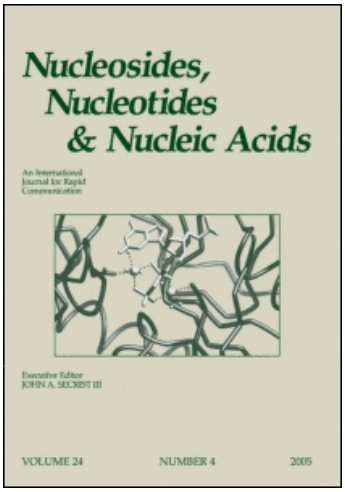

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Role of Metal Ions In The Cleavage Mechanism by The E. Coli Rnase P Holoenzyme

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ROLE OF METAL IONS IN THE CLEAVAGE MECHANISM BY THE E. COLI RNASE P HOLOENZYME

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ABSTRACT. Processing of a precursor tRNA carrying an Rp-phosphorothioate modification at the RNase P cleavage site is most efficiently promoted by Cd^{2+} ions in reactions catalyzed by the *E. coli* RNase P holoenzyme and the RNA subunit alone.

Ribonuclease P (RNase P) is an essential structure-specific endoribonuclease that generates the mature 5'-ends of tRNAs. The *E. coli* RNase P holoenzyme is composed of an RNA and protein subunit, both of which are essential *in vivo*. Yet *in vitro*, RNA subunits of bacterial RNase P enzymes were shown to be catalytically active in the absence of the protein subunit[1]. Processing of precursor tRNAs (ptRNAs) by RNase P is an essentially irreversible reaction yielding 3´-OH and 5´-phosphate termini. Mg^{2+} or Mn^{2+}-ions are essential cofactors in this reaction, and a solvated metal hydroxide is assumed to act as the nucleophile in an S_N2 in-line displacement mechanism[2, 3].

We recently investigated processing of a bacterial $ptRNA^{Gly}$, carrying an Rp-phosphorothioate modification at the RNase P cleavage site, in the reaction catalyzed by *E. coli* RNase P RNA in the absence of the protein subunit[4]. The study was performed at pH 6.0, where the chemical step is rate-limiting. The following results were obtained: (i) The Rp-phosphorothioate modification reduced the rate of processing by *E. coli* RNase P RNA at least 1000-fold in the presence of Mg^{2+} as the only divalent cation, while having no effect on ptRNA ground state binding to RNase P RNA. (ii) Processing of the modified substrate was largely restored in the presence of the "thiophilic" Cd^{2+} as the only divalent metal ion; a rescue of activity was also observed in the presence of Mn^{2+}, although to a lower extent. These results demonstrated that catalysis by *E. coli* RNase P RNA involves direct metal ion coordination to the *(pro)*-Rp substituent at the RNase P cleavage site[4].

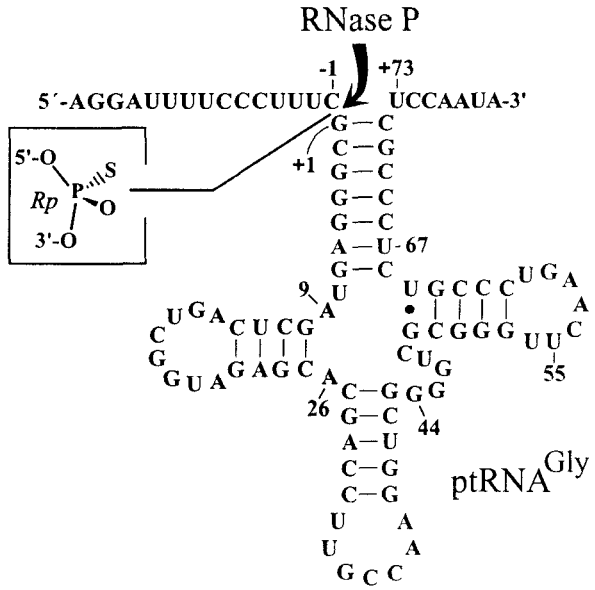

FIG. 1. Secondary structure of the glycine-specific ptRNA[5]. *In vitro* transcripts of ptRNA^{Gly} used in this study carried Rp-phosphorothioate modifications 5′ of every G residue (all-Rp ptRNA^{Gly}). Only the modification at the RNase P cleavage site is depicted. The substrate was synthesized by T7 RNA polymerase in the presence of Sp-GTPαS instead of GTP[6]. The arrow indicates the canonical RNase P cleavage site.

In the study presented here, we addressed the question whether the protein subunit of *E. coli* RNase P, named C5, may alter the metal ion dependence of cleavage of the Rp-phosphorothioate-modified ptRNA substrate (FIG. 1). We have recently shown that ptRNA substrates, carrying either a single Rp-phosphorothioate-modification at the RNase P cleavage site or multiple Rp-phosphorothioate-modifications 5′of every G residue including the RNase P cleavage site, behave very similar with respect to the metal ion requirements of cleavage by *E. coli* RNase P RNA[4]. Thus, we synthesized a ptRNA^{Gly} substrate modified 5′of every G residue including the RNase P cleavage site (termed all-Rp ptRNA^{Gly}) by *in vitro* transcription[4] (FIG. 1), using the dinucleotide ApG as initiator of transcription in order to facilitate 5′-^{32}P-end-labeling of ptRNAs[7].

Processing was analyzed in single turnover kinetic experiments, using trace amounts of 5′-^{32}P-end labeled all-Rp ptRNA^{Gly} or unmodified ptRNA^{Gly}, 1 μM *E. coli* RNase P RNA and 1 μM C5 protein. Assays were performed at pH 7.0 in the presence of 100 mM NH_4OAc and 10 mM divalent metal ions. The pH of 7.0 was chosen because the

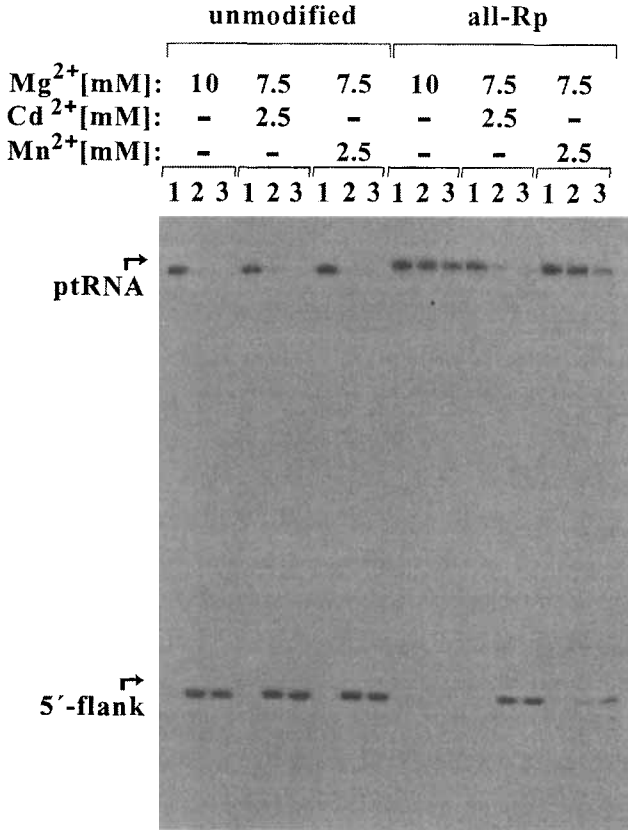

FIG. 2. Time courses of processing of 1-10 nM 5′-^{32}P-end labeled unmodified ptRNA^{Gly} (left part) or all-Rp ptRNA^{Gly} (right part) by the *E. coli* RNase P holoenzyme (1 μM RNase P RNA, 1 μM C5 protein[8]) in the presence of 40 mM TrisOAc (pH 7.0), 0.1 mM EDTA, 100 mM NH_4OAc and 10 mM divalent metal ions at 37°C. Substrates and enzyme were preincubated separately for 10 min (ptRNAs) and 60 min (RNase P holoenzyme) at 37°C under processing assay conditions. Lanes 1, substrate incubated in the absence of enzyme for 20 min at 37°C; lanes 2 and 3, extent of RNase P-catalyzed processing after 5.5 min (lanes 2) and 20 min (lanes 3). Reactions were stopped as described before[5], and samples were analyzed on a 25% polyacrylamide/urea gel. Bands corresponding to ptRNAs and 15-nt long 5′-cleavage products (5′-flank) are indicated on the left.

dissociation constant for the C5 protein-RNase P RNA interaction was reported to be essentially independent of pH in the range of pH 7.0 to 9.5[9]. Although the pH of 7.0 is assumed to be in the range where another step than chemistry becomes rate-limiting at least in the RNA alone reaction[5], we observed very similar metal ion effects with the RNase P holoenzyme at pH 7.0 (FIG. 2) as with the catalytic RNA alone under conditions of rate-limiting chemistry (pH 6.0)[4], as outlined below.

At 10 mM Mg^{2+} as the sole metal ion, processing of all-Rp ptRNA^{Gly} was severely impaired in the RNase P holoenzyme reaction, resulting in not more than 5% product formation after 20 min at 37°C (FIG. 2). Mn^{2+} can replace Mg^{2+} in numerous metal ion-assisted enzyme reactions, and its relatively higher affinity for sulfur[10] in comparison with Mg^{2+} has been the reason for the use of Mn^{2+} ions in metal ion rescue experiments in order to identify sites of direct metal ion coordination to nonbridging phosphate oxygens in RNA molecules[6]. Partial replacement of Mg^{2+} with Mn^{2+} (7.5 mM Mg^{2+}/2.5 mM Mn^{2+}) resulted in a significant rescue of all-Rp ptRNA^{Gly} cleavage in the RNase P holoenzyme reaction. A much stronger rescue of cleavage was observed in the presence of the "thiophilic" Cd^{2+} (7.5 mM Mg^{2+}/2.5 mM Cd^{2+}). Under these conditions, processing of all-Rp ptRNA^{Gly} was essentially complete within 5.5 min (FIG. 2). The metal ion rescue effects, which are qualitatively the same as those observed for the RNA alone reaction[4], support the notion that direct metal ion coordination occurs to the (*pro*)-Rp substituent at the scissile phosphodiester in the RNase P processing reaction[4].

Based on numerous biochemical studies, the protein subunit C5 of *E. coli* RNase P is thought to interact with several helical regions of the catalytic RNA on the opposite side of the surface to which substrates bind[11, 12]. The differential roles of Mg^{2+}, Mn^{2+} and Cd^{2+} ions in catalysis of cleavage of unmodified and Rp-phosphorothioate-modified substrates followed essentially the same rules in the RNase P RNA alone[4] and in the RNase P holoenzyme reaction (FIG. 2). This indicates that the protein cofactor of RNase P is not involved in the catalytic step, which is in line with the lack of experimental evidence for contacts between RNase P substrates and the protein cofactor of bacterial RNase P enzymes[11, 12]. However, the protein is assumed to stabilize the active RNA conformation[12], which may also involve a structural stabilization of binding sites for catalytic metal ions.

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