Specific phosphorothioate substitutions probe the active site of *Bacillus subtilis* ribonuclease P

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ABSTRACT

Ribonuclease P (RNase P) is a ribonucleoprotein that requires magnesium ions to catalyze the 5' maturation of transfer RNA. To identify interactions essential for catalysis, the properties of RNase P containing single sulfur substitutions for nonbridging phosphodiester oxygens in helix P4 of *Bacillus subtilis* RNase P were analyzed using transient kinetic experiments. Sulfur substitution at the nonbridging oxygens of the phosphodiester bond of nucleotide U51 only modestly affects catalysis. However, phosphorothioate substitutions at A49 and G50 decrease the cleavage rate constant enormously (300–4,000-fold for P RNA and 500–15,000-fold for RNase P holoenzyme) in magnesium without affecting the affinity of pre-tRNA^{Asp}, highlighting the importance of this region for catalysis. Furthermore, addition of manganese enhances pre-tRNA cleavage catalyzed by *B. subtilis* RNase P RNA containing an S_P phosphorothioate modification at A49, as observed for *Escherichia coli* P RNA [Christian et al., *RNA*, 2000, 6:511–519], suggesting that an essential metal ion may be coordinated at this site. In contrast, no manganese rescue is observed for the A49 S_P phosphorothioate modification in RNase P holoenzyme. These differential manganese rescue effects, along with affinity cleavage, suggest that the protein component may interact with a metal ion bound near A49 in helix P4 of P RNA.

Keywords: magnesium; ribozyme; RNA ligation; RNase P; transient kinetics

INTRODUCTION

Bacillus subtilis ribonuclease P (RNase P) is composed of a ~400-nt RNA component and a 15-kDa protein component, both of which are required for efficient processing of the 5' end of pre-tRNA in vivo (reviewed in Kirsebom, 1995; Pace & Brown, 1995; Frank & Pace, 1998; Kurz & Fierke, 2000). However, in vitro, the RNA component of the bacterial RNase P (P RNA) can catalyze cleavage of the 5' termini of the precursor tRNA (pre-tRNA) in the absence of the protein component. RNase P requires divalent metal ions, preferably

magnesium, for folding, binding, and chemistry (Smith & Pace, 1993; Pan, 1995; Beebe et al., 1996; Kurz & Fierke, 2002). The essential metal ion(s) are likely coordinated to specific binding pockets within the catalytic RNA. The protein component of RNase P (P protein) binds to P RNA near the catalytic active site and enhances the affinity of pre-tRNA by directly interacting with the leader sequence (Kurz et al., 1998; Niranjanakumari et al., 1998b). Furthermore, the P protein decreases the concentration of magnesium required for optimal RNase P activity (Gardiner et al., 1985; Reich et al., 1988) by enhancing the affinity of magnesium sites in the holoenzyme•pre-tRNA complex (Kurz & Fierke, 2002).

Nucleic acids consist of numerous potential metal ligands, including phosphate oxygens, ribose 2' hydroxyl groups, and the ring oxygens and nitrogens of the purine and pyrimidine bases. Specific metal ion binding sites have been observed previously by X-ray crystallography and NMR spectroscopy in RNA, including tRNA and substructures of the *Tetrahymena* group I intron (e.g., Cate & Doudna, 1996; Cate et al., 1997; Shi & Moore, 2000). In addition to these sites, metal ions interact electrostatically with anionic polymers in a nonspecific fashion, known as the polyelectrolyte effect

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Abbreviations: 56-P RNA: circularly permuted P RNA with the 5' end at nt 56; 5-tRNA: pre-tRNA containing a 5-nt leader sequence; E: P RNA or RNase P holoenzyme; EDTA: (ethylenedinitrilo)-tetraacetic acid; MES: 2-(N-morpholino)-ethanesulfonic acid; P RNA: RNA component of RNase P; P protein: protein component of RNase P; pre-tRNA: precursor tRNA; RNase P: ribonuclease P; S or pre-tRNA^{Asp}: precursor tRNA^{Asp}; TCEP: Tris (2-carboxyethyl)phosphine; Tris, Tris-(hydroxymethyl)-aminomethane

(reviewed in Anderson & Record, 1995; Manning & Ray, 1998; Misra & Draper, 1998). This effect, exemplified by RNase P, which binds more than 100 magnesium ions nonspecifically (Beebe et al., 1996; Kurz & Fierke, 2002), makes the identification and study of specific metal binding sites in ribozymes difficult. However, the identification of metal ligands important for catalysis may provide a key to resolving the location and architecture of the active site of the enzyme. For both protein and RNA enzymes, interactions with metal ions have been probed by investigating the deleterious effects of phosphorothioate modifications and the ability of divalent metal ions, other than magnesium, to rescue activity (reviewed in Knowles, 1980; Eckstein, 1985; Heidenreich et al., 1993; Narlikar & Herschlag, 1997). This approach has previously been useful for elucidating the interactions between magnesium and substrate phosphodiester oxygens in ribozyme-catalyzed reactions (e.g., Piccirilli et al., 1993; Warnecke et al., 1996; Chen et al., 1997; Weinstein et al., 1997; Shan et al., 1999; Yoshida et al., 1999). Furthermore, spectroscopic studies of the hammerhead ribozyme confirm that phosphorothioate modifications alter metal affinity and specificity (Hunsicker & DeRose, 2000). However, phosphorothioate and/or soft metal ion substitutions may indirectly affect the enzyme's structure and reactivity so metal rescue experiments must be interpreted cautiously (Brautigam & Steitz, 1998; Basu & Strobel,

1999; Horton et al., 2000; Shan & Herschlag, 2000; Smith & Nikonowicz, 2000; Suzumura et al., 2000).

Modification interference experiments using random phosphorothioate modifications have demonstrated the importance for catalysis or substrate docking of at least 4, out of more than 400, *pro-R_p* oxygens in *Escherichia coli* RNase P RNA; these oxygens are located in helix P4 (Fig. 1; Hardt et al., 1995; Harris & Pace, 1995; Kazantsev & Pace, 1998; Christian et al., 2000). A high degree of phylogenetic conservation (Chen & Pace, 1997; Frank et al., 2000) as well as in vitro selection (Frank et al., 1996; Frank & Pace, 1997), and further chemical modification studies (Kazantsev & Pace, 1998) also suggest that helix P4 contains functional groups essential for the catalytic activity of RNase P. The exact role of the identified phosphodiester oxygens in the catalytic mechanism of RNase P has not yet been clearly established. Hardt et al. (1995) identified the oxygens within helix P4 as important primarily for RNA folding or tRNA binding, whereas the experiments of Harris and colleagues (Harris & Pace, 1995; Christian et al., 2000) identify them as important in substrate docking or cleavage. Phosphorothioate substitution at the cleavage site of pre-tRNA also dramatically decreases the hydrolytic rate constant (Warnecke et al., 1996, 1997, 1999, 2000; Chen et al., 1997). Furthermore, cleavage of the *R_p*-phosphorothioate substrate by *E. coli* RNase P RNA can be partially rescued by the addition of manganese

FIGURE 1. Secondary structure of *B. subtilis* RNase P RNA showing the circularly permuted P4-control RNA. The 10 nt in helix P4 that are absent in 56-P RNA and the inserted sequence covalently linking the wild-type termini are italicized and shown in gray. The positions where the P RNA is modified by incorporation of a single phosphorothioate are indicated by arrowheads.

or cadmium, suggesting that one or more catalytically relevant metal(s) may interact with pre-tRNA.

To investigate further the functional role of the phosphate oxygens within helix P4 in catalysis, we synthesized *Bacillus subtilis* RNase P RNA molecules containing a single, specific sulfur substitution at one of three phosphodiester linkages within helix P4. The use of synthetic RNA oligonucleotides allows determination of the individual effects of modification of each of the *pro-R_p* and *pro-S_p* nonbridging oxygens at the phosphodiester bond of nucleotides A49, G50, and U51. Transient kinetic experiments with phosphorothioate-modified *B. subtilis* RNase P RNA or holoenzyme indicate that substitution of sulfur for either the *pro-R_p* or *pro-S_p* oxygen atoms of U51 only modestly decreases the cleavage rate constant. On the other hand, phosphorothioate substitution at either the *pro-R_p* or the *pro-S_p* phosphodiester oxygen of nucleotide A49 or G50 decreases the rate constant for cleavage enormously without affecting binding of pre-tRNA. Therefore, these phosphate oxygens play an essential functional role, stabilizing either the catalytic transition state or a local RNA conformation that is important for cleavage; however, they are not essential for global folding or substrate binding. Furthermore, the addition of manganese ions significantly enhances the observed cleavage rate constant of a subset of the phosphorothioate modifications, including the *S_p* phosphorothioate modification at A49 of *B. subtilis* P RNA. This manganese rescue suggests that the nonbridging *pro-S_p* oxygen of the phosphodiester bond at A49 of *B. subtilis* P RNA may coordinate an essential magnesium ion. In contrast, manganese does not enhance the activity of RNase P holoenzyme containing the A49 *S_p* phosphorothioate modification. These data suggest that the binding of the protein component to P RNA in the vicinity of helix P4, as indicated by affinity cleavage data, confers additional structural constraints that decrease the affinity or reactivity of bound manganese ions at nucleotide A49.

RESULTS

Preparation and characterization of phosphorothioate-modified RNase P RNA

To prepare P RNA with single modifications, a 10-base RNA oligonucleotide was synthesized (Dharmacon, Inc.; Scaringe et al., 1998) with a sequence identical to the 5'-half of helix P4 (G46–U55) except for the incorporation of a single phosphorothioate. The residual all-oxygen containing oligonucleotide (<5%) is easily separated from the sulfur-containing oligonucleotide by anion exchange HPLC. The presence of a sulfur substituted for a nonbridging phosphodiester oxygen in each oligonucleotide was confirmed by iodine cleavage (data not shown; Schatz et al., 1991). Oligonucleotides con-

taining the *R_p* and *S_p* phosphorothioate configuration were separated by HPLC on a C18 column and the stereochemistry was determined using snake venom phosphodiesterase I (Burgers & Eckstein, 1978).

To reconstitute full-length P RNA molecules incorporating the synthetic oligonucleotide into helix P4, a circularly permuted P RNA molecule (56-P RNA, Fig. 1; Nolan et al., 1993) was prepared with the following features: (1) the 5' terminus begins at position G56 (wild-type *B. subtilis* numbering) at the end of helix P4, (2) the 3' terminus stops at G45 such that 10 nt of helix P4 are deleted, and (3) the 5' and 3' termini of wild-type P RNA are covalently attached with a linker sequence. The ligation method of Moore and Sharp (1992) was used to form a phosphodiester bond between the 3' end of the synthetic RNA oligonucleotide and the 5' end of the 56-P RNA molecule transcribed in vitro (Fig. 1). With use of this procedure, 80–90% of the 56-P RNA is consistently converted to full-length P RNA (46-P RNA), as determined by primer extension analysis, containing a single, specific modification. The ligation junction was always accurate, as determined by RNA sequencing with reverse transcriptase. Using this method, we prepared *B. subtilis* RNase P RNAs with single sulfur substitutions for either the *pro-R_p* or *pro-S_p* nonbridging oxygens at the phosphodiester bonds of nucleotides A49, G50, or U51.

Helix P4 is essential for function

Before investigating the effects of the site-specific modifications, we determined the functional properties of 46-P RNA and 56-P RNA in comparison to the wild-type P RNA. Single turnover experiments, containing 56-P RNA (100 nM) and 5-tRNA (<1 nM) in 50 mM MES, 800 mM NH₄Cl, 100 mM MgCl₂, pH 6.0, revealed that <1% mature tRNA was formed after 48 h of incubation at 37 °C. Likewise, incubation of 5-tRNA with 56-P RNA (1 μM) and P protein (1.02 μM) at pH 7.8 and 37 °C for 17 h resulted in the formation of <5% mature tRNA. In both cases, the observed rate constant for product formation decreases more than 10⁶-fold when this portion of helix P4 is deleted. This result is consistent with previous measurements of the activity of deletion mutants of RNase P (Waugh & Pace, 1993) and demonstrates that helix P4 is essential for RNase P to efficiently catalyze pre-tRNA hydrolysis.

To investigate further the importance of P4, we measured the affinity of 56-P RNA for pre-tRNA^{Asp} using gel filtration spin columns to separate free and bound substrate (Beebe & Fierke, 1994). In binding buffer (where calcium is substituted for magnesium), no 56P-RNA•pre-tRNA^{Asp} complex was observed, either with or without the P protein, even when the enzyme concentration was in excess of 1 μM, suggesting a *K_d* >> 1 μM. Consistent with this, 56-P RNA (50 nM) has no effect on the cleavage of 5-tRNA (≤1 nM) cata-

lyzed by wild-type P RNA (10 nM), implying that the P RNA lacking P4 cannot compete with wild-type P RNA for binding substrate. Finally, 56-P RNA migrates more slowly than wild-type RNase P RNA on a nondenaturing polyacrylamide gel (data not shown) suggesting that 56-P RNA does not fold into a compact structure similar to that of wild type. These data collectively demonstrate both that the presence of a small (<20%) amount of 56-P RNA will not interfere with the analysis of the activity of the modified 46-P RNAs and that the P4 helix is essential for the function of RNase P.

To investigate the functional effects of the permutation of the termini of the RNase P RNA molecule, we prepared the circularly permuted 46-P RNA both by in vitro transcription and by ligation of a wild-type RNA oligonucleotide (P4control-RNA). P4control-RNA comigrated with RNase P RNA on a nondenaturing polyacrylamide gel (data not shown), suggesting that it folds into a compact structure. Importantly, the single turnover cleavage rate constants catalyzed by both P4control- and 46-P RNA are comparable to that of wild type (Tables 1 and 2) indicating that the catalytic activity of RNase P is not altered by circular permutation at this position. Therefore, 46-P RNA may be used to determine the functional effects of site-specific modification.

Phosphorothioate modifications in P4 decrease cleavage catalyzed by RNase P holoenzyme

To investigate the functional importance of the *pro-R_p* or *pro-S_p* nonbridging oxygens at the phosphodiester

bonds of nucleotides A49, G50, and U51, we measured the single turnover rate constants for cleavage of 5-tRNA catalyzed by each of these phosphorothioate-modified P RNA/P protein complexes at concentrations of enzyme and magnesium (10 mM) that are saturating for the unmodified enzyme. Because the activity of wild-type RNase P RNA and RNase P holoenzyme is pH dependent (Smith & Pace, 1993; Kurz & Fierke, 2002), we measured the cleavage rate constant at pH 7.8 for the relatively inactive enzymes and at pH 6.0 for the active enzymes (Table 1). Under these conditions, all of the cleavage reactions are well described by a single exponential function (see Fig. 2A). Furthermore, the observed rate constants show a log-linear dependence on pH (data not shown), suggesting that cleavage is the rate-limiting step. The cleavage rate constants (Table 1) indicate that two types of effects are observed. Phosphorothioate substitutions at nucleotide U51 (U51 *R_p* or U51 *S_p*) decrease the observed cleavage rate constant modestly (2- to 15-fold) indicating that the nonbridging phosphate oxygens at this position play a small role in stabilizing the transition state for phosphodiester bond cleavage. In contrast, substitution of sulfur for the *pro-S_p* nonbridging oxygen at the phosphodiester bond of either nucleotide A49 or G50 decreases the cleavage rate constant by 5,900- or 730-fold, respectively. Likewise, sulfur substitution for the *pro-R_p* nonbridging oxygen at the phosphodiester bond of nucleotide A49 or G50 causes a 5,200-fold or 15,000-fold, respectively, decrease in the cleavage rate constant. This decreased cleavage activity is similar to (within a factor of 2) the phosphorothioate effects at homologous positions (A67 and G68) in an *E. coli* self-

TABLE 1. Rate and dissociation constants for the cleavage of 5-tRNA catalyzed by phosphorothioate-modified RNase P holoenzymes.^a

Modified holoenzyme	k_2 ($\times 10^3 \text{ s}^{-1}$)	k_2 (wt)/ k_2 (variant)	K_d (nM)	k_2^{Mn} ($\times 10^3 \text{ s}^{-1}$) 3 mM MnCl_2	k_{rel}^b
Wild-type, pH 6	150 $\pm$ 10	1	7 ^c	76 $\pm$ 9	1.6
46-P RNA, pH 6	180 $\pm$ 10	0.83 $\pm$ 0.07		57 $\pm$ 3	1
U51 <i>R_p</i> , pH 6	10 $\pm$ 1	15 $\pm$ 2	<30 ^d		
U51 <i>S_p</i> , pH 6	70 $\pm$ 30	2.1 $\pm$ 0.9	<200 ^d		
Wild-type, pH 7.8	8,800	1	7	4,000 $\pm$ 200	1.4
A49 <i>R_p</i> , pH 7.8	1.7 $\pm$ 0.2 ^e	5,200 $\pm$ 700	22 $\pm$ 11 ^e	50 $\pm$ 10 ^f	93
A49 <i>S_p</i> , pH 7.8	1.5 $\pm$ 0.3 ^e	5,900 $\pm$ 8 ^e	15 $\pm$ 8 ^e	1.2 $\pm$ 0.2 ^f	3
G50 <i>R_p</i> , pH 7.8	0.6 $\pm$ 0.1 ^f	15,000 $\pm$ 3000	7 $\pm$ 5 ^e	6 $\pm$ 2 ^e	32
G50 <i>S_p</i> , pH 7.8	12 $\pm$ 1 ^g	730 $\pm$ 80	<8 ^d	20 $\pm$ 2 ^f	5

^aSingle turnover data ($[E]/[S] > 5$; $[E] = 3 \text{ nM}$ to $10 \text{ } \mu\text{M}$) determined at 37 °C in low salt buffer (50 mM Tris, 100 mM NH_4Cl , 10 mM MgCl_2 , 1 mM TCEP) or low salt buffer supplemented with 3 mM MnCl_2 . The pH of the reactions are indicated.

^b $k_{\text{rel}} = (k_{\text{Mn}}/k_{\text{Mg}})^{\text{modified RNase P}}/(k_{\text{Mn}}/k_{\text{Mg}})^{46\text{-P}}$.

^cTaken from Kurz et al. (1998).

^dThe value of K_d is estimated from $K_d = k_{-1}/k_1$, where $k_{-1} < k_2$.

^eValues for k_2 and K_d were determined as described in the legend of Figure 2 assuming a rapid equilibrium fit (Eq. 1).

^fThe values for k_2 in the presence of Mn^{2+} are determined from single exponential fits of the single turnover data at saturating enzyme concentrations and the errors are the standard deviations determined from multiple measurements.

^gThe value of k_2 was determined by fitting the data to a mechanism of two consecutive first order exponentials (Eq. 2).

TABLE 2. Rate constants for cleavage of 5-tRNA catalyzed by RNase P RNA containing specific phosphorothioate modifications.^a

Modified RNA	k_{obs} ($\times 10^3 \text{ s}^{-1}$)	$k_{obs} \text{ (wt)}/k_{obs} \text{ (variant)}$	$K_{1/2}$ (nM)	$\frac{k_{Mn}^{obs}}{k_{obs}}$ ($\times 10^3 \text{ s}^{-1}$) 6 mM MnCl ₂	k_{rel}^b
Wild-type P RNA	220 $\pm$ 20 ^c (6,000) ^{d,e}	1	63 $\pm$ 14 ^c	77 $\pm$ 9	1
P4 control RNA	110 $\pm$ 10 ^c	2	84 $\pm$ 12 ^c		
46-P RNA	240 $\pm$ 10 ^c	0.9	154 $\pm$ 28 ^c	81 $\pm$ 6	1
A49 R _p P RNA	0.09 $\pm$ 0.05 ^{f,g} 1.3 $\pm$ 0.7 ^{f,g}	2,400	<5 ^f	0.2 $\pm$ 0.1	6 ^h
A49 S _p P RNA	0.09 $\pm$ 0.06 ^{f,g} 1.3 $\pm$ 0.7 ^{f,g}	2,400	<5 ^f	6 $\pm$ 2	190 ^h
G50 R _p P RNA	0.7 $\pm$ 0.3 ^f (1.4) ^d	310	<5 ^f	0.3 $\pm$ 2	1
G50 S _p P RNA	0.5 $\pm$ 0.1 ^f (4) ^d	440	<5 ^f	0.9 $\pm$ 0.1	5
U51 R _p P RNA	20 $\pm$ 2 ^c	11	21 $\pm$ 5 ^c		
U51 S _p P RNA	260 $\pm$ 10 ^c	0.8	150 $\pm$ 20 ^c		

^aSingle turnover data ($[E]/[S] > 5$, $[E] = 5 \text{ nM} - 2 \mu\text{M}$) at 37 °C in high salt buffer (50 mM MES, 800 mM NH₄Cl, 100 mM MgCl₂, 1 mM TCEP, pH 6) or high salt buffer containing 6 mM MnCl₂.

^b $k_{rel} = (k_{Mn}/k_{Mg})^{\text{modified P RNA}}/(k_{Mn}/k_{Mg})^{46\text{-P}}$.

^cFor these P RNAs, k_{obs} and $K_{1/2}$, reflecting $k_{cleavage}$ and $K_d^{\text{pre-tRNA}}$, respectively, are determined from a rapid equilibrium fit (Eq. 1) of the data as described in the legend of Figure 2.

^dSingle turnover data measured at pH 7.8.

^eTaken from Beebe and Fierke (1994).

^fThe observed rate constants are not dependent on the enzyme concentration down to 5 nM. The errors are the standard deviation determined at multiple enzyme concentrations.

^gTwo additive exponentials were observed for this reaction. The rate constant of the slower phase accounts for >70% of the amplitude of the reaction.

^hThe rate constant of the slow phase was used to calculate k_{rel} .

cleaving RNase P ribozyme (without the protein component; see Fig. 5A). The exception is that the S_p phosphorothioate at G68 in the *E. coli* ribozyme is less deleterious (Christian et al., 2000). These large and conserved phosphorothioate effects indicate that the nonbridging oxygens at these phosphodiester bonds play important functional roles in RNase P. This decrease in the observed rate constant could be caused by lowered catalytic activity, diminished affinity of substrate or metal ions, or incorrect folding of the macromolecule.

To investigate whether these specifically incorporated phosphorothioate substitutions alter substrate affinity, we measured the dependence of the observed single turnover cleavage rate constant on the concentration of enzyme. For pre-tRNA cleavage catalyzed by RNase P, the simplest kinetic mechanism consists of two steps (Scheme 1): substrate binding followed by

cleavage. There are two limiting conditions for this mechanism: a “rapid equilibrium mechanism,” in which the dissociation of the substrate from the E•S complex occurs more rapidly than the cleavage of the substrate to product ($k_{-1} > k_2$; Scheme 1); or two consecutive, irreversible steps in which cleavage of the substrate occurs more rapidly than substrate dissociation ($k_{-1} < k_2$; Scheme 1). For a rapid equilibrium mechanism, single exponential decays are always observed and the enzyme concentration dependence of k_{obs} simultaneously measures the cleavage rate constant at saturating enzyme, k_2 , and the dissociation constant for substrate, K_d (Fierke & Hammes, 1995). In the alternative mechanism, however, lags in the time course of cleavage are observed under conditions ($k_1 [E] \approx k_2$) where both steps are partially rate limiting (Fierke & Hammes, 1995). Furthermore, the dependence of k_{obs} on the enzyme concentration reflects a change in rate-limiting steps from substrate association ($k_1 [E]$) to cleavage (k_2).

Transient kinetic time courses for the appearance of product catalyzed by the three least active phosphorothioate-substituted RNase P enzymes (A49 R_p, A49 S_p, and G50 R_p) are well described by a single exponential fit at all enzyme concentrations assayed,

SCHEME 1.

FIGURE 2. **A:** Time course of 5-tRNA cleavage catalyzed by RNase P holoenzyme containing an *R_p*-phosphorothioate modification at G50 under single turnover conditions. 5' end-labeled 5-tRNA (1–10 nM) was mixed with 40 nM G50*R_p*-RNase P and incubated in low salt buffer (●) (50 mM MES, 100 mM NH₄Cl, 10 mM MgCl₂, 1 mM TCEP, pH 8) or low salt buffer containing 3 mM MnCl₂ (○) at 37°C. Reactions were stopped by dilution into an equal volume of 0.2 M EDTA, the products were separated by polyethylenimine thin layer chromatography, and cleavage was visualized and quantified using a PhosphorImager. The time courses for the appearance of product are fit with a single exponential and the rate constants are listed in Table 1. **B:** The observed rate constant was determined from the appearance of product formation at various concentrations of G50*R_p*-RNase P. The substrate dissociation constant and the chemical rate constant at saturating enzyme are calculated by fitting Equation 1 to these data (Table 1).

indicative of a rapid equilibrium kinetic mechanism (Fig. 2). The pre-tRNA K_d s of 7–22 nM calculated from these data (Table 1) are comparable to the value measured for wild-type RNase P holoenzyme. These data demonstrate that the nonbridging oxygens at these phosphodiester bonds are not essential for substrate binding and also imply that these phosphorothioate substitutions do not affect the global conformation of RNase P. Therefore, the large decreases in the cleavage rate constant demonstrate that the nonbridging phosphodiester oxygens at *pro-R_p* A49, *pro-S_p* A49, and *pro-R_p* G50 play a crucial role in phosphodiester bond hydrolysis catalyzed by RNase P holoenzyme. However, these data do not distinguish between direct stabilization of the transition state and stabilization of structure in P4 that is essential for forming the catalytically competent enzyme–substrate complex.

In contrast, the transient kinetic time courses for the appearance of product catalyzed by wild-type and the more active phosphorothioate-substituted RNase P en-

zymes (G50 *S_p*, U51 *R_p*, and U51 *S_p*) reveal lags that are dependent on the enzyme concentration (Fig. 3). This is diagnostic of a kinetic mechanism in which the chemical rate constant is faster than the substrate dissociation rate constant ($k_2 > k_{-1}$). At low enzyme concentrations ($[E] \ll k_2/k_1$), the cleavage reaction is well described by a first order exponential, and the observed rate constant is dependent on the enzyme concentration. Therefore, the substrate association rate constant (k_1) can be determined from either a linear fit of the dependence of the observed rate constants on subsaturating enzyme concentration or a three-dimensional fit of the dependence of the observed rate constants on both time and enzyme concentration (Eq. 2 in Materials and Methods; Crary et al., 1998; Kurz et al., 1998; Fig. 3). These analyses yield values for the association rate constants (k_1 ; Scheme 1) of $0.33 \pm 0.03 \mu\text{M}^{-1} \text{s}^{-1}$ for U51 *R_p* RNase P, $0.36 \pm 0.02 \mu\text{M}^{-1} \text{s}^{-1}$ for U51 *S_p* RNase P, and $1.2 \pm 0.6 \mu\text{M}^{-1} \text{s}^{-1}$ for G50 *S_p* RNase P. The association rate constants for phosphorothioate substitutions at U51 are more than 10-fold slower than the comparable values for wild-type RNase P holoenzyme ($6 \mu\text{M}^{-1} \text{s}^{-1}$; Crary et al., 1998). These decreases in the rate constant for substrate association may explain the observed modification-interference effects of a phosphorothioate substitution at U51 on self-cleavage (Harris & Pace, 1995). Given this kinetic mechanism, only an upper limit for the pre-tRNA dissociation constant can be estimated ($K_d = k_{-1}/k_1$ where $k_{-1} < k_2$; Table 1). These data suggest that the oxygen-to-sulfur substitution for the *pro-S_p* nonbridging oxygen at the phosphodiester bond of G50 has little effect on substrate affinity. On the other hand, the estimated K_d values for phosphorothioate-substituted RNase P at nucleotide U51 are con-

FIGURE 3. Single turnover measurement of cleavage of 5-tRNA catalyzed by RNase P holoenzyme with a sulfur modification at the *pro-R_p* nonbridging oxygen at the phosphodiester bond of nucleotide G50. Catalysis of pre-tRNA cleavage by this modified holoenzyme in low salt buffer [5 nM (◇); 10 nM (●); 20 nM (□); 40 nM (▲)] or in low salt buffer with 3 mM MnCl₂ [60 nM (○)] under single turnover conditions was analyzed as described in the legend of Figure 2. The association and cleavage rate constants were determined using a three-dimensional fit of the data for the appearance of product as a function of time (Eq. 2).

sistent with a modestly decreased pre-tRNA affinity. The slower substrate association rate constant for phosphorothioate-modified RNase P suggests that the nonbridging phosphodiester oxygens at U51 may stabilize the correct ground-state conformation of RNase P or facilitate a substrate "docking" step subsequent to the formation of an initial E•pre-tRNA complex.

Metal rescue of phosphorothioate substitutions in the holoenzyme

The previous data demonstrate that the nonbridging phosphodiester oxygens of nucleotides A49 (*pro-R_p* and *pro-S_p*) and G50 (*pro-R_p*) play a crucial role in catalysis of phosphodiester bond hydrolysis by RNase P holoenzyme. These large phosphorothioate effects are comparable to those observed at the scissile phosphates of group I introns (Piccirilli et al., 1993; Weinstein et al., 1997) and the pre-tRNA substrate of RNase P (Warnecke et al., 1996; Chen et al., 1997). Furthermore, the decreases also parallel the decreased affinity of Mg^{2+} for sulfur versus oxygen in model complexes (10^3 - to 10^4 -fold; Jaffe & Cohn, 1978; Pecoraro et al., 1984). To investigate the possibility that the phosphorothioate modifications in P4 might disrupt a magnesium site, we determined whether the addition of 3 mM manganese (in the presence of 10 mM $MgCl_2$) could "rescue" the catalytic activity of the modified RNase P holoenzymes (Table 1). Under these conditions, the single turnover rate constant for wild type is decreased two- to threefold whereas the rate constant catalyzed by RNase P containing a sulfur substitution for the *pro-S_p* phosphodiester oxygen at either nucleotide A49 or G50 is enhanced less than twofold. These results provide no evidence for metal binding at these positions. On the other hand, significant increases in the single turnover rate constant were observed for sulfur substitution for the *pro-R_p* phosphodiester oxygen at either nucleotide A49 or G50 (Figs. 2 and 4, Table 1). The addition of similar concentrations of other soft metal ions, such as cadmium(II), cobalt(II) or zinc(II), did not rescue the catalytic defect caused by any of the phosphorothioate modifications (data not shown). However, these metal ions inhibit holoenzyme significantly more than manganese (k_{obs} decreases ~ 10 -fold upon addition of 3 mM $ZnSO_4$ or $CoCl_2$ and ~ 20 -fold in 3 mM $CdCl_2$; data not shown); hence, further studies with these metals were not pursued.

To investigate further the effect of manganese on the activity of phosphorothioate-substituted RNase P, we measured the single turnover rate constant as a function of manganese concentration for the unsubstituted 46-P and the A49 *R_p*-phosphorothioate-substituted RNase P (Fig. 4). A similar trend is observed; manganese activates the phosphorothioate-substituted enzyme and inhibits the 46-P holoenzyme in a concentration-dependent manner. To correct for these

FIGURE 4. Dependence of k_{obs} on manganese ion concentration (0–5 mM $MnCl_2$, 100 mM NH_4Cl , 10 mM $MgCl_2$, 1 mM TCEP) for A49 *R_p*-holoenzyme (○, pH 7.8) or 46-P holoenzyme (●; pH 6). A hyperbola [$k_{obs} = k_{max}/(K_{1/2}/[Mn^{2+}] + 1)$] is fit to the data. Inset: To correct for the differential effects of Mn^{2+} on wild-type and phosphorothioate-substituted RNA, $k_{rel}((k_{Mn}/k_{Mg})^{A49R_p}/(k_{Mn}/k_{Mg})^{46-P})$ (■) is plotted. The data are well described by a hyperbola with a maximum activation value of 110 ± 20 and an apparent manganese affinity ($K_{Mn,app}$) of 5 ± 1 mM.

differential effects, we calculated k_{rel} , where the effect of manganese on the rate constant of the phosphorothioate-substituted enzyme is divided by the effect on the 46-P holoenzyme [$(k_{Mn}/k_{Mg})^{A49R_p}/(k_{Mn}/k_{Mg})^{46-P}$]. This analysis assumes that the modified enzyme is inhibited by Mn(II) identically to the wild-type holoenzyme and is activated by Mn(II) at an independent, second site (presumably interacting with the phosphorothioate modification). Because both magnesium and manganese ions are present, the dependence of the single turnover rate constant on manganese concentration reflects the competition between magnesium and manganese ions for interaction with P RNA. A plot of the dependence of k_{rel} on manganese (Fig. 4) is hyperbolic with a maximum activation value of 110 ± 20 and an apparent manganese affinity ($K_{Mn,app}$) of 5 ± 1 mM. Therefore, at saturating manganese, significant rescue of the single turnover activity is observed for the *R_p* phosphorothioate substitution at A49. However, rescue is not complete; the rate constant remains ~ 50 -fold slower than the wild-type RNase P cleavage rate constant at pH 7.8. One explanation of the enhanced activity of this phosphorothioate-substituted RNA in the presence of manganese is that the *pro-R_p* phosphodiester oxygen at position 49 interacts with a magnesium ion that is important for the cleavage activity.

These manganese rescue data (Table 1) contrast with similar measurements of the phosphorothioate effects at homologous positions (A67 and G68) in an *E. coli* self-cleaving RNase P ribozyme (see Fig. 5B). A similar value (65-fold) for the manganese rescue

FIGURE 5. A: A comparison of the decrease in the single turnover rate constant for pre-tRNA cleavage caused by a single phosphorothioate modification ($k_{WT}/k_{modified}$) in either: (H) *B. subtilis* holoenzyme (Table 1); (B) *B. subtilis* P RNA (Table 2); or (E) self-cleaving *E. coli* P RNA ribozyme (taken from Christian et al., 2000). **B:** A comparison of the manganese rescue of the cleavage activity ($k_{rel} = (k_{Mn}/k_{Mg})^{modified E}/(k_{Mn}/k_{Mg})^{46-P}$) of phosphorothioate-substituted enzymes, including: (H) *B. subtilis* holoenzyme (Table 1); (B) *B. subtilis* P RNA (Table 2); or (E) self-cleaving *E. coli* P RNA ribozyme (taken from Christian et al., 2000).

was observed for phosphorothioate modification at the *pro-R_p* position of A67 (homologous to A49). In contrast, little or no rescue (1.6-fold) was observed for the phosphorothioate modification at the *pro-R_p* oxygen of the phosphodiester bond at G68 (homologous to G50) and complete rescue (5,200-fold) was observed for modification at the *pro-S_p* phosphodiester oxygen of A67 (homologous to A49). There are a significant number of differences between the two experiments, including: self-cleaving versus *trans*-cleaving, P RNA versus RNase P holoenzyme, *E. coli* versus *B. subtilis* P RNA, and reaction conditions.

Phosphorothioate substitutions at G50 and U51 in P RNA

To delineate further the reason(s) for the differential metal rescue effects of phosphorothioate-substituted *E. coli* self-cleaving P RNA and *B. subtilis* RNase P holoenzyme, we investigated the functional effects of phosphorothioate modification in *B. subtilis* P RNA. We first measured the single turnover rate constants for cleavage of 5-tRNA catalyzed by P RNA containing a single sulfur substituted for either the *pro-R_p* or *pro-S_p* nonbridging oxygen at the phosphodiester bonds of nucleotides G50 and U51 at saturating magnesium concentrations (100 mM). The cleavage reactions catalyzed by P RNA containing either the *R_p* or *S_p* phosphorothioate at nucleotides G50 or U51 are each well described by a single exponential function at pH 6 (Table 2). Furthermore, these sulfur substitutions cause decreases in the cleavage rate constant, following the same general trend as that observed for *B. subtilis* RNase P holoenzyme (Tables 1 and 2, Fig. 5A) and *E. coli* self-cleaving P RNA (Christian et al., 2000). Large decreases in the single turnover rate constant at saturating enzyme concentration, k_{obs} (340- to 480-fold), are observed for substitutions at G50 and modest decreases (1- to 12-fold) are measured for changes at U51. However, the *R_p* phosphorothioate substitution at G50 has a significantly smaller deleterious effect on *B. subtilis* P RNA than either *E. coli* P RNA or RNase P holoenzyme (340-fold versus 4,000- or 15,000-fold decrease). This smaller effect is likely partially due to the inclusion of much higher concentrations of magnesium in the *B. subtilis* P RNA reaction, which may partially rescue activity.

To examine further the effect of these substitutions on P RNA activity, we measured the dependence of the observed rate constant on the concentration of enzyme. For phosphorothioate substitutions at U51, the cleavage of substrate by the modified P RNAs follows a rapid equilibrium mechanism ($k_{-1} > k_2$; Scheme 1). The dependence of the observed rate constants for product formation on the enzyme concentration reveals that the substrate affinity for U51*S_p*-P RNA is decreased modestly (approximately twofold) compared to wild-type P RNA, whereas U51*R_p*-P RNA binds 5-tRNA slightly better (threefold; Table 2). Therefore, the second order rate constant (k_2/K_d) is not significantly altered (greater than or equal to fourfold) by these modifications. Taken together, these data provide additional evidence that the oxygens in the phosphodiester bond of U51 do not play an essential functional role in P RNA.

In contrast, the single turnover rate constant catalyzed by P RNA containing either an *R_p*- or *S_p*-phosphorothioate modification at position G50 does not decrease as the enzyme concentration is lowered from 200 nM to 5 nM (data not shown). One possible expla-

nation for these data is that these modifications significantly enhance the substrate affinity such that the dependence on enzyme concentration is shifted to much lower concentrations (<5 nM), reflecting the lowered $K_d^{\text{pre-tRNA}}$. Alternatively, the substrate dissociation rate constant could decrease significantly (to ≤ 0.001 s $^{-1}$) such that the kinetic mechanism changes from rapid equilibrium to two consecutive first-order reactions. In this case, the cleavage step is rate limiting ($k_1[E] > k_2$) for all concentrations of P RNA examined. Using these data, a lower limit for the association rate constant of $0.2 \mu\text{M}^{-1} \text{s}^{-1}$ and an upper limit for $K_d^{\text{pre-tRNA}}$ of 5 nM can be estimated; therefore, these modifications enhance pre-tRNA affinity.

To investigate further the kinetics of pre-tRNA cleavage catalyzed by these phosphorothioate-substituted P RNAs, we measured the pH dependence of the single turnover rate constant. In contrast to wild-type P RNA, the observed single turnover rate constant at saturating enzyme does not increase linearly as the proton concentration decreases. In fact, as the pH increases from 6 to 7.8, k_{obs} increases less than two-fold for G50 R_p and only eightfold for G50 S_p P RNA (Table 2). Decreasing the salt concentrations to levels at which inactive conformers are destabilized for wild-type RNase P RNA (10 mM MgCl_2 , 100 mM NH_4Cl ; Beebe et al., 1996) did not affect the pH dependence of the cleavage rate constant for R_p G50-RNA (data not shown). There are two feasible explanations for the observed pH dependence. First, the catalytic pK_A could decrease from ~ 8 for wild-type P RNA (Smith & Pace, 1993) to 6–7 for these phosphorothioate-substituted P RNAs. Alternatively, the main rate-contributing step for the single turnover reaction catalyzed by phosphorothioate-substituted P RNA at G50 may be a concentration-independent step, such as a conformational change in either P RNA or the E•pre-tRNA complex prior to chemistry, and *not* substrate cleavage. The addition of up to 6 mM MnCl_2 has little effect (Table 2) on the single turnover rate constant catalyzed by P RNA containing a phosphorothioate at G50. Taken together, the minimal effect of altering the pH or the manganese concentration on the single turnover rate constant suggests that the chemical cleavage step is not rate limiting under these conditions. In summary, these data suggest that the phosphate oxygens in the phosphodiester bond at G50 are essential for forming the catalytically active E•pre-tRNA complex.

Phosphorothioate substitutions at A49 in P RNA

For phosphorothioate substitutions at A49 in P RNA, with either the R_p or S_p configuration, catalysis of product formation under single turnover conditions is best described by two additive single exponential reactions (Eq. 3; Fig. 6). The observed rate constants of $1.3 \pm$

$0.7 \times 10^{-3} \text{s}^{-1}$ (first phase) and $9 \pm 6 \times 10^{-5} \text{s}^{-1}$ (second phase; Table 2) for the two phases are nearly identical for R_p and S_p -phosphorothioate-substituted P RNA at A49. The magnitude of the faster phase was generally 10–20% of the initial substrate concentration and the magnitude of the second phase was larger ($\sim 50\%$). One possible explanation for these data is that these phases reflect the existence of two, non-equilibrating conformers of the E•pre-tRNA complex that catalyze substrate cleavage with different rate constants. In this case, phosphorothioate substitutions at A49 decrease the cleavage rate constant 100–1,000-fold, as observed for RNase P holoenzyme (Tables 1 and 2). The addition of up to 6 mM MnCl_2 has little effect on the reaction catalyzed by P RNA containing the R_p -phosphorothioate substitution at A49. However, the addition of 6 mM MnCl_2 to reactions catalyzed by P RNA containing the S_p -phosphorothioate at A49 significantly enhances the cleavage activity; under these conditions, the cleavage of pre-tRNA is described by a single exponential decay with an observed rate constant of $6 \pm 2 \times 10^{-3} \text{s}^{-1}$ (Fig. 6, inset). This increase could mainly reflect either: (1) stabilization of the more active enzyme conformer such that the amplitude of the rapid phase increases (from <0.2 to >0.7); or (2) a significant increase ($k_{\text{rel}} \sim 200$) in the observed rate constant of the slow phase (Table 2). This increase in catalytic activity by the addition of manganese is consistent with the direct coordination of a divalent metal ion at this position, although the function of this metal ion is not clearly delineated.

In summary, the observed functional effects of single phosphorothioate modifications in RNase P RNA are consistent with previous experiments (Harris & Pace,

FIGURE 6. Single turnover measurements of cleavage of 5-tRNA catalyzed by P RNA with a S_p -phosphorothioate modification at the pro- S_p oxygen of nucleotide A49. A representative time course for 5-tRNA cleavage catalyzed by 10 nM A49 S_p -RNA (■) was analyzed using Equation 3 describing two additive exponential decays where $[P]_\infty = 0.55$, $[A] = 0.07$, $k_1 = 1.3 \times 10^{-3} \text{s}^{-1}$, and $k_2 = 9 \times 10^{-5} \text{s}^{-1}$. Inset: Single turnover reactions of A49 S_p -pRNA (>100 nM) and 5-tRNA were measured in the presence (○) or absence (■) of 6 mM MnCl_2 . The time course for the appearance of product in the presence of MnCl_2 is fit with a single exponential (Table 2).

1995; Christian et al., 2000) in that the modifications significantly lower the observed catalytic efficiency (Fig. 5A). Furthermore, the largest rescue upon addition of MnCl_2 is observed for P RNA containing an S_p -phosphorothioate at A49, consistent with data previously observed for the *E. coli* ribozyme (Christian et al., 2000) and in contrast with the RNase P holoenzyme data (Fig. 5B). These data indicate that the differential metal rescue effects observed for RNase P holoenzyme are mainly due to the addition of the protein component to P RNA. However, some of this difference can potentially be explained by the measurement of different rate-limiting steps in cleavage of pre-tRNA catalyzed by phosphorothioate-substituted P RNAs and RNase P holoenzymes.

DISCUSSION

Function of phosphodiester oxygens within helix P4

Random phosphorothioate modification interference analysis previously identified several *pro-R_p* nonbridging phosphodiester oxygens in P4 of *E. coli* RNase P RNA that are functionally important (Hardt et al., 1995; Harris & Pace, 1995; Kazantsev & Pace, 1998). Large phosphorothioate effects at two positions (A67 R_p and S_p and G68 R_p) have also been measured in an *E. coli* self-cleaving RNase P ribozyme (without the protein component; Christian et al., 2000) demonstrating that sulfur modifications at these positions significantly decrease either substrate docking or cleavage. Detailed kinetic investigation of *B. subtilis* P RNA and RNase P holoenzyme containing specific phosphorothioate substitutions confirm that sulfur substitution at either the *pro-R_p* or *pro-S_p* phosphodiester oxygen of A49 or G50 causes a large deleterious effect on the cleavage rate constant whereas modifications at U51 have modest effects (Tables 1 and 2). The data also demonstrate that the catalytic defects are not caused by alterations in substrate affinity or global conformational changes. Therefore, sulfur substitution at these positions indicates that the phosphodiester oxygens of nucleotides A49 and G50 play essential functional roles in RNase P, either by directly stabilizing the transition state for cleavage or by stabilizing a local RNA conformation that is important for cleavage.

Previous investigations of the magnesium dependence of RNase P have demonstrated that multiple magnesium ions are important for function, including: ≥ 3 magnesium ions stabilize the folded P RNA structure; ≥ 2 magnesium ions enhance tRNA binding; and 1–3 magnesium ions activate the catalytic transition state (Smith & Pace, 1993; Pan, 1995; Beebe et al., 1996; Warnecke et al., 1996, 1999; Kurz & Fierke, 2002). One possible explanation of the large decreases in pre-tRNA cleavage (10^3 – 10^4 -fold) observed for phos-

phorothioate modifications in P4 is that the sulfur modification may disrupt an important magnesium binding site. Consistent with this conclusion, the observed decreases in the cleavage rate constant are comparable to the estimated decrease in magnesium affinity for sulfur compared to oxygen (Jaffe & Cohn, 1978; Pecoraro et al., 1984) and to the decreased activity of the group I introns caused by phosphorothioate substitutions (e.g., Piccirilli et al., 1993; Weinstein et al., 1997). Evidence for metal binding sites in helix P4 of P RNA has also been obtained from NMR studies of a stem-loop model of P4 (Schmitz & Tinoco, 2000) and in vitro selection experiments where a specific mutation in P4 altered the metal specificity of RNase P (Frank & Pace, 1997).

To investigate further whether phosphorothioate substitutions in RNase P disrupt magnesium binding sites, the activity in the presence of a "softer" metal ion, manganese, was determined. Metal ion specificity switch experiments have previously been successfully used to investigate metal-substrate interactions in RNase P (Warnecke et al., 1996, 1997, 1999, 2000; Chen et al., 1997). Addition of manganese to *B. subtilis* RNase P RNA containing phosphorothioate modifications at A49 R_p , G50 R_p , and G50 S_p (Table 2) has little or no effect on the observed cleavage rate constant. The absence of metal rescue, however, cannot rule out direct metal coordination, as substitution of manganese for magnesium and/or substitution of sulfur for oxygen may perturb the active site structure substantially, as observed in DNA polymerase I (Brautigam & Steitz, 1998) and RNA structures (Smith & Nikonowicz, 2000; Suzumura et al., 2000). Furthermore, in the *Tetrahymena* group I ribozyme P4-P6 domain, metal rescue of the phosphorothioate effects on stability is not observed at two sites where direct metal coordination has been observed crystallographically (Basu & Strobel, 1999).

In contrast, the defect in cleavage due to the S_p phosphorothioate substitution at A49 in P RNA is partially rescued (within a factor of 13 of the rate constant for wild-type P RNA) by the addition of MnCl_2 to the reaction (Table 2; Fig. 5). This increase in activity suggests that the original defect could be due to the decreased affinity for a directly coordinated Mg^{2+} by the modified atoms and suggests that the *pro-S_p* phosphodiester oxygen of A49 may coordinate a functionally important metal ion. However, the cleavage rate constant in the presence of manganese under these conditions remains lower than the wild-type rate constant. In the *E. coli* P RNA ribozyme, addition of manganese to phosphorothioate-substituted P RNA at the homologous position almost completely rescues catalytic activity (Christian et al., 2000). This differential rescue is likely due to both differences in the solution conditions and structures of the two RNAs. Partial manganese rescue of defects caused by sulfur modifications have been previously observed in the hammerhead ribo-

zyme, in the RNase P pre-tRNA substrate, and in phosphorothioate-substituted substrates for DNA polymerase I (Warnecke et al., 1996, 1999; Chen et al., 1997; Curley et al., 1997; Brautigam & Steitz, 1998).

The manganese rescue data suggest that a divalent metal ion may coordinate the *pro*-S_p nonbridging phosphate oxygen of the phosphodiester bond of nucleotide A49. Furthermore, the data demonstrate that this metal ion is not essential for binding substrate or for stabilizing the global fold of P RNA. However, the metal ion binding could enhance catalysis either by directly stabilizing the transition state for cleavage or by stabilizing a local RNA conformation that is important for cleavage. Our data suggest that a conformational change is the rate-limiting step for the A49 S_p phosphorothioate-substituted P RNA and suggest that Mn²⁺ may stabilize the active RNA conformer. Therefore, metal ion coordination at this site may stabilize a specific *tertiary structure* that is essential for stabilizing the cleavage step rather than directly stabilizing the catalytic transition state. This effect may be similar to the *Tetrahymena* group I ribozyme reaction where manganese rescue of phosphorothioate-modified substrates is an indirect effect due to stabilization of substrate docking (Shan & Herschlag, 2000).

In tertiary structure models of RNase P RNA (Chen et al., 1998; Massire et al., 1998), helix P4 is modeled as an A-form helix (Fig. 7). In these structures, the nonbridging phosphate oxygens of the phosphodiester

FIGURE 7. Proposed structure of the P4 helix in *B. subtilis* RNase P RNA with bound pre-tRNA taken from the model of Chen et al. (1998), showing the modeled positions of the *pro*-R_p and *pro*-S_p nonbridging oxygens of the phosphodiester bonds of nucleotides A49 and G50. The position of the 5' end of tRNA is indicated by an arrow.

bond of nucleotide A49 are near the scissile phosphodiester bond but too far (>10 Å) to chelate metal ion(s) that directly stabilize the transition state (Fig. 7). These models therefore suggest that the manganese rescue of phosphorothioate-modified P RNA may be due to an indirect effect on catalysis. However, compared to other regions of the P RNA tertiary structure, the position of helix P4 is relatively poorly constrained by intramolecular crosslinking data. Furthermore, crosslinks identified between the -1 and +1 nucleotides in pre-tRNA and J5/15 and J18/2 in RNase P RNA (Christian et al., 1998) are incongruous with the current low resolution models of RNase P RNA. Therefore, the structural models may require significant adjustments in the vicinity of the cleavage site that could decrease the distance between residues A49 and G50 and the scissile bond. The functional role of the phosphate oxygens at these positions could potentially be further delineated by investigation of the synergistic effect of phosphorothioate substitutions in the P RNA and pre-tRNA.

Metal rescue of phosphorothioate substitutions in holoenzyme compared to P RNA

The enhancement of the cleavage activity of phosphorothioate-substituted P RNA by the addition of manganese (k_{rel} in Tables 1 and 2) is significantly altered upon addition of the protein component (Fig. 5B). Significant manganese rescue ($k_{rel} \geq 20$) is observed only for the A49 S_p phosphorothioate substitution in *B. subtilis* P RNA, whereas rescue is observed for A49 R_p and G50 R_p for RNase P holoenzyme. These differences in metal rescue may partially reflect the different rate-limiting steps and magnesium concentrations in the two reactions. The pH dependence of k_2 suggests that cleavage is rate limiting for the modified RNase P holoenzymes, whereas a conformational change may be rate limiting for the RNase P RNA enzymes modified at G50.

Furthermore, in comparison to both *B. subtilis* holoenzyme and the self-cleaving *E. coli* P RNA-tRNA conjugate (Christian et al., 2000), the A49 R_p phosphorothioate in *B. subtilis* P RNA causes a smaller decrease in the cleavage rate constant and a smaller enhancement upon addition of manganese ($k_{rel} = 6$). Both of these effects may be caused by the higher experimental magnesium concentrations used to obtain optimal activity of *B. subtilis* P RNA (100 mM versus 10–25 mM Mg²⁺; Gardiner et al., 1985; Reich et al., 1988). This high magnesium concentration may partially rescue the catalytic defect caused by the phosphorothioate modification even in the absence of manganese, consistent with the proposal that this modification decreases the apparent magnesium affinity of P RNA. High concentration of Mg²⁺ has also been shown to suppress the effect of R_p-phosphorothioates

in P4 (C70 and C71) of the *E. coli* self-cleaving ribozyme (Kazantsev & Pace, 1998). In summary, these data suggest that the *pro-R_p* oxygen of the phosphodiester bond of A49 has a similar effect on the catalytic activity in both P RNA and RNase P holoenzyme.

In contrast, the difference in metal rescue of P RNA (Fig. 5B; Tables 1 and 2; Christian et al., 2000) and holoenzyme containing the A49 *S_p* phosphorothioate modification is unexpected and interesting. The low activity of both holoenzyme and P RNA containing the A49 *S_p*-modification is consistent with the loss of a coordinated metal ion at this position (Tables 1 and 2). However, manganese does not significantly enhance cleavage catalyzed by the A49 *S_p*-modified holoenzyme, whereas metal rescue ($k_{rel} = 200\text{--}5,000$) is observed for reactions catalyzed by either A49 *S_p*-modified *B. subtilis* or A67 *S_p*-modified *E. coli* P RNA (Table 2; Christian et al., 2000), despite large differences in solution conditions. One explanation for these data is that the binding of the protein component to the P RNA confers additional structural constraints that decrease the affinity or reactivity of bound manganese ions at this position. Consistent with this hypothesis, cross-linking and thermodynamic data indicate that the protein component interacts with the leader sequence of pre-tRNA (Kurz et al., 1998; Niranjanakumari et al., 1998b) and is therefore located near the catalytic active site in RNase P. Furthermore, holoenzyme reconstituted with RNase P protein modified with an affinity cleavage reagent [either iron-EDTA modification at amino acid 54 in the *E. coli* P protein (Biswas et al., 2000) or copper-phenanthroline modification at amino acid 60 or 64 in the *B. subtilis* P protein (S. Niranjanakumari & C.A. Fierke, unpubl. data)] specifically cleaves P RNA in this region of helix P4 (nt 66, 67, 72–74 in *E. coli* RNase P and nt 48–50 in *B. subtilis*). Taken together, these data suggest that the P protein interacts with a metal ion in the vicinity of the *pro-S_p* A49 phosphate oxygen. One of the effects of the P protein is to decrease the concentration of magnesium required for optimal RNase P activity (Gardiner et al., 1985; Reich et al., 1988) by enhancing the affinity of several specific Mg^{2+} sites in the holoenzyme•pre-tRNA complex (Kurz & Fierke, 2002). The mechanism of the enhanced magnesium affinity conferred by the protein component is not known but could include direct interaction with essential metal ions.

MATERIALS AND METHODS

Materials

All nucleoside triphosphates were purchased from Pharmacia. Guanosine, guanosine monophosphate, and tRNA^{xx} were purchased from Sigma. NH_4Cl , MES buffer, EDTA, and $MnCl_2$ were SigmaUltra reagents. The purest available $MgCl_2$ was purchased from Aldrich. All solutions were made with deion-

ized, metal-free H_2O and stored in plastic in order to minimize the leaching of metals into the solutions.

Unmodified RNA and protein preparations

Unmodified RNase P RNA (P RNA) and pre-tRNA^{Asp} containing a 5-nt leader (5-tRNA; Crary et al., 1998) were prepared by in vitro transcriptions using linearized plasmids with T7 RNA polymerase (Milligan & Uhlenbeck, 1989; Beebe & Fierke, 1994), as previously described (Beebe & Fierke, 1994). 5-tRNA was transcribed in the presence of guanosine to create an RNA molecule with a 5'-hydroxyl group (Harris & Pace, 1995) and subsequently labeled at the 5' hydroxyl by incubation with $\gamma\text{-}^{32}P\text{-ATP}$ and T4 polynucleotide kinase (New England Biolabs). Recombinant P protein, prepared as previously described (Niranjanakumari et al., 1998a), was a generous gift from Dr. S. Niranjanakumari.

Purification of phosphorothioate-containing oligonucleotides

RNA oligonucleotides (5'-GAAAGUCCAU-3') containing single phosphorothioate modifications were either purchased or a kind gift from Dharmacon Research, Inc. (Boulder, CO; Scaringe et al., 1998). Oligonucleotides were deprotected by incubation in acetic acid, according to the manufacturer's recommendations, purified on a 20% polyacrylamide/7 M urea gel, resuspended in buffer A (20 mM Tris-Cl, pH 7, 3% acetonitrile), and filtered through a 0.22 μm nylon membrane. To separate the phosphorothioate-containing oligonucleotide from any containing only oxygen (<5% of the total), the oligonucleotide (500 μL ; <100 μM) was loaded onto a strong anion exchange HPLC column (Macrosphere SAX, 7 μm , 250 $\times$ 4.6 mm; Alltech) in buffer A, washed for 5 min, and then eluted with a gradient of increasing buffer B (20 mM Tris-Cl, pH 7, 3% acetonitrile, 1 M NaCl). Fractions were pooled, precipitated, and redissolved in buffer C (20 mM ammonium acetate, pH 7, 2% acetonitrile). The *R_p* and *S_p* phosphorothioate configurations were separated on a C18 column (Nucleosil C18, 5 μm , 250 $\times$ 4.6 mm; Alltech); the oligonucleotide (500 μL ; <70 μM) was loaded and washed with buffer C before elution with a gradient of buffer D (20 mM ammonium acetate, pH 7, 20% acetonitrile). Oligonucleotides with the *R_p* phosphorothioate configuration eluted from the C18 column prior to those with the *S_p* phosphorothioate.

Oligonucleotide analysis

Oligonucleotides were labeled at the 5' hydroxyl by incubation with $\gamma\text{-}^{32}P\text{-ATP}$ and T4 polynucleotide kinase (New England Biolabs) and purified through a C18 Sep-Pak column (Waters). The presence of sulfur within the RNA oligonucleotides was confirmed by incubating trace amounts of RNA oligonucleotide for 1 min at 95°C with an equal volume of 1 mM iodine and assaying for cleavage of the oligonucleotide on a 20% polyacrylamide/7 M urea gel (Schatz et al., 1991). The position of the sulfur modification was confirmed by comparison to an alkaline hydrolysis ladder of an all-oxygen-containing RNA oligonucleotide.

The configuration of the sulfur substitution at each phosphodiester bond was determined by incubating a trace amount

of RNA oligonucleotide with snake venom phosphodiesterase I (0.31 U/20 μ L reaction; Pharmacia) in 50 mM Tris-Cl, 0.1 mM EDTA, pH 8.16, with 25% glycerol and 0.25 mg/mL tRNA^{XX} at 37 °C, quenching the reaction by the addition of formamide and assaying cleavage on a 20% polyacrylamide/7 M urea gel (Burgers & Eckstein, 1978).

Preparation of 46-P RNA and 56-P RNA

Plasmid p2PRNA containing tandem repeats of RNase P RNA (Nolan et al., 1993) was prepared by ligating the following two pieces of DNA: (1) the *Nco*I-*Dra*I fragment of plasmid pDW25 encoding RNase P RNA and (2) pDW25 digested with *Bam*HI, incubated with the Klenow fragment of DNA polymerase I to fill in the single-strand overhang (Sambrook et al., 1989) and then digested with *Nco*I. Plasmid p2PRNA was used as the template for a polymerase chain reaction amplification with *Pfu* polymerase and primers (5'-TAA TAC GAC TCA CTA TAG CTC GCA CGG TGC TGA GAT GCC-3'; 5'-ACC GCT CGA GGT CTC ACT CTA CAG ATT CAA GAT CTG C-3') to create a DNA fragment beginning with a T7 RNA promoter followed by RNase P RNA nucleotide G56 and ending with nucleotide G45 of RNase P RNA followed by the sequence recognized by the restriction enzyme *Bsa*I. In this molecule, the native 3' and 5' termini are joined by the sequence 3'-ACAUUU-5' (Fig. 1). This DNA fragment was subcloned into plasmid pUC18 at the blunt site created by digestion with *Sma*I to create plasmid p56PRNA. Following the linearization of plasmid p56PRNA with *Bsa*I, in vitro transcription reactions produced 56-P RNA, a molecule beginning with a 5' monophosphate group at G56 and ending at G45, with the usual 5' and 3' termini of RNase P RNA covalently connected. 46-P RNA, containing a 5' triphosphate group at G46 and ending at G45, was prepared by similar methods (using the primers 5'-TAA TAC GAC TCA CTA TAG AAA GTC CAT GCT CGC-3'; 5'-ATG GAG GTA CCT CTA CAG ATT CAA GAT CTG C-3'; plasmid p46PRNA was linearized with *Kpn*I).

Ligation reactions

The phosphorothioate-containing oligonucleotides were coupled to 56-P RNA as follows (Moore & Sharp, 1992). 56P RNA (>2 μ M) and a 10-fold molar excess of both the RNA oligonucleotide and a DNA splint were annealed in 10 mM Tris-Cl, 1 mM EDTA, pH 8, 5 mM NaCl, by heating to 95 °C for 3 min, slowly cooling to 40 °C, and incubating at room temperature for 30 min. The DNA splint was complementary to all of the RNA oligonucleotide and the first 23 nt of 56-P RNA (5'-GGG CAT CTC AGC ACC GTG CGA GCA TGG ACT TTC A-3'). After addition of an equimolar concentration of T4 DNA ligase (>1 μ M), purified from an overexpressing strain kindly provided by Dr. S. Strobel, the reaction was incubated in 66 mM Tris-Cl, pH 7.6, 6.6 mM MgCl₂, 10 mM dithiothreitol, 66 μ M ATP, and 30–35% glycerol at room temperature for 4–6 h. The ligated RNA was purified on a gel containing 7 M urea and either 6% or a gradient of 6–20% polyacrylamide. The eluted RNA was precipitated and resuspended in 10 mM Tris, pH 8, 1 mM EDTA, 1 mM Tris(2-carboxyethyl)phosphine (TCEP). The product yield was determined using an ϵ_{260} of $3.94 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ (Beebe & Fierke, 1994).

Determination of ligation efficiency and specificity

The efficiency of the RNA ligation reaction was determined by primer extension analysis (Perrotta & Been, 1998), using a ³²P-labeled primer (5'-GCC CTA GCT TAT GAC TTC GC-3') that anneals to nt 114 to 95 (Fig. 1). The primer was annealed to RNase P RNA and then incubated in reaction buffer (80 mM Tris-Cl, pH 8.3, 63 mM NaCl, 15 mM MgCl₂, 2.5 mM MnCl₂, 8.5 mM dithiothreitol) containing 0.65 U avian myeloblastosis viral reverse transcriptase (Invitrogen) and 0.8 mM each dGTP, dATP, dCTP, and dTTP, at 37 °C for 1 min followed by 60 °C for 1 min, prior to addition of 1.4 vol of quench (formamide containing 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.25 mg/mL tRNA^{XX}). The extension products were separated on a 6% polyacrylamide/7 M urea gel, visualized with a PhosphorImager, and quantified with ImageQuant software (Amersham). The sequence of the RNA through the ligation junction was determined using a protocol similar to that for primer extension, with the addition of 0.08 mM of a given dideoxynucleotide in the extension step (Perrotta & Been, 1998).

Native gel electrophoresis

56-P RNA or wild-type P RNA was denatured by heating to 95 °C for 3 min prior to the addition of buffer (final concentration, 10 mM Tris-Cl, pH 8, 25 mM NaCl, and 10 mM MgCl₂). The RNAs were allowed to refold for 15 min at room temperature, then an equal volume of gel loading buffer (50 mM Tris-acetate, 25 mM NaCl, 6 mM MgCl₂, 0.05% xylene cyanol, 0.05% bromophenol blue) was added, and the RNAs were loaded on a nondenaturing gel (17.5 cm $\times$ 13.5 cm $\times$ 1 mm) containing 6% polyacrylamide (39:1) in 50 mM Tris-acetate, 25 mM NaCl, and 6 mM MgCl₂ (Perrotta & Been, 1998). The positions of the RNA species were visualized by staining with Stains-All (USB).

Single turnover experiments

Single turnover experiments were performed under conditions of excess enzyme concentration ($[E]/[S] > 5$; $[E] = 5 \text{ nM}$ to $1 \mu\text{M}$; $[S] = 0.1 \text{ nM}$ to 10 nM) at 37 °C, in either low salt buffer (50 mM MES or Tris, 100 mM NH₄Cl, 10 mM MgCl₂, 1 mM TCEP) or high salt buffer (50 mM MES or Tris, 800 mM NH₄Cl, 100 mM MgCl₂, 1 mM TCEP) at pH 6.0 to 8.9. In some cases, single turnover reactions were supplemented with 0.1 to 6 mM MnCl₂, CdCl₂, CoCl₂, or ZnSO₄. All RNAs were denatured at 95 °C for 3 min in 10 mM Tris, pH 8, 1 mM EDTA, diluted into reaction buffer, and then incubated at 37 °C for 10 min. Holoenzyme was reconstituted by the addition of a slight molar excess of P protein to the RNase P RNA solution and incubation for 5–10 min. Reactions were performed and analyzed as previously described (Crary et al., 1998) using 0.2 M EDTA, pH 8.0, to quench the reaction and polyethylenimine cellulose thin layer chromatography plates (Merck) to separate the 5-nt product from pre-tRNA. The separated reactants and products were visualized and quantified using a PhosphorImager (Amersham). For rapid reactions ($k_{\text{obs}} > 0.2 \text{ s}^{-1}$), a mechanical quench-flow apparatus (Model RQF-3, Kintek Instruments) was used (Beebe & Fierke, 1994).

Measurement of pre-tRNA dissociation constants

RNase P holoenzyme or 56-P RNA was incubated with 5-tRNA ($[E]/[S] > 5$; $[E] = 10$ to 100 nM; $[S] = 0.2$ to 10 nM) for 10–20 min at 37°C in low salt binding buffer (50 mM MES, 50 mM Tris, 10 mM CaCl_2 , 100 mM NH_4Cl , pH 6.0, 1 mM TCEP) or high salt binding buffer (50 mM MES, 50 mM Tris, 100 mM CaCl_2 , 800 mM NH_4Cl , pH 6.0, 1 mM TCEP). Bound and unbound substrates were separated using spin column chromatography (Beebe & Fierke, 1994) and quantified using Cerenkov scintillation counting. The fraction of substrate bound was determined as the fraction of total radioactivity that is found in the eluate. These data were fit with a standard binding isotherm (Beebe & Fierke, 1994).

Data analysis

Single turnover data were fit with KaleidaGraph (Synergy Software) or Systat (Systat, Inc.). The chemical rate constants were approximated by fitting the equation describing a single exponential decay to the time course for the appearance of product. This fit is ideal for all reactions in which the bound enzyme–substrate complex equilibrates rapidly with free enzyme and substrate before it is converted to enzyme and product. Under these conditions, values for k_2 and K_d can be determined by fitting Equation 1 to the observed rate constants measured over a range of enzyme concentrations.

$$k_{\text{obs}} = (k_2[E]) / ([E] + K_d) \quad (1)$$

However, when the chemical rate constant is significantly faster than the substrate dissociation rate constant, Equation 2, describing two consecutive irreversible steps (Fierke & Hammes, 1995), is used to fit the data. At saturating enzyme concentrations ($[E] \gg k_2/k_1$), the chemical rate constant can be estimated by a single exponential fit of the time course for the appearance of product.

$$[P]_t = [\text{pre-tRNA}]_0 \left[1 + \frac{1}{k_1[E] - k_2} (k_2 e^{-k_1[E]t} - k_1[E] e^{-k_2t}) \right] \quad (2)$$

For some of the modified RNAs, two additive exponential decays were observed. These data were fit using Equation 3 where: $[P]_\infty$ is the overall endpoint of the reaction; $[A]$ and k_1 are the amplitude and rate constant of the first phase; and $([P]_\infty - [A])$ and k_2 are the amplitude and rate constant of the second phase.

$$[P]_t = [P]_\infty - [A]e^{-k_1t} - ([P]_\infty - [A])e^{-k_2t} \quad (3)$$

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REFERENCES

- Anderson CF, Record M Jr. 1995. Salt–nucleic acid interactions. *Annu Rev Phys Chem* 46:657–700.
- Basu S, Strobel SA. 1999. Thiophilic metal ion rescue of phosphorothioate interference within the *Tetrahymena* ribozyme P4-P6 domain. *RNA* 5:1399–1407.
- Beebe JA, Fierke CA. 1994. A kinetic mechanism for cleavage of precursor tRNA^{Asp} catalyzed by the RNA component of *Bacillus subtilis* ribonuclease P. *Biochemistry* 33:10294–10304.
- Beebe JA, Kurz JC, Fierke CA. 1996. Magnesium ions are required by *Bacillus subtilis* ribonuclease P RNA for both binding and cleaving precursor tRNA^{Asp}. *Biochemistry* 35:10493–10505.
- Biswas R, Ledman DW, Fox RO, Altman S, Gopalan V. 2000. Mapping RNA–protein interactions in ribonuclease P from *Escherichia coli* using disulfide-linked EDTA–Fe. *J Mol Biol* 296:19–31.
- Brautigam CA, Steitz TA. 1998. Structural principles for the inhibition of the 3′-5′ exonuclease activity of *Escherichia coli* DNA polymerase I by phosphorothioates. *J Mol Biol* 277:363–377.
- Burgers PM, Eckstein F. 1978. Absolute configuration of the diastereomers of adenosine 5′-O-(1-thiotriphosphate): Consequences for the stereochemistry of polymerization by DNA-dependent RNA polymerase from *Escherichia coli*. *Proc Natl Acad Sci USA* 75:4798–4800.
- Cate JH, Doudna JA. 1996. Metal-binding sites in the major groove of a large ribozyme domain. *Structure* 4:1221–1229.
- Cate JH, Hanna RL, Doudna JA. 1997. A magnesium ion core at the heart of a ribozyme domain. *Nat Struct Biol* 4:553–558.
- Chen JL, Nolan JM, Harris ME, Pace NR. 1998. Comparative photocrosslinking analysis of the tertiary structures of *Escherichia coli* and *Bacillus subtilis* RNase P RNAs. *EMBO J* 17:1515–1525.
- Chen JL, Pace NR. 1997. Identification of the universally conserved core of ribonuclease P RNA. *RNA* 3:557–560.
- Chen Y, Li X, Gegenheimer P. 1997. Ribonuclease P catalysis requires Mg^{2+} coordinated to the pro-R_P oxygen of the scissile bond. *Biochemistry* 36:2425–2438.
- Christian E, Kaye NM, Harris ME. 2000. Helix P4 is a divalent metal ion binding site in the conserved core of the ribonuclease P ribozyme. *RNA* 6:511–519.
- Christian EL, McPheeters DS, Harris ME. 1998. Identification of individual nucleotides in the bacterial ribonuclease P ribozyme adjacent to the pre-tRNA cleavage site by short-range photocrosslinking. *Biochemistry* 37:17618–17628.
- Crary SM, Niranjanakumari S, Fierke CA. 1998. The protein component of *Bacillus subtilis* ribonuclease P increases catalytic efficiency by enhancing interactions with the 5′ leader sequence of pre-tRNA^{Asp}. *Biochemistry* 37:9409–9416.
- Curley JF, Joyce CM, Piccirilli JA. 1997. Functional evidence that the 3′-5′ exonuclease domain of *Escherichia coli* DNA polymerase I employs a divalent metal ion in leaving group stabilization. *J Am Chem Soc* 119:12691–12692.
- Eckstein F. 1985. Nucleoside phosphorothioates. *Annu Rev Biochem* 54:367–402.
- Fierke CA, Hammes GG. 1995. Transient kinetic approaches to enzyme mechanisms. *Methods Enzymol* 249:3–37.
- Frank DN, Adamidi C, Ehringer MA, Pitulle C, Pace NR. 2000. Phylogenetic-comparative analysis of the eukaryal ribonuclease P RNA. *RNA* 6:1895–1904.
- Frank DN, Ellington AE, Pace NR. 1996. In vitro selection of RNase P RNA reveals optimized catalytic activity in a highly conserved structural domain. *RNA* 2:1179–1188.
- Frank DN, Pace NR. 1997. In vitro selection for altered divalent metal specificity in the RNase P RNA. *Proc Natl Acad Sci USA* 94:14355–14360.
- Frank DN, Pace NR. 1998. Ribonuclease P: Unity and diversity in a tRNA processing ribozyme. *Annu Rev Biochem* 67:153–180.

- Gardiner KJ, Marsh TL, Pace NR. 1985. Ion dependence of the *Bacillus subtilis* RNase P reaction. *J Biol Chem* 260:5415–5419.
- Hardt WD, Warnecke JM, Erdmann VA, Hartmann RK. 1995. R_p-phosphorothioate modifications in RNase P RNA that interfere with tRNA binding. *EMBO J* 14:2935–2944.
- Harris ME, Pace NR. 1995. Identification of phosphates involved in catalysis by the ribozyme RNase P RNA. *RNA* 1:210–218.
- Heidenreich O, Pieken W, Eckstein F. 1993. Chemically modified RNA: Approaches and applications. *FASEB J* 7:90–96.
- Horton TE, Maderia M, DeRose VJ. 2000. Impact of phosphorothioate substitutions on the thermodynamic stability of an RNA GAAA tetraloop: An unexpected stabilization. *Biochemistry* 39:8201–8207.
- Hunsicker LM, DeRose VJ. 2000. Activities and relative affinities of divalent metals in unmodified and phosphorothioate-substituted hammerhead ribozymes. *J Inorg Biochem* 80:271–281.
- Jaffe EK, Cohn M. 1978. Divalent cation-dependent stereospecificity of adenosine 5'-O-(2-thiotriphosphate) in the hexokinase and pyruvate kinase reactions. The absolute stereochemistry of the diastereoisomers of adenosine 5'-O-(2-thiotriphosphate). *J Biol Chem* 253:4823–4825.
- Kazantsev AV, Pace NR. 1998. Identification by modification-interference of purine N-7 and ribose 2'-OH groups critical for catalysis by bacterial ribonuclease P. *RNA* 4:937–947.
- Kirsebom LA. 1995. RNase P—A "Scarlet Pimpernel." *Mol Microbiol* 17:411–420.
- Knowles JR. 1980. Enzyme-catalyzed phosphoryl transfer reactions. *Annu Rev Biochem* 49:877–919.
- Kurz JC, Fierke CA. 2000. Ribonuclease P: A ribonucleoprotein enzyme. *Curr Opin Chem Biol* 4:553–558.
- Kurz JC, Fierke CA. 2002. The affinity of magnesium binding sites in the *Bacillus subtilis* RNase P•pre-tRNA complex is enhanced by the protein subunit. *Biochemistry*. In press.
- Kurz JC, Niranjankumari S, Fierke CA. 1998. Protein component of *Bacillus subtilis* RNase P specifically enhances the affinity for precursor-tRNA^{Asp}. *Biochemistry* 37:2393–2400.
- Manning GS, Ray J. 1998. Counterion condensation revisited. *J Biomol Struct Dyn* 16:461–476.
- Massire C, Jaeger L, Westhof E. 1998. Derivation of the three-dimensional architecture of bacterial ribonuclease P RNAs from comparative sequence analysis. *J Mol Biol* 279:773–793.
- Milligan JF, Uhlenbeck OC. 1989. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol* 180:51–62.
- Misra VK, Draper DE. 1998. On the role of magnesium ions in RNA stability. *Biopolymers* 48:113–135.
- Moore MJ, Sharp PA. 1992. Site-specific modification of pre-mRNA: The 2'-hydroxyl groups at the splice sites. *Science* 256:992–997.
- Narlikar GJ, Herschlag D. 1997. Mechanistic aspects of enzymatic catalysis: Lessons from comparison of RNA and protein enzymes. *Ann Rev Biochem* 66:19–59.
- Niranjanakumari S, Kurz JC, Fierke CA. 1998a. Expression, purification and characterization of the recombinant ribonuclease P protein component from *Bacillus subtilis*. *Nucleic Acids Res* 26:3090–3096.
- Niranjanakumari S, Stams T, Crary SM, Christianson DW, Fierke CA. 1998b. Protein component of ribozyme ribonuclease P alters substrate recognition by directly contacting pre-tRNA. *Proc Natl Acad Sci USA* 95:15212–15217.
- Nolan JM, Burke DH, Pace NR. 1993. Circularly permuted tRNAs as specific photoaffinity probes of ribonuclease P RNA structure. *Science* 261:762–765.
- Pace NR, Brown JW. 1995. Evolutionary perspective on the structure and function of ribonuclease P, a ribozyme. *J Bacteriol* 177:1919–1928.
- Pan T. 1995. Higher order folding and domain analysis of the ribozyme from *Bacillus subtilis* ribonuclease P. *Biochemistry* 34:902–909.
- Pecoraro VL, Hermes JD, Cleland WW. 1984. Stability constants of Mg²⁺ and Cd²⁺ complexes of adenine nucleotides and thionucleotides and rate constants for formation and dissociation of MgATP and MgADP. *Biochemistry* 23:5262–5271.
- Perrotta AT, Been MD. 1998. A toggle duplex in hepatitis delta virus self-cleaving RNA that stabilizes an inactive and a salt-dependent pro-active ribozyme conformation. *J Mol Biol* 279:361–373.
- Piccirilli JA, Vyle JS, Caruthers MH, Cech TR. 1993. Metal ion catalysis in the *Tetrahymena* ribozyme reaction. *Nature* 361:85–88.
- Reich C, Olsen GJ, Pace B, Pace NR. 1988. Role of the protein moiety of ribonuclease P, a ribonucleoprotein enzyme. *Science* 239:178–181.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Scaringe SA, Wincott FE, Caruthers MH. 1998. Novel RNA synthesis method using 5'-O-silyl-O-orthoester protecting groups. *J Am Chem Soc* 120:11820–11821.
- Schatz D, Leberman R, Eckstein F. 1991. Interaction of *Escherichia coli* tRNA^{Ser} with its cognate aminoacyl-tRNA synthetase as determined by footprinting with phosphorothioate-containing tRNA transcripts. *Proc Natl Acad Sci USA* 88:6132–6136.
- Schmitz M, Tinoco I Jr. 2000. Solution structure and metal-ion binding of the P4 element from bacterial RNase P RNA. *RNA* 6:1212–1225.
- Shan SO, Herschlag D. 2000. An unconventional origin of metal-ion rescue and inhibition in the *Tetrahymena* group I ribozyme reaction. *RNA* 6:795–813.
- Shan SO, Yoshida A, Sun S, Piccirilli JA, Herschlag D. 1999. Three metal ions at the active site of the *Tetrahymena* group I ribozyme. *Proc Natl Acad Sci USA* 96:12299–12304.
- Shi H, Moore PB. 2000. The crystal structure of yeast phenylalanine tRNA at 1.93 Å resolution: A classic structure revisited. *RNA* 6:1091–1105.
- Smith D, Pace NR. 1993. Multiple magnesium ions in the ribonuclease P reaction mechanism. *Biochemistry* 32:5273–5281.
- Smith JS, Nikonowicz EP. 2000. Phosphorothioate substitution can substantially alter RNA conformation. *Biochemistry* 39:5642–5652.
- Suzumura K, Warashina M, Yoshinari K, Tanaka Y, Kuwabara T, Orita M, Taira K. 2000. Significant change in the structure of a ribozyme upon introduction of a phosphorothioate linkage at P9: NMR reveals a conformational fluctuation in the core region of a hammerhead ribozyme. *FEBS Lett* 473:106–112.
- Warnecke JM, Furste JP, Hardt WD, Erdmann VA, Hartmann RK. 1996. Ribonuclease P (RNase P) RNA is converted to a Cd²⁺-ribozyme by a single R_p-phosphorothioate modification in the precursor tRNA at the RNase P cleavage site. *Proc Natl Acad Sci USA* 93:8924–8928.
- Warnecke JM, Green CJ, Hartmann RK. 1997. Role of metal ions in the cleavage mechanism by the *E. coli* RNase P holoenzyme. *Nucleosides Nucleotides* 16:721–725.
- Warnecke JM, Held R, Busch S, Hartmann RK. 1999. Role of metal ions in the hydrolysis reaction catalyzed by RNase P RNA from *Bacillus subtilis*. *J Mol Biol* 290:433–445.
- Warnecke JM, Sontheimer EJ, Piccirilli JA, Hartmann RK. 2000. Active site constraints in the hydrolysis reaction catalyzed by bacterial RNase P: Analysis of precursor tRNAs with a single 3'-S-phosphorothiolate internucleotide linkage. *Nucleic Acids Res* 28:720–727.
- Waugh DS, Pace NR. 1993. Gap-scan deletion analysis of *Bacillus subtilis* RNase P RNA. *FASEB J* 7:188–195.
- Weinstein LB, Jones BC, Cosstick R, Cech TR. 1997. A second catalytic metal ion in group I ribozyme. *Nature* 388:805–808.
- Yoshida A, Sun S, Piccirilli JA. 1999. A new metal ion interaction in the *Tetrahymena* ribozyme reaction revealed by double sulfur substitution. *Nat Struct Biol* 6:318–321.