

Chloroplast ribonuclease P does not utilize the ribozyme-type pre-tRNA cleavage mechanism

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ABSTRACT

The transfer RNA 5' maturation enzyme RNase P has been characterized in Bacteria, Archaea, and Eukarya. The purified enzyme from all three kingdoms is a ribonucleoprotein containing an essential RNA subunit; indeed, the RNA subunit of bacterial RNase P RNA is the sole catalytic component. In contrast, the RNase P activity isolated from spinach chloroplasts lacks an RNA component and appears to function as a catalytic protein. Nonetheless, the chloroplast enzyme recognizes a pre-tRNA substrate for *E. coli* RNase P and cleaves it as efficiently and precisely as does the bacterial enzyme. To ascertain whether there are differences in catalytic mechanism between an all-RNA and an all-protein RNase P, we took advantage of the fact that phosphodiester bond selection and hydrolysis by the *E. coli* RNase P ribozyme is directed by a Mg^{2+} ion coordinated to the nonbridging *pro-R_p* oxygen of the scissile bond, and is blocked by sulfur replacement of this oxygen. We therefore tested the ability of the chloroplast enzyme to process a precursor tRNA containing this sulfur substitution. Partially purified RNase P from spinach chloroplasts can accurately and efficiently process phosphorothioate-substituted pre-tRNAs; cleavage occurs exclusively at the thio-containing scissile bond. The enzymatic throughput is fivefold slower, consistent with a general chemical effect of the phosphorothioate substitution rather than with a metal coordination deficiency. The chloroplast RNase P reaction mechanism therefore does not involve a catalytic Mg^{2+} bonded to the *pro-R_p* phosphate oxygen, and hence is distinct from the mechanism of the bacterial ribozyme RNase P.

Keywords: catalytic mechanism; enzyme; evolution of catalysis; magnesium ion; thiosubstitution; tRNA processing

INTRODUCTION

Transfer RNA (tRNA) molecules are synthesized as precursors possessing both 5'-leader and 3'-trailer extensions. Ribonuclease P (RNase P) is a ubiquitous enzyme required for maturation of precursor tRNA (pre-tRNA) by endonucleolytic cleavage between the 5'-leader and the mature tRNA domain. In all varieties of RNase P studied previously, the enzyme is composed of an RNA subunit and a protein subunit. In bacteria, it is the RNA component alone that is responsible for catalysis (Guerrier-Takada et al., 1983), whereas in eukaryal and archaeal varieties of RNase P, both subunits are required for activity (for review, see Pace & Brown, 1995). The bacterial RNase P protein subunit

contains an average of 120 amino acids and the RNA subunit is 350–400 nt long (Brown & Pace, 1992). For example, the *Escherichia coli* RNase P protein has a chain length of 119 nt (13.8 kDa) whereas the RNA subunit is 377 nt (146 kDa). The role of the protein subunit is to facilitate binding of the anionic substrate and enzyme in vivo; and to alter substrate versus product-binding kinetics to permit enzyme discrimination between substrate and product (Reich et al., 1988; Tallsjö & Kirsebom, 1993; Kurz et al., 1998).

Bacterial RNase P is a metalloenzyme, requiring divalent metal cations for activity. The divalent ion functions directly in catalysis, as the RNA subunit can bind substrate in the presence of monovalent ions alone, and displays a burst of activity once a divalent species is added (Smith et al., 1992). Three Mg^{2+} ions participate in catalysis (Hardt et al., 1993; Smith & Pace, 1993; Beebe et al., 1996; Chen et al., 1997). Magnesium(II) is thought to promote catalysis in two ways; by coordinating phosphate oxygen molecules and polarizing the phosphorus center, and by activating the nucleophilic water molecule that attacks the scissile bond (Smith & Pace, 1993; Fersht, 1985).

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In striking contrast to all other types of RNase P characterized, preliminary studies of RNase P from chloroplasts indicated that it lacked an associated RNA component (Wang et al., 1988; Wang, 1991; Li, 1992). The buoyant density of partially purified RNase P, measured by isopycnic centrifugation in CsCl, is 1.28 ± 0.03 g/mL, and the distribution of activity in the gradient is absolutely coincident with that of total chloroplast protein (Wang et al., 1988). This density establishes an upper limit of $\leq 7\%$ RNA (mass/mass) (Hamilton, 1971), corresponding to only ≤ 15 –20 nt RNA per enzyme molecule of 85 ± 15 kDa (as measured by gel filtration with or without 2 M urea; Gao, 1995). However, in partially purified samples of chloroplast RNase P that were deproteinized and dephosphorylated, no RNA species of any size were detected by 3'-end labeling with [32 P]pCp and RNA ligase (Li, 1992). Finally, pretreatment of crude or partially purified preparations of chloroplast RNase P with micrococcal nuclease had no effect on activity, even at nuclease concentrations 50-fold greater than needed to completely inactivate *E. coli* RNase P (Wang et al. 1988; Li, 1992).

We have achieved a 1,500-fold purification of spinach chloroplast RNase P (Thomas, 1996; a preliminary report appeared in Thomas et al., 1995). Pre-tRNA 5'-processing activity contains no detectable RNA, and cofractionates with one major and 4–5 minor polypeptides, although which polypeptide(s) contain RNase P activity has not been determined. Taken together, these data indicate that plant chloroplast RNase P is a conventional protein enzyme.

The differences between a ribozyme-catalyzed and a protein-catalyzed reaction pose some intriguing questions. Like the bacterial enzyme, chloroplast RNase P requires divalent cations for activity: although the enzyme can bind tRNA in the absence of Mg^{2+} ions (D. Stomp, unpubl.), pre-tRNA cleavage is absolutely dependent on Mg^{2+} (Wang et al., 1988; Li, 1992). Almost all ribozyme-catalyzed reactions rely upon the proper positioning of metal ions that bear the burden of catalysis (Peebles et al., 1986; Dahm et al., 1993; Piccirilli et al., 1993). The *E. coli* RNase P reaction involves at least three catalytically essential Mg^{2+} ions that stabilize the transition state and generate the nucleophilic hydroxide (Hardt et al., 1993; Smith & Pace, 1993; Beebe et al., 1996). At least one of these Mg^{2+} ions is coordinated directly by the *pro-R_P* phosphate oxygen of the scissile bond (Chen et al., 1997).

One useful approach toward elucidating the role(s) of metal ions in RNA-processing reactions is the use of phosphorothioate-containing RNA substrates. These modified RNAs are particularly valuable in reactions where Mg^{2+} ions are coordinated directly by phosphodiester oxygens (Dahm & Uhlenbeck, 1991; Slim & Gait, 1991; Christian & Yarus, 1993; Piccirilli et al., 1993; Harris & Pace, 1995). Incorporation of the *S_P* isomer of nucleoside 5'-[α -thio]triphosphates (NTP α S) during in

vitro transcription reactions produces *R_P*-phosphorothioate-containing RNAs (Eckstein, 1985); this incorporation does not significantly alter the structure of the RNA (Herschlag et al., 1991). In the bacterial RNase P RNA reaction, sulfur replacement of the *pro-R_P* non-bridging oxygen at the scissile phosphodiester bond in pre-tRNA decreases the hydrolytic rate constant (k_{chem}) by greater than 10^4 -fold. This inhibition can partially be reversed by substituting Mn^{2+} for Mg^{2+} (Chen et al., 1997).

To compare one aspect of chloroplast RNase P catalysis to this well-defined facet of ribozyme RNase P chemistry—the interaction of divalent cation(s) with the scissile phosphodiester—we tested a substantially purified preparation of spinach chloroplast RNase P for its ability to process a precursor tRNA containing an *R_P*-phosphorothioate substitution at the scissile bond. The results define one fundamental difference between the protein-only and the RNA-only activities.

RESULTS

Partial purification of chloroplast RNase P

RNase P was purified from spinach chloroplasts essentially as described by Thomas (1996), using a modification of the procedure of Li (1992). A hypotonic lysate of crude spinach chloroplasts is freed of endogenous nucleic acids by precipitation with polyethyleneimine and passage over a column of DEAE-cellulose. The most dramatic purification, about 100-fold, is afforded by chromatography on the cation-exchanger S-Sepharose FF. Subsequent purification by hydroxyapatite-agarose (HA-Ultrogel) was modified from Li (1992) so that the high-salt S-Sepharose eluate was applied directly to the HA column, nonspecifically bound protein eluted with 1 M KCl, and RNase P activity then eluted with a linear phosphate gradient. Enzyme at this stage is approximately 800- to 900-fold purified over the chloroplast lysate. During further purification by Reactive Blue-agarose or heparin-agarose pseudo-affinity chromatography, activity cofractionates closely with one major polypeptide of ~ 30 kDa and to a lesser extent with 4–5 minor polypeptides of ~ 50 –90 kDa. At this time an assignment of activity to any polypeptide(s) is not yet possible. We are confident that the purified fractions contain only protein, because there is essentially no difference between the pattern of polypeptides detected by silver staining and that detected by Coomassie staining of polypeptides transferred to PVDF membrane (not shown). Details of the purification will be published separately.

Chloroplast RNase P cleaves a phosphorothioate-containing pre-tRNA

A semisynthetic precursor to yeast tRNA^{Phe} (Sampson & Uhlenbeck, 1988) is accurately processed both by

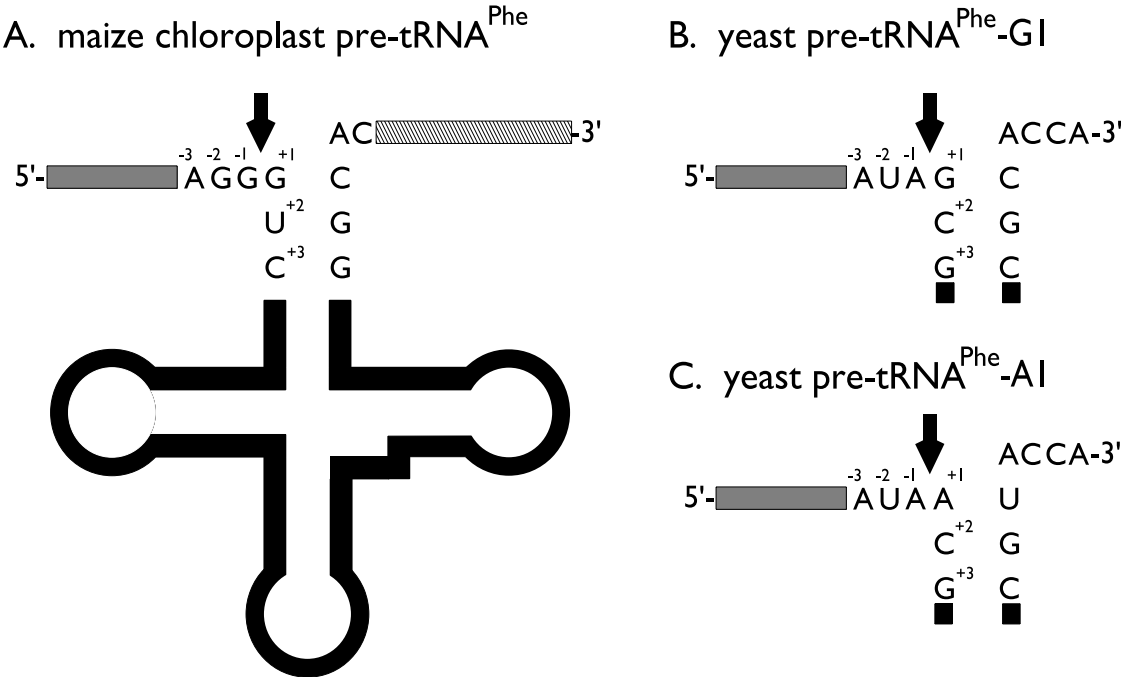


FIGURE 1. Schematic representation of precursor tRNA substrates. The mature tRNA domain starts with nucleotide +1. The large arrow indicates the RNase P cleavage site. **A:** Maize chloroplast pre-tRNA^{Phe}. The shaded box represents the 143-nt 5' leader sequence, the striped box represents the 116-nt 3'-trailer RNA, and the filled line indicates the 74-nt mature tRNA domain. **B:** Yeast pre-tRNA^{Phe} (substrate pre-G₁Phe) contains a 43-nt 5' leader RNA (shaded box) and a 73-nt mature tRNA^{Phe} domain followed by the 3' terminal CCA. **C:** Substrate pre-A₁Phe is identical to pre-G₁Phe except that G1:C72 is changed to A1:U72.

E. coli and by spinach chloroplast RNase P, and was used previously to ascertain the phosphorothioate sensitivity of the bacterial enzyme (Chen et al., 1997). For this reason, we employed the same substrate with chloroplast RNase P. As shown in Figure 1B, RNase P cleaves this pre-tRNA 5' to the phosphoryl of guanine at position +1 of the mature tRNA sequence. To control for the effects of phosphorothioate substitutions other than at the scissile bond, two substrates were employed: the wild-type yeast pre-tRNA^{Phe}, here referred to as pre-G₁Phe (Fig. 1B), in which the scissile bond is A₋₁↓pG₊₁, and a variant, called pre-A₁Phe (Fig. 1C), which is identical except that the scissile bond is A₋₁↓pA₊₁ (Chen et al., 1997). In this strategy, pre-G₁Phe uniformly substituted with GMPαS (called pre-G₁Phe[GαS]) or pre-A₁Phe uniformly substituted with AMPαS (pre-A₁Phe[AαS]) possess thiosubstitutions both at the scissile bond and at numerous other positions, so the corresponding substrates pre-A₁Phe[GαS] or pre-G₁Phe[AαS] control for the effects of thiosubstitution at positions other than the scissile bond.

To quantify the effect of phosphorothioate substitutions, initial reaction velocities were measured. Typical processing assays are presented in Figure 2, and the quantitative results are shown in Table 1. Unsubstituted pre-G₁Phe and pre-A₁Phe both are effective substrates for chloroplast RNase P (Fig. 2, compare lanes 2 and 7). The G₊₁ substrate is cleaved about twofold faster than the A₊₁ substrate, as indicated by the data in

Table 1. Cleavage of pre-G₁Phe substituted either with AMPαS or GMPαS is reduced to the same extent (Fig. 2, lanes 1 and 3, respectively), to 0.6–0.7 nM/min (see Table 1). This represents a five- to sixfold reduction

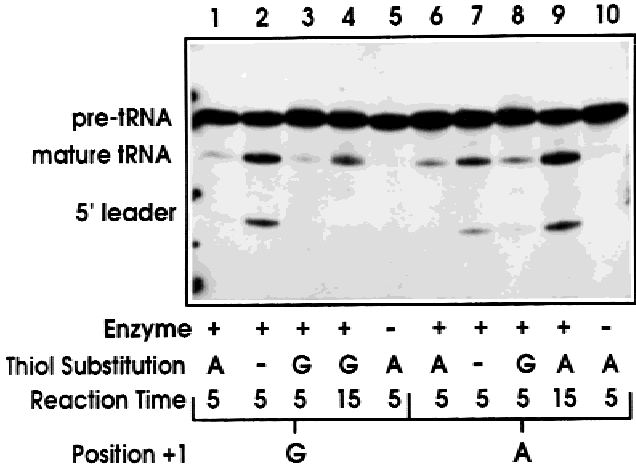


FIGURE 2. Chloroplast RNase P processing of unsubstituted or phosphorothioate-substituted yeast pre-tRNA^{Phe}. Precursor tRNAs were either unsubstituted or uniformly substituted with GMPαS and were labeled with [³²P]UMP. Each 20-μL reaction contained ~0.33 U of a 790-fold pure sample of hydroxyapatite-purified chloroplast RNase P, and 80–120 nM yeast precursor tRNA^{Phe}. The reaction was incubated at 37 °C for 5 or 15 min, and reaction products were separated by electrophoresis on a 10% polyacrylamide/7 M urea gel, an autoradiograph of which is shown.

TABLE 1. RNase P initial velocities with substrates pre-G₁Phe and pre-A₁Phe.

Precursor tRNA		
Nucleotide at position +1	Phosphorothioate nucleotide	Initial reaction velocity (nM tRNA formed min ⁻¹)
G	none	3.6
	GMP α S	0.6
	AMP α S	0.7
A	none	1.8
	GMP α S	0.6
	AMP α S	0.9

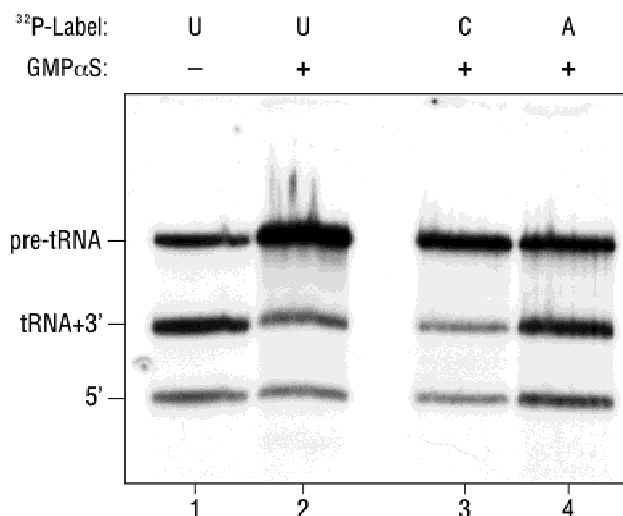
Initial velocities were calculated from the experiment shown in Figure 2, assuming linear reaction for the first 15 min.

from the cleavage rate of the unsubstituted precursor. Correspondingly, cleavage of pre-A₁Phe substituted either with AMP α S or GMP α S (Fig. 2, lanes 6 and 8) is reduced to a similar rate, 0.6–0.9 nM/min (Table 1) or about two- to threefold slower than the unsubstituted precursor. These results demonstrate that phosphorothioate substitution in pre-tRNA affects RNase P cleavage to the same extent whether or not the scissile bond contains a phosphorothioate. In other words, little if any of the reduced cleavage can be attributed to the thio-substitution at the scissile bond itself.

Precise cleavage of a phosphorothioate bond by chloroplast RNase P

These results indicated that chloroplast RNase P cleaves phosphorothioate-containing bonds. To determine whether cleavage occurs at the authentic RNase P site, the sequence surrounding the cleavage site was determined in processing reactions performed with a homologous substrate. We previously determined the nucleotide sequence at the cleavage site in maize chloroplast pre-tRNA^{Phe} (precursor I of Wang et al., 1988), the substrate used for purification of chloroplast RNase P. Cleavage normally occurs 5' to the phosphoryl of G₊₁ within the sequence –Ap_{–2}Gp_{–1}G_{OH}↓p₊₁Gp₊₂UpC–. The products resulting from RNase P cleavage occurring at this position are a 143-nt 5' leader RNA and a 190-nt species containing mature tRNA plus 3' trailer RNA. As Figure 3 shows, equivalent-sized products are generated from substrates that were unsubstituted (lane 1) or uniformly substituted with GMP α S (lanes 2–4), consistent with chloroplast RNase P cleavage at the correct site in both substrates.

To precisely localize the position of chloroplast RNase P cleavage, tRNA + trailer reaction products were recovered and subjected to 5'-end analysis. Ribonuclease T2 hydrolyzes 5'-mature tRNA to produce a unique ribonucleoside 3', 5' bisphosphate (pNp) from the 5' end of the tRNA, and ribonucleoside 3' monophosphates (Np) from all internal positions. The RNase

**FIGURE 3.** Preparative-scale processing by chloroplast RNase P of phosphorothioate-substituted chloroplast pre-tRNA^{Phe}. Precursor tRNAs were either unsubstituted or were uniformly substituted with GMP α S and were labeled singly with [³²P]UMP, [³²P]AMP, or [³²P]CMP. Each 100- μ L reaction contained ~2 U HA-Ultrogel-purified RNase P and ~5 nM maize chloroplast pre-tRNA^{Phe}. The reaction was incubated at 37 °C for 45 min; products were separated by electrophoresis and detected by autoradiography.

T2 digestion products were separated using anion-exchange thin-layer chromatography. To detect products resulting from cleavage at positions other than +1, pre-tRNAs were labeled singly with [³²P]UMP, [³²P]AMP, or [³²P]CMP. Table 2 enumerates the possible outcomes of 5'-end analysis with the labeled precursor tRNAs used in this study.

The results of this analysis are shown in Figure 4. Cleavage products from unsubstituted [³²P]UMP-labeled pre-tRNA^{Phe} contain only 5'-terminal pGp, indicating that chloroplast RNase P processing occurred at posi-

TABLE 2. Potential products of tRNA 5' end analysis.

Substrate	³² P label	Potential cleavage position(s)	Resulting 5' nucleotide
Unsubstituted	p*U	+1	pGp*
		+2	p*Up
		–3, –2, –1, +3	NL ^a
G α S-substituted	p*U	+1	p _(S) Gp* ^b
		+2	p*Up
		–3, –2, –1, +3	NL
	p*C	–3, –2, –1, +1	NL
		+2	pUp*
		+3	p*Cp
	p*A	–2, –1, +1, +2	NL
		–3	p*Ap
		+3	pCp*

*: the ³²P-labeled phosphate.

^aNL: no nucleotide would be labeled under these conditions.

^bp_(S)Gp* is guanosine 3'-[³²P]phosphate, 5' phosphorothioate.

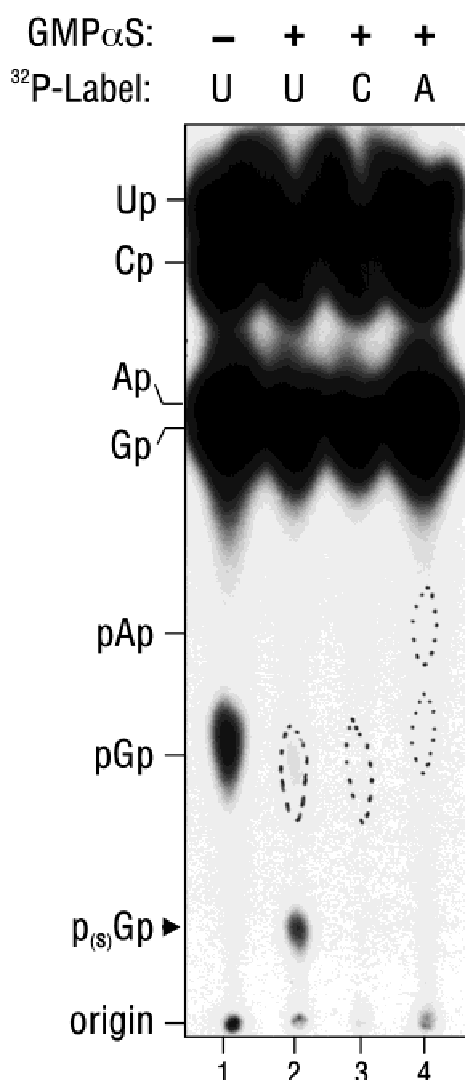


FIGURE 4. Determination of RNase P cleavage site in chloroplast pre-tRNA^{Phe}. The tRNA + 3' trailer products from a preparative-scale processing reaction (shown in Fig. 3) were digested with RNase T2. The resulting nucleotides were separated on PEI-cellulose thin-layer plates developed with 1.6 M LiCl and were detected by autoradiography. The migration positions of unlabeled pAp and pGp, added as internal standards, are indicated by dotted lines. The identity of p(s)Gp was determined as described in Materials and Methods.

tion +1. In RNase P products from GMP α S-substituted precursor labeled with [³²P]AMP (Fig. 4, lane 4) or [³²P]CMP (Fig. 4, lane 3), no nucleoside bisphosphates are visible, indicating that cleavage of phosphorothioate-substituted pre-tRNA^{Phe} did not occur 5' to phosphates at positions -3, +2, or +3.

From [³²P]UMP-labeled, GMP α S-substituted tRNA + trailer RNA, RNase T2 digestion releases two pNp species: a low-abundance species that comigrates with pGp as well as a prominent, slowly migrating species near the chromatographic origin (Fig. 4, lane 2). We identified the slowly migrating species as guanosine 3'-[³²P]phosphate, 5'-O-(thiomonophosphate) [p(s)Gp*].

The identity of this species is based on two lines of evidence: first, its migration is identical to that of commercially synthesized p(s)Gp. Second, when the slowly migrating material was eluted from the thin-layer plate and treated with iodoethanol to hydrolyze phosphorothioate-containing bonds, guanosine 3'-[³²P]monophosphate was released (see Methods; data not shown). The small amount of labeled material comigrating with internal marker pGp (Fig. 4, lane 2) corresponds most likely to pGp derived by oxidation of the phosphorothioate during handling (see also Chen et al., 1997).

Metal coordination at the scissile *pro-R_P* oxygen is unlikely to be rate limiting for chloroplast RNase P

To further investigate the mechanism whereby uniform phosphorothioate substitution reduces cleavage velocity, we determined Michaelis-Menten steady-state kinetic constants. We expect that an inhibition resulting from decreased Mg²⁺ coordination will reduce the catalytic rate constant, k_{chem} , by an amount commensurate with the decreased affinity of Mg²⁺ for sulfur versus for oxygen, or about 10⁻³- to 10⁻⁴-fold if the catalytic step is the rate-limiting component of k_{cat} (Pecoraro et al., 1984; discussed in Chen et al., 1997). Even if catalysis is not rate limiting, thiosubstitution will produce a profound inhibition of multiple-turnover reactions (cf. Chen et al., 1997). On the other hand, if there is no metal coordination by the *pro-R_P* oxygen, we expect an extent of inhibition commensurate with that of the reduced reactivity of sulfur-linked phosphorus, which has been measured at 4- to 11-fold for model reactions (Herschlag et al., 1991). To this end we compared substrate dependence in kinetic assays using unsubstituted chloroplast pre-tRNA^{Phe} and GMP α S-containing pre-tRNA^{Phe}. The kinetic constants obtained from the best-fitting Michaelis-Menten curves are summarized in Table 3. Unsubstituted pre-tRNA^{Phe} is cleaved by chloroplast RNase P with a V_{max} of 2.7 nM 5' leader formed min⁻¹. The observed K_M , 16 nM pre-tRNA, is similar to that for bacterial RNase P (Stark et al., 1978;

TABLE 3. Kinetic constants for unsubstituted and GMP α S-substituted chloroplast pre-tRNA^{Phe}.

Thiosubstitution	K_M (nM)	V_{max} (nM min ⁻¹)	V_{max}/K_M (min ⁻¹)
none	16. \pm 3	2.7 \pm 0.1	0.17 \pm 0.02
GMP α S	12. \pm 3	0.38 \pm 0.03	0.032 \pm 0.006

Substrate titrations were performed using pre-tRNAs that were either unsubstituted or 100% substituted with GMP α S. Each 20- μ L reaction contained 0.1 U of HA-Ultrogel-purified RNase P and increasing amounts of precursor tRNA. Reactions were performed and analyzed as described in Materials and Methods.

McClain et al., 1987; Reich et al., 1988; Smith & Pace, 1993). In comparison, for pre-tRNA^{Phe} uniformly substituted with GMP α S, V_{max} is reduced about sevenfold, to 0.38 nM 5' leader formed min⁻¹, although K_M remains almost unchanged at 12 nM pre-tRNA. The overall throughput of chloroplast RNase P in processing these two pre-tRNAs was estimated from the value of V_{max}/K_M , which, as indicated in Table 3, decreases fivefold upon phosphorothioate substitution. Chloroplast RNase P is hence fivefold less efficient when processing a phosphorothioate-substituted pre-tRNA than an unsubstituted precursor. This reduction in V_{max} and in V_{max}/K_M is markedly less than that expected for a disruption of metal coordination, but is of a magnitude consistent simply with reduced chemical reactivity of the phosphorothioate center.

DISCUSSION

Chloroplast RNase P was previously shown to be a protein enzyme, lacking the RNA subunit common to all other characterized RNase Ps (Wang et al., 1988; Li, 1992). Further purification as reported here corroborates the protein nature of this enzyme. On several grounds, we believe that this activity is an authentic plastid-specific RNase P. First, only one peak of cleavage activity against chloroplast pre-tRNA is detected during chromatographic purification (Li, 1992; Thomas, 1996). Second, the activity we have purified displays a substrate specificity consistent with that expected for a plastid-specific enzyme. The partially purified enzyme exhibits approximately equal activity against maize chloroplast pre-tRNA^{Phe} and yeast nuclear pre-tRNA^{Phe}-YF0 ($V_{max}/K_M = 0.077 \pm 0.043$ min⁻¹ vs. 0.073 ± 0.018 min⁻¹, respectively, when assayed with 0.05 U HA-Ultrogel-purified enzyme [Li, 1992]), whereas the nuclear/cytoplasmic RNase P from wheat embryo is inactive against the chloroplast precursor (see Fig. 25 of Oommen, 1991). Finally, the chloroplast pre-tRNA^{Phe} used for purification assays is not a substrate for *E. coli* RNase P RNA (Li, 1992), presumably because it lacks an encoded 3'-CCA sequence.

To compare the catalytic mechanism of a protein RNase P to that of an all-RNA RNase P, we tested partially purified preparations of chloroplast RNase P for their ability to cleave a substrate molecule containing a phosphorothioate substitution at the scissile bond. Catalysis by the RNA subunit of *E. coli* RNase P depends absolutely upon the presence of a Mg²⁺ or Mn²⁺ ion coordinated by the *pro-R_P* nonbridging oxygen of the scissile bond in the bacterial RNase P reaction. Indeed, in the presence of Mg²⁺, *E. coli* RNase P RNA cleaves phosphorothioate-substituted pre-tRNA at the unsubstituted bond closest to the correct position, albeit with a 10⁴-fold reduced rate constant (Chen et al., 1997). In striking contrast, RNase P from spinach chloroplasts can accurately and efficiently process a pre-

cursor tRNA possessing a *pro-R_P* phosphorothioate substitution. Analysis of the reaction products demonstrates that chloroplast RNase P cleavage of phosphorothioate-containing pre-tRNA occurs at the correct position, 5' to the thiophosphoryl of nucleotide +1, the first nucleotide of the mature tRNA domain. Cleavage at alternate, nonthiolated, bonds was not detected. The ability of the chloroplast enzyme to hydrolyze an *R_P*-sulfur-containing phosphodiester bond suggests that Mg²⁺ is not coordinated, directly or indirectly, by the substrate *pro-R_P* oxygen during the chloroplast RNase P reaction.

Because the rate-limiting step of steady-state catalysis is unknown for the chloroplast enzyme, these results do not directly inform us of the effect of thiosubstitution on the chemical step, but they suggest that the rate-limiting step, chemical or not, is unaffected by thiosubstitution. Our data are most consistent with the rate-limiting step in the chloroplast RNase P reaction being a chemical step involving the *pro-R_P* oxygen without direct metal-ion coordination. We cannot yet exclude the possibility that the chemical step involves metal coordination and that upon thiosubstitution the rate-limiting step changes from a nonchemical to a chemical step.

Because RNA and protein phosphodiesterase and related activities display a diversity of reaction mechanisms, we use the presence or absence of *R_P*-thiosensitivity not to differentiate between RNA enzymes and protein enzymes as classes, but rather as a hallmark of the RNA-catalyzed RNase P reaction. To explain the catalytic chemistry of chloroplast RNase P, it is useful to compare it to other protein enzymes which catalyze phosphodiester bond hydrolysis, generating products with 3'-hydroxyl and 5'-phosphoryl termini, and for which phosphorothioate sensitivity is known. These fall generally into two classes. The first class contains those enzymes that are *R_P*-specific (cleave *R_P*-thiosubstituted but not *S_P*-thiosubstituted scissile phosphodiester), and consists of helix-specific hydrolases and 3'-exonucleases. The second class contains enzymes that are *S_P*-specific (*R_P*-thiosensitive), and consists mostly of single-strand-specific endonucleases.

Members of the *R_P*-specific class of protein nucleases include the duplex-specific RNase III (*E. coli*: Nicholson et al., 1988) and RNase H1 (*E. coli*: Uchiyama et al., 1994) and restriction endonucleases *EcoRI* (Connolly et al., 1984), *EcoRV* (Grasby & Connolly, 1992), *SfiI*, *HpaII*, and the MuA transposase (Mizuuchi et al., 1999). Scissile *S_P*-phosphorothioate inhibition of RNase H, *SfiI*, *HpaII*, and MuA transposase was not reversed by addition of Mn²⁺ (Uchiyama et al., 1994; Mizuuchi et al., 1999), consistent with complex enzyme-substrate or enzyme-metal-substrate interactions. The exonuclease group includes the 3' → 5' proofreading exonuclease activity of DNA polymerase (*E. coli polI*: Brautigam & Steitz, 1998; phage T4: Gupta et al., 1982), *E. coli*

exonuclease I (Brody & Doherty, 1985), and human serum 3'-exonuclease (Koziolekiewicz et al., 1997).

The S_P -specific class of nucleases is more limited, and consists of the single-strand-specific Zn(II)-dependent endonucleases (P1 nuclease: Potter et al., 1983a; S1 nuclease: Potter et al., 1983b; mung bean nuclease: Hamblin et al., 1987), as well as the second, hydrolytic, step of the snake venom exonuclease reaction (Burgers et al., 1979). A few nucleases are known to be inhibited both by R_P - and S_P -thiosubstitution, but of these, only DNase I (Spitzer & Eckstein, 1988) is comparable to RNase P in generating 5'-P and 3'-OH termini.

No direct comparison can be made between these nucleases and chloroplast RNase P, because its S_P -thiosensitivity has not been tested. However, comparison among these activities is instructive. Current proposed mechanisms for the *E. coli* RNase P RNA-catalyzed reaction invoke three catalytic Mg^{2+} ions (Hardt et al., 1993; Smith & Pace, 1993; Beebe et al., 1996). One of these Mg^{2+} ions clearly coordinates the *pro-R_P* oxygen (Chen et al., 1997), and was proposed to coordinate the attacking hydroxide. On the other hand, the 3' \rightarrow 5' exonuclease domain of *E. coli* DNA polymerase I (Beese & Steitz, 1991; Steitz & Steitz, 1993) does not employ a metal ion in the vicinity of the *pro-R_P* oxygen of the scissile bond. Instead, the hydroxyl moiety of tyrosine residue 497 can hydrogen bond to and help position the attacking nucleophilic hydroxide ion, and might interact with the *pro-R_P* oxygen of the scissile bond. We speculate on this basis that chloroplast RNase P is capable of hydrolyzing an R_P -phosphorothioate bond because it uses amino-acid side chains to coordinate the attacking nucleophile in a similar manner.

MATERIALS AND METHODS

Enzymes and reagents

Bacteriophage RNA polymerases were purchased from U.S. Biochemicals. RNase inhibitor (InhibitAce, now called Prime RNase Inhibitor) was purchased from 5' \rightarrow 3' Inc. Restriction endonucleases were from New England Biolabs or Promega. All DNA modifying enzymes were used with their supplied buffers. All chemicals were reagent or ultrapure grade. Organic-free deionized water (Barnstead Nanopure, 0.2 mm filtered) was used throughout.

Preparation of pre-tRNA substrates

The precursor to chloroplast pre-tRNA^{Phe}, designated pre-NXPhe, consists of maize chloroplast tRNA^{Phe} (74 nt) plus the chloroplast-encoded 143-nt 5' extension and 116-nt 3' extension ("precursor I" of Wang et al., 1988). This substrate was transcribed from plasmid pTUC8-NXPhe (Wang et al., 1988) linearized with *Hind*III. Precursors to yeast tRNA^{Phe} in which the first base of the mature tRNA was G (wild-type, pre-G₁Phe) or A (pre-A₁Phe) were prepared by transcription

of *Bst*NI-linearized plasmids pBSYF0 or pBSYF0-A1U72 (Chen et al., 1997).

Transcription reactions were performed as described (Chen et al., 1997) with 50 μ g DNA template/mL, 800 U RNA polymerase/mL, and 100–500 μ Ci [α -³²P]rNTP (New England Nuclear)/mL. RNAs were separated by electrophoresis on 10% polyacrylamide/7 M urea gels and purified essentially as described (Wang et al., 1988). Eluted RNA was ethanol precipitated from 2 M NH₄OAc, dried, and resuspended in sterile water.

The correct label and presence of R_P phosphorothioate in the transcribed RNA were confirmed in two ways. First, samples of most transcripts, and one sample of gel purified product, were digested with nuclease P1, which cannot cleave R_P phosphorothioate-containing phosphodiester bonds. The reaction products were separated by thin-layer chromatography on PEI-cellulose (described below). Both unsubstituted and substituted RNAs yielded a single nucleoside 3'-monophosphate identical with the one used to label the RNA during transcription. Substituted RNAs also contained nuclease-resistant mono-, di-, and trinucleotides. Second, the purified RNAs were treated with 20–80 mM iodoethanol (2 min, 95 °C) and analyzed by denaturing gel electrophoresis. Unsubstituted RNA was unaffected by this treatment, whereas thiolated RNA was extensively fragmented.

Chloroplast RNase P processing assays

One unit of chloroplast RNase P activity is defined as the amount of enzyme giving a reaction velocity of 1 nM 5'-leader released min⁻¹ under standard assay conditions (linear with respect both to time and to enzyme concentration in a 5-min reaction). Standard processing assays (20 μ L) were done with ~0.3–3 U RNase P and ~5 nM [³²P]pre-tRNA in processing buffer (Wang et al., 1988) containing 50 mM KCl and 15 mM MgCl₂. Reactions were incubated for 5–10 min at 37 °C, then stopped and analyzed by gel electrophoresis as described (Wang et al., 1988). RNA species were quantitated by measuring Cerenkov radiation in gel slices corresponding to each RNA and correcting for background in each lane.

The same procedure was utilized for kinetic assays, except that pre-tRNA^{Phe} concentrations ranged from 1–120 nM (0.06–7.5 times wild-type K_M) and incubation times were 1 or 5 min at 37 °C. Only reactions giving <20% cleavage were used. Using data from more than four assays, kinetic constants were calculated by nonlinear curve fitting of the Michaelis-Menten and Hill equations using Prism (GraphPad Software) software.

Preparation of chloroplast RNase P

Spinach chloroplast RNase P was purified by a modification (Thomas, 1996) of the procedures of Wang et al. (1988) and Li (1992). (A preliminary account appeared in Thomas et al. [1995].) Similar results were obtained when buffers other than those containing phosphate were prepared either with EDTA or EGTA. Crude chloroplasts were isolated essentially as described (Wang et al., 1988; Gegenheimer, 1990) starting with 2–3 kg supermarket spinach leaves. Twice-washed chloroplasts were lysed by resuspension in Hypotonic Lysis buffer (Gegenheimer, 1990) containing 0.1 \times protease inhibitor mix

(1× protease inhibitors is 1 mM benzamidine, 1 mM PMSF, 1 mM benzamide, and 5 mM ϵ -amino-*n*-caproic acid). The lysate was brought to 0.15 M $(\text{NH}_4)_2\text{SO}_4$ (4% of saturation at 4°C) and large nucleic acids were precipitated with 0.1% polyethyleneimine. The supernatant from this step was further fractionated by raising the $(\text{NH}_4)_2\text{SO}_4$ saturation to 60% at 4°C. The resulting pellet was resuspended in F-buffer (20 mM Tris-HCl [pH 8.0 at 20°C], 1 mM NaEGTA [pH 8.0], 20% glycerol [v/v], 0.5% Genapol X-080 [Fluka], 1 mM DTT, 0.1× protease inhibitors) containing 50 mM KCl, and was then dialyzed against two changes of the same buffer. The dialysate was fractionated on a DEAE-cellulose column (Whatman DE-52) equilibrated at pH 8.0 with the above buffer. RNase P activity was eluted from the column using a linear gradient of 50 mM to 300 mM KCl in F-buffer. Fractions with RNase P activity were pooled and dialyzed against two changes of H-buffer containing 100 mM KCl and 40% glycerol (H-buffer is 20 mM HEPES-KOH [pH 7.0], 0.1 mM NaEDTA [pH 8.0], 20% glycerol [v/v], 0.5% Genapol X-080, 1 mM DTT, 0.1× protease inhibitors). The dialysate was fractionated on S-Sepharose FF (Pharmacia) equilibrated with the same buffer and eluted with a linear gradient from 100 mM to 500 mM KCl in H-buffer. Fractions with RNase P activity were pooled and loaded directly onto a column of HA-Spectra/Gel (Spectrum) equilibrated with H-buffer containing sufficient KCl to raise the conductivity to that of the input pool (200–300 mM KCl). The column was washed to baseline absorbance with 1 M KCl H-buffer and RNase P was eluted with a linear gradient from 50 to 500 mM P-buffer (K- PO_4 [pH 7.0], 20% glycerol, 0.1 mM NaEDTA [pH 8.0], 0.5% Genapol X-080, 1 mM DTT, 0.1× protease inhibitors). Fractions with RNase P activity were pooled and dialyzed against two changes of H-buffer containing 20 mM KCl. The analyses reported here were performed with four independent preparations of RNase P, purified to the S-Sepharose step (preparation P19-S, ~300-fold purified) or to the HA-Ultrogel stage (~500–800-fold purified: P10-HA, P19-HA2, and P21-HA).

5'-end analysis

Cleavage with RNase T2 was performed as in Chen et al. (1997) but in a 0.5-mL microcentrifuge tube, using between 1 and 10 U RNase T2 (Calbiochem) for 2–4 h at 50°C. The reaction mix was spotted onto pre-washed (Chen et al., 1997) polyethyleneimine-cellulose thin-layer plates (Polygram Cel 300 MN PEI, Brinkman Instruments), which were then developed with 1.6 M LiCl and visualized by autoradiography.

Individual nucleotide species on the thin-layer plate were recovered (Volckaert et al., 1976) for further analysis by elution into 2 M triethylammonium acetate (pH 7.6). The eluate was dried under vacuum, dried twice from 50 μL of sterile water, and resuspended in 15 μL of sterile water. To test for the presence of phosphorothioate, selected nucleotides were treated with 2-iodoethanol (Sigma): 10 μL of resuspended nucleotide was mixed with 1 μL of concentrated 2-iodoethanol (~13 M) and incubated at 95°C for 2 min. The mixture was then separated on PEI-cellulose thin-layer plates developed with 1.6 M LiCl, and visualized by autoradiography. Control reactions demonstrated that unsubstituted pGp and pAp were unaffected by this treatment.

Authentic guanosine 5'-phosphorothioate, 3'-monophosphate, $p_{(S)}\text{Gp}$, was synthesized by Macromolecular Resources,

Inc. (Colorado State University, Fort Collins, Colorado, USA). After synthesis, $p_{(S)}\text{Gp}$ was precipitated with ethanol in the presence of 15 mM MgCl_2 .

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