Modular construction for function of a ribonucleoprotein enzyme: the catalytic domain of *Bacillus subtilis* RNase P complexed with *B.subtilis* RNase P protein

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ABSTRACT

The bacterial RNase P holoenzyme catalyzes the formation of the mature 5′-end of tRNAs and is composed of an RNA and a protein subunit. Among the two folding domains of the RNase P RNA, the catalytic domain (C-domain) contains the active site of this ribozyme. We investigated specific binding of the *Bacillus subtilis* C-domain with the *B.subtilis* RNase P protein and examined the catalytic activity of this C-domain–P protein complex. The C-domain forms a specific complex with the P protein with a binding constant of ~0.1 μM. The C-domain–P protein complex and the holoenzyme are equally efficient in cleaving single-stranded RNA (~0.9 min^{-1} at pH 7.8) and substrates with a hairpin–loop 3′ to the cleavage site (~40 min^{-1}). The holoenzyme reaction is much more efficient with a pre-tRNA substrate, binding at least 100-fold better and cleaving 10–500 times more efficiently. These results demonstrate that the RNase P holoenzyme is functionally constructed in three parts. The catalytic domain alone contains the active site, but has little specificity and affinity for most substrates. The specificity and affinity for the substrate is generated by either the specificity domain of RNase P RNA binding to a T stem–loop-like hairpin or RNase P protein binding to a single-stranded RNA. This modular construction may be exploited to obtain RNase P-based ribonucleoprotein complexes with altered substrate specificity.
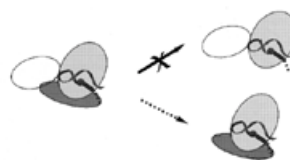
INTRODUCTION

Bacterial RNase P is responsible for the generation of the mature 5′-ends of all tRNAs in the cell (1,2). The RNase P holoenzyme is composed of an RNA of ~330–420 nt and a protein of ~120 amino acids. The *Bacillus subtilis* RNase P RNA (P RNA) contains two folding domains that also have distinct functions (3–6): one binds the T stem–loop region of a pre-tRNA substrate (the specificity domain or S-domain) and the other contains the active site (the catalytic domain or C-domain). Previous work has identified the primary function of the S-domain as providing specificity and affinity to this ribozyme reaction.

The P protein in the holoenzyme has been shown to bind a single-stranded region in the 5′-leader of a pre-tRNA substrate (7). This ability of P protein binding to a single-stranded RNA turns the holoenzyme into an excellent enzyme that cleaves a wide range of substrates as simple as single-stranded RNA (8). In previous studies on the holoenzyme it was thought that both domains of P RNA are required for P protein binding to P RNA (9–11). Therefore, it is not known how the holoenzyme is partitioned functionally.

This work demonstrates that the catalytic domain of *B.subtilis* P RNA alone forms a specific complex with *B.subtilis* P protein and that the C-domain–P protein complex is a very efficient enzyme (Fig. 1). The C-domain–P protein complex cleaves single-stranded and hairpin–loop RNA substrates with the same efficiency as the holoenzyme. For cleavage of a pre-tRNA substrate the holoenzyme is much more efficient and much less sensitive to variations in the 5′-leader length/sequence. These results indicate that a ribonucleoprotein enzyme as exemplified by the RNase P holoenzyme is functionally constructed in multiple parts.
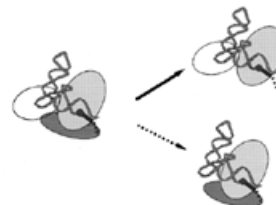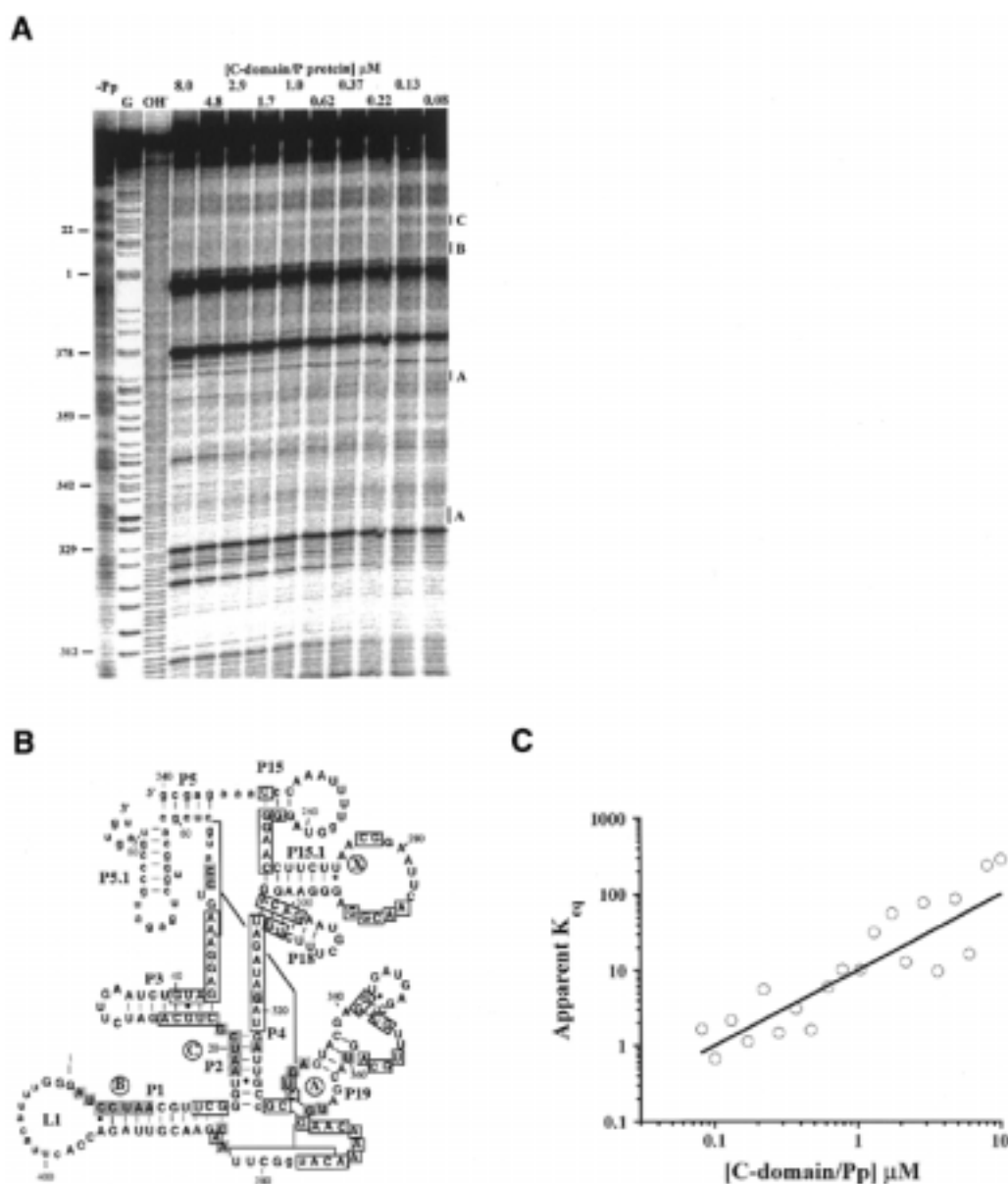

Figure 1. Effects of P protein depletion [previous results summarized in Loria and Pan (19)] (solid arrows) or S-domain deletion (this work, dashed arrows) on catalytic activity. (**A**) P RNA cleaves a single-stranded or a hairpin–loop substrate at least 10 000-fold less efficiently under high salt conditions (data not shown). In contrast, the C-domain–P protein complex has the same catalytic efficiency for a hairpin–loop or a single-stranded RNA substrate as the holoenzyme. (**B**) P RNA effectively binds and cleaves a pre-tRNA substrate. The C-domain–P protein complex also binds and cleaves a pre-tRNA substrate, but less efficiently than a hairpin–loop substrate.

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Figure 2. The *B.subtilis* C-domain forms a specific complex with the *B.subtilis* P protein in the absence of substrate. (**A**) Hydroxyl radical protection at varying concentrations of C-domain and P protein, always at a 1:1 molar ratio. All reactions contained 20 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$ and the reaction time was 30 min at 37°C. G, partial nuclease T1 digestion; OH⁻, partial alkaline hydrolysis; −Pp, 8 µM C-domain alone. (**B**) The protected residues upon P protein binding superimposed on the phylogenetically derived secondary structure (33). Only residues that have protection factors of >1.5 are considered to be protected. Residues protected upon P protein binding are shaded. Residues protected upon Mg^{2+}-induced folding are boxed (5). Region X (residues 270−280) was only protected in the holoenzyme (11). Nucleotides shown in lower case could not be analyzed either due to gel resolution or due to autolytic cleavage (around nt 405). (**C**) Determination of the binding constant of the C-domain−P protein complex [apparent $K_{eq} = y/(1 − y)^2$, curve fitted to equation **1b**]. The protection factors (*PF*) for regions A−C are averaged. The fraction of C-domain bound with P protein corresponds to 1/*PF* and is plotted against the C-domain and P protein concentration, always at a 1:1 molar ratio.

MATERIALS AND METHODS

Preparation of the RNA and P protein

The C-domain of the *B.subtilis* P RNA, containing nt 240–409 + 1–85, was constructed as described (5). The RNA was prepared by the standard *in vitro* transcription method using T7 RNA polymerase (12). The single-stranded and hairpin–loop substrates were chemically synthesized using 2′-ortho-ester-protected phosphoramidites (Dharmacon Research, Boulder, CO). The pre-tRNA substrates were prepared by enzymatic ligation (13) of a synthetic oligonucleotide to an RNA transcript composed of nt 10–76 of yeast tRNA^{Phe} (14). The *B.subtilis* P protein was prepared by overexpression as described by Fierke and co-workers (15).

Hydroxyl radical protection of the C-domain–P protein complex

Hydroxyl radical protection was carried out using the standard Fe(II)–EDTA method in 20 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$ at 37°C (16,17). Briefly, the C-domain alone in buffer was heated at 85°C for 2 min, followed by incubation for 3 min at room temperature. Mg^{2+} was added to designated concentrations. The mixture was incubated for 5 min at 50°C. An equimolar ratio of P protein was added. The mixture was further incubated for 5 min at 37°C. Ascorbic acid and dithiothreitol were added to final concentrations of 1 and 5 mM, respectively. The reaction was immediately initiated by addition of 1 mM Fe(II), 1.2 mM EDTA. The reaction proceeded for 30 min at 37°C and was quenched upon addition of 10 mM thiourea. The reaction mixture was separated on polyacrylamide gels containing 7 M urea and the hydroxyl radical cleavage products quantitated by phosphorimaging.

In this work the C-domain and P protein were always kept at a 1:1 molar ratio to prevent non-specific binding by P protein. Under these conditions the fraction of the C-domain protected (y) as a function of total RNA concentration (x) can be described by applying equilibria equations:

$$y = 1 + K_d/2x - \sqrt{(K_d^2/4x^2 + K_d/x)} \qquad \textbf{1a}$$

where K_d is the binding constant of the complex. Another way to present this type of data is to plot the apparent equilibrium constant K_{eq} [$= y/(1 - y)^2$] as a function of total RNA concentration:

$$y/(1 - y)^2 = K_{eq} = x/K_d \qquad \textbf{1b}$$

Kinetics of the cleavage reaction

All reactions were performed under single turnover conditions at 0.02–25 μM enzyme and <2 nM ^{32}P-labeled substrate. The C-domain–P protein complex was reconstituted as described above. The 5′-^{32}P-labeled RNA substrates were renatured as described previously (14). The cleavage reaction was initiated upon mixing an equal volume of the ribozyme and the substrate. Aliquots were taken at designated time points and mixed with an excess of 9 M urea, 100 mM EDTA to stop the cleavage reaction. The reaction products were separated from the unreacted substrates on denaturing gels containing 7 M urea. The amounts of products and substrates were determined by phosphorimaging using a Fuji Phosphorimager. Reaction rates were obtained by fitting the amount of cleavage products over time to a single exponential.

RESULTS AND DISCUSSION

The C-domain forms a specific complex with P protein

Hydroxyl radical protection was performed to determine whether the C-domain forms a specific complex with P protein (Fig. 2). Hydroxyl radicals cleave the solvent-accessible ribose–phosphate positions in RNA. Extensive protection was observed upon folding of the C-domain alone (Fig. 2A, lane –Pp). Since the P protein has a strong tendency to bind RNA non-specifically (10,11,15,18), a 1:1 molar ratio of C-domain and P protein was maintained to minimize protections arising from non-specific binding by P protein (data not shown).

Additional protections at specific regions in the C-domain are observed upon addition of the P protein (Fig. 2A, compare lane –Pp or 0.08 with 8; Fig. 2B, shaded nucleotides). Because it is not possible to distinguish direct protein contacts and conformational changes in the C-domain on the basis of footprinting alone, only regions that had more than five protected residues are further discussed. Protected regions A and C are present in the C-domain–P protein complex and in the holo-enzyme (11), suggesting that they are contact sites for P protein. Region B in the C-domain–P protein complex could
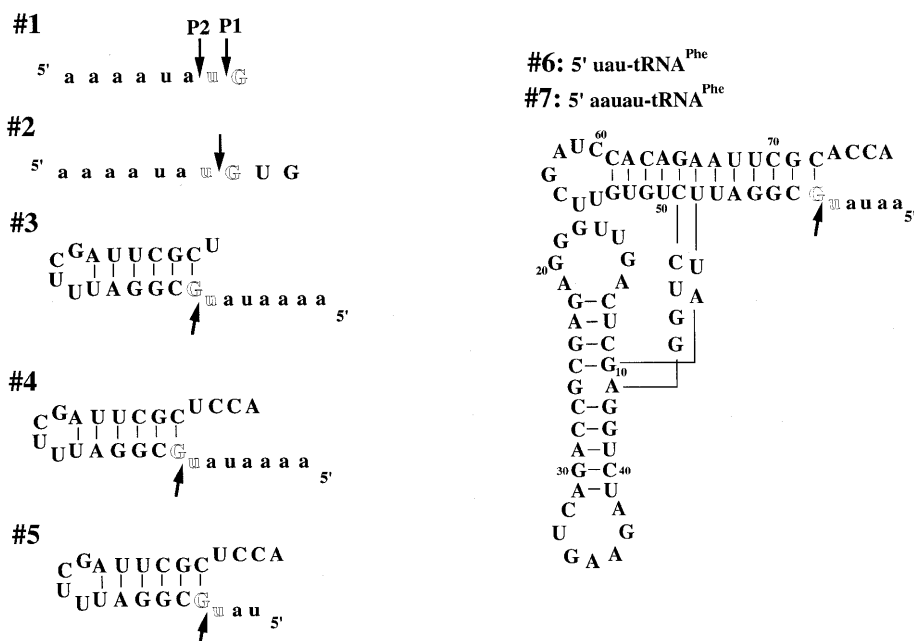
Figure 3. Substrates used in this study with the intended cleavage site shown between the highlighted residues. The observed cleavage sites are indicated by arrows. The 5′-leader regions are shown in lower case.

Table 1. Catalysis by the C-domain–P protein complex

Substrate	k_{cl} (min^{-1})[a]	$K_{1/2}$ (μM)[b]
1	0.057 ± 0.002 (P2)	7.1 ± 0.6
	0.012 ± 0.002 (P1)	
2	0.92 ± 0.04	5.6 ± 0.6
3	31 ± 5	4.6 ± 1.8
4	35 ± 3	1.8 ± 0.5
5	17 ± 3	13 ± 4
6	0.90 ± 0.06	1.9 ± 0.4
7	8.1 ± 0.4	0.70 ± 0.15

[a]Cleavage rate at saturating enzyme concentration under single turnover conditions. Conditions for all substrates: 50 mM Tris–HCl pH 7.78, 10 mM MgCl$_2$, 2% glycerol, 37°C.
[b]The enzyme concentration at $k_{cl}/2$.

Figure 4. (**A**) Cleavage of 5′-^{32}P-labeled substrates 1, 2 and 4 by 1 μM holoenzyme (lane 1) and 1 μM C-domain–P protein complex (lane 2) under single turnover conditions. The control reaction was performed with 1 μM P protein alone (lane 3). All reactions contained 20 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$, 2% glycerol. (**B**) pH dependence of the cleavage rate at saturating concentrations of the C-domain–P protein complex (>10 μM) for substrates 2, 4, 6 and 7. The pH dependencies for cleavage of substrates 2 and 4 have slopes of 1.1 and 0.6 for the C-domain–P protein complex, similar to those for the holoenzyme (1.0 for substrate 2 and 0.7 for substrate 4; 8).

not be analyzed in the holoenzyme due to its proximity to the 5′-end of P RNA. Region X (residues 270–280) was protected in the holoenzyme, but no protection was present in the C-domain–P protein complex. Therefore, protection of region X in the holoenzyme is likely derived from either interdomain RNA–RNA interactions or from additional protein binding that can only occur in the presence of the S-domain.
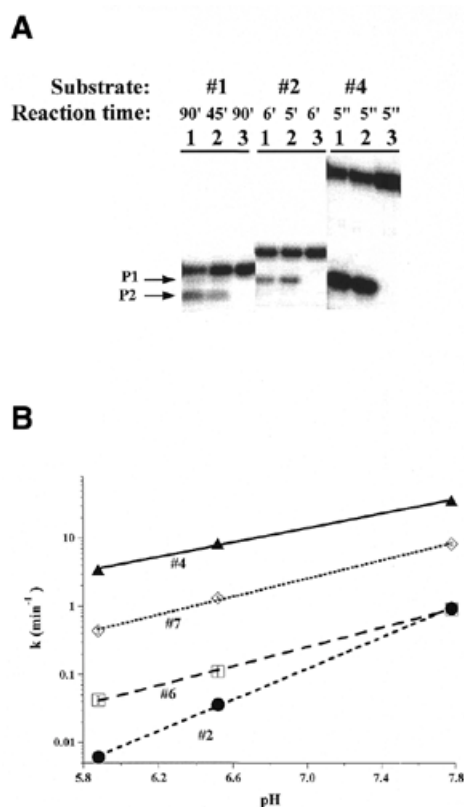
The binding constant (K_d) of the C-domain–P protein complex can be obtained from the extent of protection as a function of C-domain and P protein concentration (Fig. 2C, where the slope = $1/K_d$). Because the RNA and protein are always equimolar, the fraction of C-domain bound with P protein is fitted by **1b**, assuming that the complex contains one C-domain and one P protein subunit. The K_d of the C-domain–P protein complex is 0.099 ± 0.015 μM, ~200-fold weaker than the K_d of the holoenzyme (table 3 in Talbot and Altman; 18).

In summary, the C-domain forms a specific complex with the P protein. Compared to the holoenzyme, this specific C-domain–P protein complex has decreased regions of protection and lower binding affinity.

Catalytic activity of the C-domain–P protein complex

The catalytic activity of the C-domain–P protein complex was demonstrated briefly in a previous study but no detailed analyses were carried out (19). In order to compare substrate recognition and catalytic efficiency of the C-domain–P RNA complex and the holoenzyme, cleavage of seven substrates was determined (Fig. 3 and Table 1). All seven substrates had similar 5′-leader sequences but different RNA structure on the 3′-side of the cleavage site: single-stranded (1 and 2), hairpin–loop (3–5) or tRNA (6 and 7).
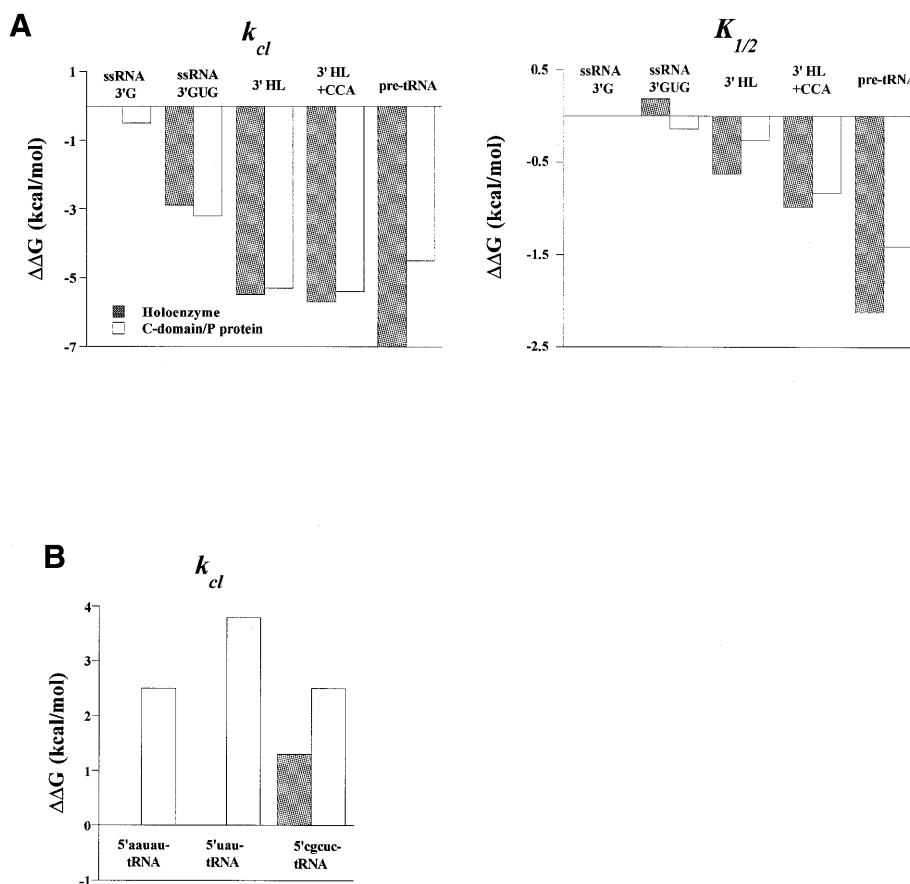
The C-domain–P protein complex accurately and efficiently cleaved all seven substrates (Fig. 4 and Table 1). Catalysis by the C-domain–P protein complex and by the holoenzyme had remarkable similarities. Both complexes cleaved all substrates at the same sites (see for example Fig. 4A), suggesting similar specificities in substrate binding, and showed the same pH dependencies (see for example Fig. 4B), suggesting a similar rate limiting step in the catalysis. Both the cleavage rate (k_{cl}) and the ribozyme concentration at $k_{cl}/2$ ($K_{1/2}$) are within 2-fold ($\Delta\Delta G < 0.4$ kcal/mol) for the single-stranded and the hairpin–loop substrates (Fig. 5A).

The C-domain–P protein complex cleaved pre-tRNA substrates significantly less efficiently compared to the holoenzyme (Fig. 5B). The quantitative difference in the cleavage rates, however, strongly depended on the length and sequence of the 5′-leader. When the 5′-leader was 5′-aauau (substrate 7) the holoenzyme had an ~50-fold faster cleavage rate ($\Delta\Delta G \approx$ 2.4 kcal/mol). Truncation of 5′-aauau to 5′-uau (substrate 6) made no difference for the holoenzyme reaction, but decreased the cleavage rate by the C-domain–P protein ~10-fold ($\Delta\Delta G \approx$ 3.8 kcal/mol). Changing 5′-aauau to 5′-cgcuc decreased the cleavage rate by the holoenzyme ~8-fold, but had no effect on the C-domain–P protein reaction ($\Delta\Delta G \approx 1.2$ kcal/mol). These results show that holoenzyme interactions with the T stem–loop of the tRNA generate faster rates, less sensitivity to the 5′-leader length and a stronger preference for an A/U-rich 5′-leader sequence. This result is consistent with a preference for the 5′-leader sequence and length of the natural *B.subtilis* tRNA precursors (20).

As for binding of pre-tRNA substrates, the $K_{1/2}$ values measured in Mg^{2+} from single turnover reactions did not approximate the binding constant as determined by pulse–chase experiments (8; data not shown). However, the $K_{1/2}$ values

Figure 5. (**A**) Changes in the cleavage rate (k_{cl}) and Michaelis constant ($K_{1/2}$) upon successive addition of RNA structure/nucleotides 3′ to the cleavage site. The holoenzyme data are taken from Loria and Pan (8). The $\Delta\Delta G$ values [left, $\Delta\Delta G = -RT \ln(k_{2-7}/k_{1(P1)})$; right, $\Delta\Delta G = -RT \ln(K_{2-7}/K_1)$] are normalized to the cleavage site P1 of substrate 1 in the holoenzyme reaction. The pre-tRNA substrate data is for substrate 7, 5′-aauau-tRNA[Phe]. (**B**) Changes in the cleavage rate upon altering the 5′-leader of pre-tRNA[Phe] substrates. The $\Delta\Delta G$ values are normalized to 5′-aauau-tRNA[Phe] in the holoenzyme reaction. The data for the 5′-cgcuc-tRNA[Phe] are taken from Loria and Pan (19).

measured from single turnover reactions carried out in Ca^{2+} can be used to approximate the binding constant of a pre-tRNA substrate. Compared to the holoenzyme, the C-domain–P protein complex bound the 5′-aauau-tRNA substrate at least 100-fold weaker in Ca^{2+} (data not shown).

In summary, the S-domain–T stem–loop interaction increased the binding affinity of a pre-tRNA by >100-fold and increased the cleavage rate by up to 500-fold. The catalytic activities of the C-domain–P protein complex and the holoenzyme were essentially identical for any other substrate that only interacts with the C-domain and the P protein.

Modular construction for function of a ribonucleoprotein enzyme

This work shows that bacterial RNase P is a modular enzyme, functionally partitioned among the two P RNA domains and the P protein. The catalytic domain contains the active site and is a very efficient ribozyme for substrates that can interact with the L1 loop (21), although it cleaves pre-tRNA and other substrates very poorly. The specificity domain contains the binding site for a specific hairpin–loop, e.g. the T stem–loop in tRNA or hairpin–loops in several selected substrates (22), and

it can specifically bind a pre-tRNA substrate at micromolar affinity (unpublished results). The P protein contains a substrate-binding site for single-stranded RNA plus two other potential binding sites, presumably for binding to P RNA (23).

Starting from the C-domain, the addition of either the S-domain or the P protein or both produces different enzymes. The C-domain linked with the S-domain is P RNA, a ribozyme capable of specific recognition and cleavage of a pre-tRNA substrate, but works very poorly with single-stranded and hairpin–loop substrates (Fig. 1A and B, top). For P RNA the efficiency and specificity is conferred by the S-domain interaction with a portion of the substrate 3′ to the cleavage site. Association of the C-domain and P protein produces the C-domain–P protein complex, a ribonucleoprotein enzyme capable of cleaving single-stranded and hairpin–loop substrates but which cannot distinguish pre-tRNA from hairpin–loop substrates (Fig. 1A and B, bottom). For the C-domain–P protein complex the efficiency and a large portion of the specificity is conferred by the P protein interaction with a portion of the substrate 5′ to the cleavage site. The desired specificity and catalytic efficiency for a pre-tRNA substrate is only achieved by combination of all three modules to produce the holoenzyme.

Our results establish a parallel for ribonucleoprotein and protein enzymes. Examples of functional division in protein enzymes abound. The restriction enzyme *Fok*I contains two domains: one responsible for specific binding of its recognition site, the other responsible for endonucleolytic cleavage 5–9 bp from the recognition site (24,25). Many aminoacyl-tRNA synthetases contain more than one domain: one responsible for recognition of anticodon nucleotides, the other responsible for aminoacylation (26–31). The Tn*5* transposase contains three domains: one binds to DNA, another cleaves the DNA strand and the third is responsible for protein–protein contacts in the synaptic complex (32).

The modular construction of RNase P function may be exploited to obtain RNase P-based ribonucleoprotein enzymes with altered specificity. We have previously demonstrated that the S-domain can be substituted by a randomly chosen RNA structure and that this altered ribozyme efficiently cleaves only RNA substrates that contain matching structures to the S-domain replacement (6). Likewise, the sequence of the 5′-leader binding-site in the P protein may be changed and a C-domain–altered P protein complex may efficiently cleave only substrates that contain matching structures to the altered binding site in the P protein mutant.

ACKNOWLEDGEMENTS

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Figure 2. The *B.subtilis* C-domain forms a specific complex with the *B.subtilis* P protein in the absence of substrate. (**A**) Hydroxyl radical protection at varying concentrations of C-domain and P protein, always at a 1:1 molar ratio. All reactions contained 20 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$ and the reaction time was 30 min at 37°C. G, partial nuclease T1 digestion; OH⁻, partial alkaline hydrolysis; −Pp, 8 µM C-domain alone. (**B**) The protected residues upon P protein binding superimposed on the phylogenetically derived secondary structure (33). Only residues that have protection factors of >1.5 are considered to be protected. Residues protected upon P protein binding are shaded. Residues protected upon Mg^{2+}-induced folding are boxed (5). Region X (residues 270−280) was only protected in the holoenzyme (11). Nucleotides shown in lower case could not be analyzed either due to gel resolution or due to autolytic cleavage (around nt 405). (**C**) Determination of the binding constant of the C-domain−P protein complex [apparent $K_{eq} = y/(1 − y)^2$, curve fitted to equation **1b**]. The protection factors (*PF*) for regions A−C are averaged. The fraction of C-domain bound with P protein corresponds to 1/*PF* and is plotted against the C-domain and P protein concentration, always at a 1:1 molar ratio.

Figure 3. Substrates used in this study with the intended cleavage site shown between the highlighted residues. The observed cleavage sites are indicated by arrows. The 5′-leader regions are shown in lower case.

Figure 4. (**A**) Cleavage of 5′-^{32}P-labeled substrates 1, 2 and 4 by 1 µM holoenzyme (lane 1) and 1 µM C-domain–P protein complex (lane 2) under single turnover conditions. The control reaction was performed with 1 µM P protein alone (lane 3). All reactions contained 20 mM Tris−HCl pH 7.5, 10 mM MgCl$_2$, 2% glycerol. (**B**) pH dependence of the cleavage rate at saturating concentrations of the C-domain−P protein complex (>10 µM) for substrates 2, 4, 6 and 7. The pH dependencies for cleavage of substrates 2 and 4 have slopes of 1.1 and 0.6 for the C-domain−P protein complex, similar to those for the holoenzyme (1.0 for substrate 2 and 0.7 for substrate 4; 8).

Figure 5. (**A**) Changes in the cleavage rate (k_{cl}) and Michaelis constant ($K_{1/2}$) upon successive addition of RNA structure/nucleotides 3′ to the cleavage site. The holoenzyme data are taken from Loria and Pan (8). The $\Delta\Delta G$ values [left, $\Delta\Delta G = -RT \ln(k_{2-7}/k_{1(P1)})$; right, $\Delta\Delta G = -RT \ln(K_{2-7}/K_1)$] are normalized to the cleavage site P1 of substrate 1 in the holoenzyme reaction. The pre-tRNA substrate data is for substrate 7, 5′-aauau-tRNA^{Phe}. (**B**) Changes in the cleavage rate upon altering the 5′-leader of pre-tRNA^{Phe} substrates. The $\Delta\Delta G$ values are normalized to 5′-aauau-tRNA^{Phe} in the holoenzyme reaction. The data for the 5′-cgcuc-tRNA^{Phe} are taken from Loria and Pan (19).

Table 1. Catalysis by the C-domain–P protein complex

Substrate	k_{cl} (min^{-1})[a]	$K_{1/2}$ (µM)[b]
1	0.057 ± 0.002 (P2)	7.1 ± 0.6
	0.012 ± 0.002 (P1)	
2	0.92 ± 0.04	5.6 ± 0.6
3	31 ± 5	4.6 ± 1.8
4	35 ± 3	1.8 ± 0.5
5	17 ± 3	13 ± 4
6	0.90 ± 0.06	1.9 ± 0.4
7	8.1 ± 0.4	0.70 ± 0.15

[a]Cleavage rate at saturating enzyme concentration under single turnover conditions. Conditions for all substrates: 50 mM Tris–HCl pH 7.78, 10 mM MgCl$_2$, 2% glycerol, 37°C.
[b]The enzyme concentration at $k_{cl}/2$.