REPORT

Helix P4 is a divalent metal ion binding site in the conserved core of the ribonuclease P ribozyme

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ABSTRACT

The ribonuclease P ribozyme (RNase P RNA), like other large ribozymes, requires magnesium ions for folding and catalytic function; however, specific sites of metal ion coordination in RNase P RNA are not well defined. To identify and characterize individual nucleotide functional groups in the RNase P ribozyme that participate in catalytic function, we employed self-cleaving ribozyme–substrate conjugates that facilitate measurement of the effects of individual functional group modifications. The self-cleavage rates and pH dependence of two different ribozyme–substrate conjugates were determined and found to be similar to the single turnover kinetics of the native ribozyme. Using site-specific phosphorothioate substitutions, we provide evidence for metal ion coordination at the pro-P phosphate oxygen of A67, in the highly conserved helix P4, that was previously suggested by modification-interference experiments. In addition, we detect a new metal ion coordination site at the pro-S phosphate oxygen of A67. These findings, in combination with the proximity of A67 to the pre-tRNA cleavage site, support the conclusion that an important role of helix P4 in the RNase P ribozyme is to position divalent metal ions that are required for catalysis.

Keywords: metal ion coordination; phosphorothioate; ribozyme; RNase P RNA

INTRODUCTION

Like many protein enzymes, large ribozymes (group I and group II introns and ribonuclease P [RNase P]) utilize magnesium ions to catalyze transesterification and phosphodiester bond hydrolysis (Pyle, 1996). For both RNA and protein enzymes, the ability of divalent metal ions, other than magnesium, to rescue the deleterious effects of phosphorothioate modifications provides a biochemical signature for metal ion interactions with phosphate oxygens (Knowles, 1980; Eckstein, 1985; Narlikar & Hershlag, 1997). This approach, combined with analysis of the reaction kinetics of modified RNAs, has been particularly useful for elucidating how magnesium ions are coordinated to substrate phosphates in ribozyme-catalyzed reactions (e.g., Piccirilli et al., 1993; Warnecke et al., 1996; Weinstein et al., 1997; Scott & Uhlenbeck, 1999; Shan et al., 1999; Yoshida et al., 1999). In contrast to the detailed perspective on interactions of metal ions at scissile phosphates, less is known about the coordination of essential divalent metal ions within the catalytic centers of the large ribozymes themselves.

Ribonuclease P is a widespread and essential ribonucleoprotein endonuclease that generates the mature tRNA 5’ end during tRNA maturation (for reviews, see Frank & Pace, 1998; Altman & Kirsebom, 1999). The RNA subunits from many bacterial RNase P enzymes are catalytic, and can bind to pre-tRNA and catalyze hydrolysis at the appropriate phosphodiester bond in the absence of protein in vitro, and so comprise one class of large ribozymes (Guerrier-Takada et al., 1983). Optimal substrate binding in vitro requires a small RNase P-associated protein or elevated concentrations of monovalent ions (Reich et al., 1988; Kurz et al., 1998). However, the RNase P ribozyme, whether in the presence or absence of its associated protein, requires millimolar concentrations of magnesium to catalyze phosphodiester bond hydrolysis (Guerrier-Takada et al., 1986; Smith et al., 1992; Beebe et al., 1996).

Like group I and group II introns, the RNase P ribozyme has a dual requirement for magnesium ions. Magnesium is essential for folding into the active conformation (Pan, 1995; Zarrinkar et al., 1996; Pan & Sosnick, 1997), and is also likely to participate directly in catalysis (Guerrier-Takada et al., 1986; Smith & Pace, 1988).
The RNase P ribozyme is composed of two domains that can fold independently (Pan, 1995; Loria & Pan, 1996). The S-, or specificity, domain is composed of helices P7–P14 and contacts the T-stem of the pre-tRNA substrate, as demonstrated by kinetic (Pan et al., 1995; Loria & Pan, 1998) and photocrosslinking studies (Nolan et al., 1993; Chen et al., 1998). The C-, or catalytic, domain is composed of helices P1–6 and P15–18, and contains the majority of nucleotide positions that are conserved among all known RNase P RNAs (Chen & Pace, 1997). The C-domain forms efficient photocrosslinks to photoagents positioned at the pre-tRNA cleavage site (Burgin & Pace, 1990; Kufel & Kirsebom, 1996; Christian & Harris, 1999). In addition, the binding site for the substrate 3′ CCA sequence is located in a region of non-Watson–Crick base pairing included in the C-domain (Kirsebom & Svard, 1994). Also, the C-domain alone can catalyze the cleavage of RNA substrates selected in vitro (Pan & Jakacka, 1996). Taken together these results demonstrate that the C-domain contains the active site of the native ribozyme. However, the individual chemical groups that comprise the active site and the roles they play are not yet defined.

Helix P4, located in the C-domain of the ribozyme, has been suggested to contain functional groups and sites of metal ion coordination central to ribozyme activity. This suggestion is based in part on phylogenetic comparative analysis of RNase P RNA sequences that demonstrated that nucleotides in helix P4, and adjoining single-stranded regions, are universally conserved in bacterial, archael, and eukaryal RNase P RNAs (Chen & Pace, 1997). In addition, nucleotide analog interference experiments identified functional groups in P4 that are important for both substrate binding and catalysis (Hardt et al., 1995, 1996; Harris & Pace, 1995; Kazantsev & Pace, 1998; Heide et al., 1999; Siew et al., 1999). Preliminary evidence for thiophilic metal ion rescue of phosphorothioate effects in P4 (Hardt et al., 1995; Harris & Pace, 1995) and P15 (Kufel & Kirsebom, 1998) have been reported. However, the high concentrations of thiophilic metal ions used in these experiments or the lack of a quantitative assessment of the effects makes a definitive interpretation difficult.

To examine more closely the role of nonbridging phosphate oxygens in helix P4, we measured the effects of site-specific phosphorothioate substitution, focusing on positions that displayed large modification-interference effects on the cleavage reaction (Harris & Pace, 1995). In addition, we examined the effect of sulfur substitution of the corresponding pro-Sp nonbridging phosphate oxygens that are not generally analyzed by conventional modification-interference. These studies provide, for the first time, a quantitative assessment of phosphorothioate interference effects in the universally conserved core of the RNase P ribozyme. Importantly, we report confirmation of one previously suggested position of metal ion coordination and evidence for a new site of metal ion coordination with a substantially larger thiophilic metal rescue of the phosphorothioate-induced defect on catalysis. The current work, taken together with available biochemical and phylogenetic information, provides direct evidence that helix P4 is an important divalent metal ion binding site in the conserved core of the RNase P ribozyme.

RESULTS AND DISCUSSION

In the current analysis, the use of two different self-cleaving ribozyme constructs [PT50 and PT332(-5)], facilitated site-specific functional group modifications in helix P4 (Fig. 1). These self-cleaving constructs allow site-specific functional group modifications to be introduced into the conserved core of the ribozyme by ligation of short synthetic oligonucleotides (Moore & Sharp, 1992). Importantly, these reagents make possible the kinetic characterization of modified ribozymes that, in a number of cases, could not be obtained in sufficient quantities to be analyzed under standard single-turnover conditions. PT50 and PT332(-5) contain tRNA sequences appended to circularly permuted RNase P RNAs. The design of PT50 and PT332(-5) is based on structural analyses demonstrating that nucleotide −10 in the pre-tRNA 5′ leader sequence is adjacent to helix P3 (Christian & Harris, 1999), and that positions −2 to −5 in the pre-tRNA 5′ leader sequence are adjacent to J18/2 of the RNase P ribozyme (Burgin & Pace, 1990). Modifications to the 5′ half of helix P4 were introduced into PT50, whereas those adjacent to the 3′ half of helix P4 were incorporated into PT332(-5).

Effective interpretation of site-specific functional group modification effects on ribozyme catalysis requires an understanding of the reaction kinetics of the RNA under investigation. We therefore defined reaction conditions (25 mM Mg2+, 50 mM PIPES, 3 M NaCl, 0.1 mM EDTA, pH 5.5, 50 °C) under which the PT50 and PT332(-5) self-cleavage reactions reflect the single-turnover kinetic behavior of the native ribozyme. PT332(-5) was previously shown to accurately cleave the tethered pre-tRNA substrate (Frank et al., 1994). Similarly, intramolecular cleavage of PT50 generates two products: a small fragment with mobility on denaturing gels similar to mature tRNA, and a larger fragment with mobility of RNase P RNA (Fig. 2A). The reaction of PT50 is thus consistent with a single endo-
nucleolytic cleavage at the pre-tRNA cleavage site. To establish the accuracy of PT50 cleavage, a radiolabeled DNA oligonucleotide complementary to the last 20 nt of the tRNA product was annealed to the 3' cleavage fragment. Hybrids were extended by reverse transcriptase, and the extension products were compared to dideoxy sequencing standards derived from primer extension of the uncleaved RNA (Fig. 2B). The run-off primer extension product corresponded in size to that expected for the mature tRNA 5' end, demonstrating that PT50 cleavage occurred at the authentic cleavage site.

A time course of product formation demonstrates that the PT50 and PT332(-5) cleavage reactions are described by a single exponential function (Fig. 2C). The observed pseudo-first-order rate constant for cleavage ($k_{obs}$) of both tethered constructs are similar to each other [0.74 min$^{-1}$ for PT50 and 0.73 min$^{-1}$ for PT332(-5)], and to that catalyzed by native RNase P RNA (0.86 min$^{-1}$). The observed rate constants for PT50 and PT332(-5) reactions are also insensitive to dilution over a broad range of RNA concentrations (0.02–20 nM), indicating that the reactions are genuinely intramolecular in nature (Frank et al., 1994; data not shown). It was shown previously that a plot of log $k_{obs}$ for the native enzyme versus pH is linear between pH 5.5 and pH 7.5 with a slope near one (Smith & Pace, 1993), suggesting that the reaction rate is limited by hydroxide-ion concentration, and that $k_{obs}$ reflects phosphodiester bond hydrolysis. Although the rate of PT332(-5) self-cleavage is known to be sensitive to pH (Kazantsev & Pace, 1998), the precise pH dependence of the reaction was not determined. Importantly, we find that log $k_{obs}$ for both PT332(-5) and PT50 cleavage are also linearly dependent on pH, with slopes similar to that of the native ribozyme (0.88 for PT332(-5) and 0.81 for PT50; Fig. 2D). PT332(-5) and PT50 thus appear to be useful and accurate tools for analysis of native RNase P RNA catalysis.

Using PT50 and PT332(-5) we incorporated site-specific phosphorothioate substitutions at, and adjacent to, positions with dominant phosphorothioate modification-interference effects (Harris & Pace, 1995), reasoning that these functional groups were more likely to play a direct role in the hydrolysis reaction. These substitutions are located in, or adjacent to, the 5' (A65 to U69) or 3' (A352) strands of helix P4 (Fig. 1A), and their effects on ribozyme catalysis are shown in Table 1. The strongest inhibitory effects from pro-RP phosphate oxygen substitutions were observed at A67 and G68, where phosphorothioate modifications resulted in 10,000- and 4,000-fold decreases in $k_{obs}$, respectively. At A352 and U69 there were comparatively smaller, but significant, decreases of 1,500- and 7-fold upon sulfur substitution of the pro-RP nonbridging phosphate oxygen. In addition, we identified two SP phosphorothioate sensitive positions (Table 1). SP phosphorothioate modification at A67 and G68 resulted in 4,000- and 33-fold decreases in $k_{obs}$, whereas no significant effect was observed at A352 or U69. Little (less than three-
fold) or no effect on $k_{\text{obs}}$ was observed for $R_P$ and $S_P$ phosphorothioate modifications at nucleotides A65 and A66, consistent with previous modification-interference studies (Hardt et al., 1995; Harris & Pace, 1995; Table 1). The large phosphorothioate effects observed in this analysis are of the same order of magnitude as those observed at the scissile phosphates of the hammerhead ribozyme (Scott & Uhlenbeck, 1999), group I introns (Piccirilli et al., 1993; Weinstein et al., 1997), and the pre-tRNA substrate of RNase P RNA (Warnecke et al., 1996), and thus are consistent with perturbation of functional groups that may be directly involved in catalytic function.

To determine whether a metal ion binding site was disrupted by phosphorothioate modification in P4, we introduced manganese into reactions to a final concentration of 5 mM along with 20 mM magnesium, to maintain a constant concentration (25 mM) of divalent metal ion. Although most of the positions tested showed less than a threefold increase in $k_{\text{obs}}$ due to the presence of...
manganese, significant rescue (65-fold) of the effect of phosphorothioate modification was observed at the pro-R_p position of A67, confirming the partial rescue observed at this position in modification-interference experiments. Surprisingly, we find a dramatic (5,200-fold) rescue of the newly identified S_p phosphorothioate effect at A67 (Table 1). Notably, under these conditions the rescued rate constant is within experimental error of the unmodified control, and is thus complete. To confirm these effects in the presence of a second thiophilic metal ion, we also examined phosphorothioate rescue in the presence of cadmium. Again, little if any effect is observed with cadmium on the unmodified control, but significant rescue of k_obs was found in ribozymes modified at A67 (Table 1). The metal ion rescue effects that we observe do not appear to be due to indirect stabilization of ribozyme structure by manganese or cadmium, as the changes in rate constants of the phosphorothioate-modified RNAs are much larger than that in the unmodified controls. Taken together, these findings indicate a biochemical signature for coordination of divalent metal ion(s) at A67.

An intriguing difference in the metal ion rescue results reported here compared to analyses in other systems is that both the R_p and S_p phosphorothioate modifications at the same phosphate give rise to metal ion rescues. One possibility for these effects is that two different metal ions are coordinated to the two nonbridging phosphate oxygens of A67. An example of metal ions coordinated to both nonbridging oxygens at the same phosphate is found in an A-rich bulge in the P4–P6 domain of the group I intron from Tetrahymena (Cate et al., 1997). Alternatively, the thiophilic metal rescue effects seen at A67 could be induced. That is, a thiophilic metal ion is “recruited” to A67 when a sulfur atom is incorporated. Although this explanation may account for the partial rescue of the R_p phosphorothioate modification at A67, it is less likely to account for the large and complete rescue of activity we have identified at the pro-S_p phosphate oxygen of A67. It is important to note that the absence of a thiophilic metal ion rescue does not exclude metal ion binding at the other positions where we observe phosphorothioate effects, as disruption of metal ion binding by phosphorothioate modification is not always ameliorated by inclusion of thiophilic metal ions (e.g., Basu & Strobel, 1999; Brautigam & Steitz, 1998).

Interestingly, the site(s) of metal ion binding observed here are adjacent to the pre-tRNA substrate phosphate in the current three-dimensional models of the RNase P ribozyme–substrate complex (Chen et al., 1998; Massire et al., 1998). These models are based on a large collection of phylogenetic comparative and inter- and intramolecular crosslinking data that are sufficient to position the sites of divalent metal ion binding observed here within 10–15 Å (approximately the resolution of the models themselves) of the pre-tRNA cleavage site (Fig. 3C). The positions of metal ion coordination identified here thus appear to be adjacent to functional groups shown to be involved in metal ion binding at the scissile phosphate (Warnecke et al., 1996; Chen et al., 1997). Consistent with a direct involvement of P4 in catalysis, the strong phosphorothioate effects we observe are adjacent to nucleotides conserved in all three phylogenetic kingdoms (Chen & Pace, 1997; Fig. 3A) and are on the same helical face across the major groove of the P4 helix (Harris & Pace, 1995; Fig. 3B). Struc-

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### TABLE 1. Effects of phosphorothioate substitutions on RNase P ribozyme catalysis.

<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>k_obs(Mg)</th>
<th>k_modified/k_unmodified</th>
<th>k_obs(Mn)</th>
<th>k_modified(k_obs(Mg))</th>
<th>k_obs(Cd)</th>
<th>k_modified(k_obs(Mg))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT50</td>
<td>2.0 ± 0.9</td>
<td>1.0</td>
<td>3.7 ± 0.6</td>
<td>1.8</td>
<td>2.1 ± 0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>A65 Rp</td>
<td>1.5 ± 0.1</td>
<td>1.3</td>
<td>1.8 ± 0.2</td>
<td>1.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>A65 Sp</td>
<td>1.3 ± 0.2</td>
<td>1.5</td>
<td>1.9 ± 0.2</td>
<td>1.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>A66 Rp</td>
<td>1.1 ± 0.2</td>
<td>1.8</td>
<td>2.7 ± 1.0</td>
<td>2.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>A66 Sp</td>
<td>1.0 ± 0.4</td>
<td>1.3</td>
<td>3.5 ± 0.4</td>
<td>2.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>A67 Rp</td>
<td>0.00020 ± 0.00009</td>
<td>10000</td>
<td>0.013 ± 0.005</td>
<td>65</td>
<td>0.00093 ± 0.00011</td>
<td>4.7</td>
</tr>
<tr>
<td>A67 Sp</td>
<td>0.0005 ± 0.0003</td>
<td>4000</td>
<td>2.6 ± 1.8</td>
<td>52000</td>
<td>0.11 ± 0.02</td>
<td>220</td>
</tr>
<tr>
<td>G68 Rp</td>
<td>0.0005 ± 0.0003</td>
<td>4000</td>
<td>0.0008 ± 0.0007</td>
<td>0.15</td>
<td>0.0011 ± 0.0006</td>
<td>2.2</td>
</tr>
<tr>
<td>G68 Sp</td>
<td>0.06 ± 0.04</td>
<td>33</td>
<td>0.08 ± 0.04</td>
<td>1.3</td>
<td>0.03 ± 0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>U69 Rp</td>
<td>0.3 ± 0.1</td>
<td>6.7</td>
<td>0.7 ± 0.4</td>
<td>2.3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>U69 Sp</td>
<td>2.5 ± 1.2</td>
<td>0.8</td>
<td>4.8 ± 0.4</td>
<td>1.9</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>PT332(-5)</td>
<td>1.7 ± 0.7</td>
<td>1.2</td>
<td>2.6 ± 0.2</td>
<td>1.5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>A352 Rp</td>
<td>0.0013 ± 0.0006</td>
<td>1500</td>
<td>0.0016 ± 0.0001</td>
<td>1.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>A352 Sp</td>
<td>2.2 ± 0.5</td>
<td>0.9</td>
<td>2.4 ± 2.1</td>
<td>1.1</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Units for k_obs are min⁻¹. Errors are one standard deviation determined from the average of at least three measurements. nd indicates not determined. k_modified indicates the fold change in reaction rate constant in the presence of MgCl₂ and a phosphorothioate substitution. k_unmodified indicates the fold change in reaction rate constant measured in 20 mM MgCl₂ and 5 mM MnCl₂, and k_obs(Mg) indicates the reaction rate constant measured in 20 mM MgCl₂ and 5 mM MnCl₂, and k_obs(Cd) indicates the reaction rate constant measured in 20 mM MgCl₂ and 5 mM CdCl₂. k_modified(k_obs(Mg)) is the fold change in reaction rate constant in the presence of Mn, and k_modified(k_obs(Mg)) is the fold change in reaction rate constant in the presence of Cd.
tural analyses of divalent metal ion binding sites in RNA indicates that the major groove is a common site for magnesium ion coordination (e.g., Cate & Doudna, 1996; Correll et al., 1997), and it is clear that helix junctions and noncanonical base pairs can alter helical geometry sufficiently to allow interactions in the relatively deep and inaccessible major groove of A-form helices (Weeks & Crothers, 1993; Patel, 1999). Major groove recognition is clearly important for binding the guanosine cofactor during group I intron self-splicing (Michel et al., 1989), and Pyle and coworkers have proposed that the major groove of the conserved domain V of group II intron ribozymes may function directly in catalysis (Konforti et al., 1998). Interestingly, in helix P4 the site(s) of metal ion binding are adjacent to position C70, where a C-to-U mutation results in a dramatic increase in the utilization of calcium as a catalytic metal ion (Frank & Pace, 1997), indicating that the geometry of functional groups within this region of the ribozyme is particularly important for utilization of metal ions for catalysis. Thus, in the context of available biochemical and phylogenetic information, the evidence presented here demonstrates that helix P4 is an important divalent metal ion binding site in the catalytic core of the RNase P ribozyme, and may comprise a portion of the active site itself.

MATERIALS AND METHODS

Plasmid construction and RNA synthesis

Plasmids pPT332(-5) and pPT50 were derived from plasmid pTAN (Nolan et al., 1993). Plasmid pPT332(-5) was generously supplied by the laboratory of Dr. Norman Pace. Plasmid pPT50 was synthesized from two separate rounds of polymerase chain reaction (PCR) (Sarkar & Sommer, 1990). Specifically, the tRNA portion of PT50 was generated using PCR with primer PT50F (5'-CTCTTCGGGGAGACGACTACTACCCAAAATGGTCCGGTAG-3') and primer PT30CP3 (5'-CGGGATCCGAAGACAGTGGCGGTCCGGACGGGACTCG-3') and plasmid pDW152 (also supplied by Dr. Norman Pace) as the target DNA. The circularly permuted RNase P RNA portion of the ribozyme was similarly generated using PCR with primers PT50R (5'-CGTCTCCCCGAAGAG-3') and PT30CPF (5'-CGGGATCCGAAGACAGTGGCGGTCCGGACGGGACTCG-3'). PCR products were gel purified on 2% agarose (TAE) gels. The full-length PT50 ribozyme was subsequently synthesized by taking advantage of the regions of complementarity between the tRNA and RNase P PCR products in a second round of PCR amplification. pG73PT50 (see below) was generated from pPT50 by a single round of PCR using primers GAP73PT50 (5'-GGAATTCTAATACGACTCACTATAGGCTCCATAGGGUGC-3') and PT30CP3 under the conditions above. Similarly, pG364PT332 (see below) was generated from pPT332(-5)
by a single round of PCR using primers GAP364PT33 (5'-GGAATTCATAACTGCTACTATAGGTCTAATTTAACGTTAATGA TTAA-3') and PT30CP31. PT50, PT332(-5), G73PT50, and G364PT332 PCR products were digested with EcoR1 and BamHI (New England Biolabs) to generate ends suitable for ligation into pUC18.

Uniformly labeled RNA was synthesized by in vitro transcription of linearized plasmids [plasmids were digested with BbsI for pPT50, FokI for pPT332(-5), and BsrNI for pDw152] by T7 RNA polymerase (80 U) in 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT (polymerase and buffer provided by Ambion), 1 mM rATP, rGTP, and rUTP, 0.4 mM rGTP, 100 μCi [α-32P]GTP at 37 °C for 6 h. To generate RNAs suitable for 5'-end labeling or ligation [e.g., G73PT50 and G364PT332(-5)], transcription conditions were as described except guanosine was added to 6 mM and rNTP levels were increased to 1 mM. RNAs were subsequently ethanol precipitated, resuspended in 10 mM 1 mM EDTA and 10 μL of loading buffer (98% Formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol FF, 0.025% bromphenol blue), heated to 90 °C for 2 min and purified on a 19% (19:1) polyacrylamide (8 M urea) gel. Labeled RNAs were visualized by autoradiography and unlabeled RNAs were visualized by UV shadowing. RNA transcripts were excised from the gel and eluted in a 5–10-fold volume excess (800 μL) of elution buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M sodium acetate, and 0.1% sodium dodecyl sulfate) for 4–12 h. Eluted products were extracted twice with an equal volume of a 1:1 mixture of phenol and chloroform, extracted once with an equal volume of chloroform and then precipitated with three volumes of ethanol. The 5’ ends of G73PT50 and G364PT332 RNAs were subsequently phosphorylated with T4 polynucleotide kinase (Gibco-BRL) and ATP, and gel purified as described above prior to use.

PT50 and PT332 cleavage reactions

Although experimental conditions were varied with regard to metal ion concentration and pH, standard reactions were performed in the following manner. Six microliters of transcribed or ligated RNA (2 mM) dissolved in 1 mM EDTA (and 0.1% NP-40 for ligated RNAs) were mixed with 48 μL of reaction buffer (3.75 M NaCl, 62.5 mM PIPES, pH 5.5 at 50 °C) and annealed in a PTC-100 programmable thermal controller (MJ Research; 85 °C for 1 min, cooled to 65 °C at 0.2 °C per second, maintained at 65 °C for 5 min, cooled to 50 °C at 0.25 °C per second and incubated for 3.5 h at 50 °C). Reactions were then initiated by adding 6 μL 250 mM MgCl₂. The final reaction conditions were 0.2 mM RNA, 25 mM MgCl₂, 3 M NaCl, 50 mM PIPES, pH 5.5, 50 °C. MgCl₂ was mixed into the solution by rapid pipetting for 5 s. For high-pH or high-MgCl₂ experiments, mixing typically lasted 2 s. Six microliter aliquots were removed at various times and quenched by mixing with formamide gel-loading buffer containing a 2:1 excess of EDTA relative to MgCl₂ (typically 10 mM EDTA, 98% formamide). Products were loaded directly onto 6% polyacrylamide (8 M urea) gels and the products visualized by phosphorimaging analysis (Molecular Dynamics). Experiments where metal concentrations were varied were performed as described above, but with corresponding changes in the concentration of MgCl₂ and MnCl₂ used to start the reaction. Similarly, reactions at various pHs were as described but with reaction buffer pH adjusted accordingly.

Primer extension analysis

Ten picomoles of unlabeled PT50 RNA were reacted under the conditions described above, except the temperature was reduced to 37 °C just prior to the addition of MgCl₂ and the reaction volume was 400 μL. After 1 min, the reaction was quenched with 33 mM EDTA (twice the MgCl₂ concentration) to a final volume of 600 μL and the RNA precipitated with ethanol. One picomole of gel-purified 5'-32P-end-labeled 3' tRNA<sup>44</sup> (primer 5'-TGCGGTCCGGACGGGAC-3') was annealed with 1.4 pmol of reacted or unreacted (for sequencing ladders) PT50 RNA by heating to 65 °C for 2 min in buffer (50 mM Tris-HCl, pH 8.3, 15 mM NaCl, 10 mM dithiothreitol) frozen on dry ice for 2 min, and allowed to thaw on wet ice for approximately 5 min. After addition of MgCl₂ (6 mM in a total volume of 15 μL), five 2-μL reactions of uncleaved RNA and one of cleaved RNA were aliquoted. To four of the uncleaved reactions, 1 μL of dideoxynucleotides (either ddATP, ddCTP, ddGTP, or ddTTP) was added to a final concentration of 400 μM (to generate a sequencing ladder). The remaining uncleaved aliquot (a control for polymerase stops caused by RNA structure) and the reacted PT50 aliquot were supplemented with 1 μL of water. To all reactions, 1 μL of a dNTP mix was added to a final concentration of 5 mM. Annealed primers were extended for 5 min at 47 °C by the addition of 4 U (1 μL) of AMV reverse transcriptase (Boehringer Mannheim). Reactions were terminated by freezing on dry ice for 2 min. Samples were then thawed on wet ice, combined with 4 μL gel-loading buffer, heated to 95 °C for 2 min and set on ice. Aliquots of 2 μL were loaded onto 6% (19:1) polyacrylamide (8 M urea) gels. Gels were run approximately 1.5 h at 50 W, dried, and the bands resolved by autoradiography.

Site-specific functional group modifications

Phosphorothioate modifications at individual nucleotide positions were made during the ligation of two sets of RNA fragments to generate the corresponding PT50 and PT332(-5) self-cleaving constructs (Moore & Sharp, 1992). Specifically for PT50, phosphorothioate modifications at positions A66, A66, A67, G68, or U69 were incorporated by chemical synthesis (Dharmacon) into an 11-nt RNA fragment (5'P4GapB) corresponding to positions 63–73 of the Escherichia coli RNase P ribozyme. R₆ or S₆ phosphorothioate stereo isomers of the RNA fragments were separated by reverse phase HPLC as described by Slim and Gait (1991), and the individual stereoisomers verified by digestion with snake venom phosphodiesterase (Burgers et al., 1979). 5’P4GapB oligonucleotides were subsequently phosphorylated with T4 polynucleotide kinase (Gibco-BRL) and ATP.

Purified RNA fragments of 5’P4GapB with no modification or R₆ or S₆ phosphorothioate modifications as noted above were then ligated by the method of Moore and Sharp (1992) to a 5'-32P-end-labeled 11-nt synthetic RNA fragment (5’P4GapA) containing positions 52–63 of E. coli RNase P RNA and a large RNA transcript (G73PT50, see above) containing the remainder of the PT50 ribozyme. Equal amounts (10 pmol) of 5'-32P-end-labeled 5’P4GapA, 5’P4GapB,
G73PT50, and a DNA oligonucleotide (complementary to 5’P4GapA, 5’P4GapB, and the first 20 nt of G73PT50) were combined in a total volume of 6.8 μL (in distilled water), heated to 60°C for 2 min and then immediately frozen on dry ice for at least 2 min. Samples were then thawed on wet ice and supplemented with 1 μL 10× T4 ligase buffer (New England Biolabs) 1 μL 50% 8,000 g/mol polyethylene glycol (Fluka), 0.5 μL (200 U) rRNasin (Promega) and 0.7 μL (200 U) T4 DNA ligase (New England Biolabs). Samples were then incubated for 2 h at 30°C, combined with an equal volume (10 μL) of gel-loading buffer, heated to 90°C for 2 min and purified on a 6% (19:1) polyacrylamide (8 M urea) gel. Ligated RNAs were visualized by autoradiography and were excised from the gel and recovered as described above.

Site-specific phosphorothioate substitution at position A352 was made in the context of four RNA fragments that gave rise to the self-cleaving conjugate PT332(-5). Three of the four RNA fragments, 3’P4GapA, 3’P4GapB, and 3’P4GapC, corresponding to the nucleotides 332–343, 344–353, and 354–363, were synthesized (Dharmacon) with 3’P4GapB in the presence or absence of a phosphorothioate 5’ to position 352. Prior to ligation 3’P4GapA was 5’ 32P end-labeled with 150 μCi of [γ-32P]-ATP (New England Nuclear) and T4 polynucleotide kinase (Gibco-BRL) and 3’P4GapB and 3’P4GapC were phosphorylated as described above. The forth fragment G364PT332, corresponding to the PT332(-5) ribozyme beginning at position 364, was produced by in vitro transcription (see above). G364PT332(-5) was hybridized along with 3’P4GapA, 3’P4GapB, and 3’P4GapC to a bridging DNA oligonucleotide complementary to positions 332–376 and ligated as described above.

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