mixture bound to the immobilized substrate. (U, G, C, A) Reverse transcriptase sequencing of the circular P RNA. The ribose phosphate positions are indicated by the residue numbers at the right.

secondary structure and phylogeny of P RNA. Similar results have been obtained for circularly permuted $tRNA^{Phe}$ in which numerous breaks in the core region of the tertiary structure also resulted in properly folded tRNAs (Pan et al., 1991).

Analysis of Individual CP Isomers. Since CPA was carried out using a mixture of RNA molecules, it was necessary to confirm the results by characterizing individual CP isomers. For *B. subtilis* P RNA, binding constants for substrates in solution are virtually identical to the K_Ms in the kinetic analysis (Smith & Pace, 1993). Two CP P RNAs were chosen for further study. On the basis of the CPA results, both isomers should have a similar affinity for substrate compared to the wild-type ribozyme. Initially, the RNA transcripts contained a 5′-triphosphate. Kinetics showed that

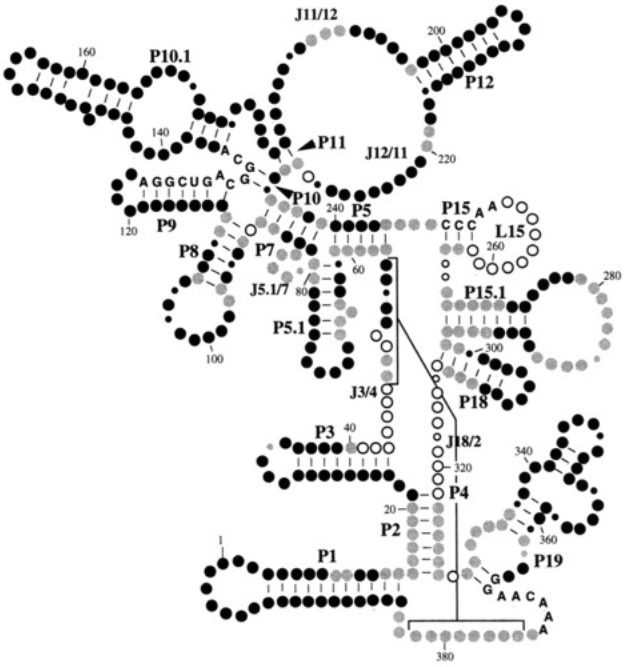

FIGURE 4: Summary of the CPA results (The P RNA figure provided by James W. Brown and the Ribonuclease P Sequence Database.). The nomenclature of the helical structures is according to N. Pace and co-workers (Haas et al., 1994). Every 20th nucleotide is marked. The depletion factor at each position is indicated by filled circles (0.9−1.5), shaded circles (1.5−2.0), and open circles (2.0−4.0). Filled circles correspond to circularly permuted P RNAs most capable of substrate binding, while open circles correspond to CP molecules least capable of substrate binding. When the depletion factors are less certain due to reverse transcriptase stops, the positions are marked by circles of smaller sizes.

Table 1: K_M and k_{cat} Values of the Circularly Permuted P RNAs

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (10^7 M^{-1} min^{-1})
wild type	0.57	20	3.5
$CP62^{5'OH}$ [a]	0.63	20	3.2
$CP62^{5'ppp}$ [b]	2.8	49	1.8
$CP240^{5'OH}$	0.74	3.7	0.5
$CP240^{5'ppp}$	3.6	8.1	0.2

[a] CP isomer with the 5′ end at nucleotide 62 containing a 5′-hydroxyl group. [b] CP isomer with the 5′ end at nucleotide 62 containing a 5′-triphosphate.

the K_M of the reaction was 4−7-fold higher than the wild-type K_M (Table 1). Since the 5′ end of the CP RNAs in the CPA experiment was a hydroxyl group, not a triphosphate, the 5′-triphosphates were removed with alkaline phosphatase. Consistent with the CPA results, the K_M was now substantially reduced to within 1.3-fold of the wild-type K_M (Table 1). k_{cat}, which reflects the rate of dissociation of the tRNA product (Reich et al., 1988; Smith & Pace, 1993), was also comparable for the CP isomer with the 5′ end at nucleotide 62. For the CP isomer with the 5′ end at nucleotide 240, k_{cat} was, however, 5.5-fold slower. Since CPA only selects for substrate binding at equilibrium, the method cannot separate CP isomers with slower k_{cat}.

DISCUSSION

We have described the effect of backbone breakage on the substrate binding of the catalytic RNA from *B. subtilis* RNAse P by circular permutation analysis. Similar to the folding of yeast $tRNA^{Phe}$ (Pan et al., 1991), many phos-

phodiester positions in P RNA can be broken with little effect on binding. This result confirms once again that the energy of RNA folding is sufficiently high to compensate for single phosphodiester bond cleavage. Many permissive breaks occur in several helical stem-loops that are present in all P RNAs but contain few conserved nucleotides. These include P1, P3, P9, P12, and their corresponding loops. Thermodynamic studies of co-axial stacked helices show that a single break in a RNA helix can notably increase the stability of the helical structure. Such a break in the helix may provide flexibility for the RNA to adapt more stable conformations (Walter et al., 1994). Thus, despite a break in these helices of P RNA, the helical structures are likely to be maintained. As expected, breaks in the nonconserved stem-loops P10.1 and P19 have little effect on substrate binding. However, a large number of breaks in the structural elements of P5.1, J5.1/7, and P15.1, which are not present in many other P RNAs, e.g., *E. coli* P RNA, cause notable decrease in the binding affinity. It has been postulated that, in P RNAs containing P5.1 and J5.1/7, a structural domain is formed by these stem-loops together with P5, P7, J5/15, and P15 (Haas et al., 1991). In P RNAs without P5.1 and J5.1/7, these structural elements are substituted by three other helical structures, P6, P16, and P17 (Haas et al., 1994). Our findings are consistent with this proposal, and they suggest that perhaps P15.1 is also a part of this structural domain.

Many breaks with severe effects on binding are located in the regions of conserved sequences (J3/4, most of P4, J18/2). Phylogenetic conservation of these nucleotides suggests that they play crucial roles in the structure and function of P RNA (Haas et al., 1994). Disruption of a phosphodiester bond in these regions is likely to result in misfolded CP isomers. A second group of breaks with strong effects are located in the regions known to interact with the pre-tRNA substrate in biochemical experiments. They include nucleotides 253−261 in loop L15, which directly interact with the 3′-CCA nucleotides in tRNA (LaGrandeur et al., 1994). Nucleotide 91 appears to be directly involved in substrate binding; this nucleotide in *E. coli* P RNA can be UV cross-linked to the substrate (Guerrier-Takada et al., 1989). Since CPA is supposed to identify backbone positions that play a role in substrate binding, breaking the backbone in regions that directly contact the substrate is expected to decrease binding. Therefore, these positions can be considered as important controls for the success of our CPA experiment. It is interesting to note that several breaks in the 3′ half of conserved stem-loop P4 have little effect, while breaks in the 5′ half or in the complementary strand substantially decrease the affinity of P RNA in binding. Such a polarity may indicate some asymmetry in the structural or functional requirement of this helix.

In addition to J3/4 and J18/2, the loops of J11/12 and J12/11 also contain a large number of phylogenetically conserved nucleotides. However, far fewer breaks in J11/12 and J12/11 have large effects on binding (Figure 4). Such a contrast to the severe effects of breaking phosphodiester bonds in J3/4 and J18/2 suggests that they play different roles in the function of P RNA. Perhaps the regions of J11/12 and J12/11 fold into a much more stable structure to overcome the effect of single backbone breaks. Another possible scenario is that residues in J11/12 and J12/11 are more involved in the chemical step of the cleavage reaction rather than in substrate binding. A third possibility is that the regions of J11/12 and J12/11 primarily provide the template for the protein component required for RNAse P activity *in vivo*. Indeed, part of the equivalent loop of J12/11 in *E. coli* P RNA becomes protected against chemical and enzymatic modification in the presence of *E. coli* RNAse P protein (Vioque et al., 1988). It will be interesting to perform CPA with an eubacterial RNAse P protein to test this hypothesis.

If the K_Ms of the two individual CP isomers can be taken as a guide, then the relative effects on binding for all CP isomers are not very high. Even in the worst cases, this effect amounts to less than an order of magnitude (Table 1 and Figure 4). It is reassuring to find that, in several circularly permuted *E. coli* P RNAs, backbone breaks only cause decrease in substrate binding of less than an order of magnitude (Harris et al., 1994). Perhaps changing a 5′,3′-phosphodiester bond to a 2′,3′-cyclic phosphate and a 5′-hydroxyl group would not destabilize the P RNA to the extent that the resulting CP isomer is completely inactive. In the CPA experiment of the bacteriophage R17 coat protein binding site which consists of a bulged hairpin loop, the maximum effect of a single backbone break on RNA folding was estimated to be two orders of magnitude (Gott et al., 1993). If we take this value as the average for a RNA with a single stem-loop structure, folding of the P RNA, a RNA with more than 18 helices and corresponding loops, should be able to compensate for the loss of free energy from a single backbone break to a larger extent. To carry these results one step further, it is tempting to suggest that backbone breaks in catalytic RNAs of comparable complexity will also cause decrease in substrate binding by less than an order of magnitude. It should be noted that such a prediction can only be applied to the property of substrate binding. It gives no information on how the CP isomers are folded in the absence of substrate nor on the effect of backbone breaks on the chemical step. Most of the known CP *E. coli* P RNAs, however, appear to fold correctly in the absence of substrate (Harris et al., 1994).

The method we have used here enables physical separation of active ribozymes from a mixture of RNA molecules in an intermolecular fashion. In principle, the method can be easily adopted for *in vitro* selection of catalytic RNAs that work in *trans* with respect to their substrates. *In vitro* selection of active P RNA sequence variants is now in progress to locate nucleotide residues in P RNA important for interacting with the pre-tRNA substrate.

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