Bimodal Action of Alkaline Earth Cations on *Dictyostelilum discoideum* Ribonuclease P Activity†

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ABSTRACT: The ribonucleoprotein ribonuclease P (RNase P) cleaves all tRNA precursors endonucleolitically to produce the mature 5′-end. *Dictyostelium discoideum* RNase P displays an absolute requirement for Mg^{2+}. Only the alkaline earth cations Ca^{2+}, Sr^{2+}, and Ba^{2+}, under appropriate conditions can substitute to some extent for Mg^{2+}. The transition metals Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} are efficient inhibitors of the enzyme activity. Ca^{2+}, Sr^{2+} and Ba^{2+}, in the presence of Mg^{2+}, exhibit a bimodal action at the kinetic phase of the reaction. Kinetic analysis of the activation phase revealed that Ca^{2+}, Sr^{2+}, or Ba^{2+} attached on a specific site of RNase P act as nonessential-noncompetitive activators. Further additions of Ca^{2+}, Sr^{2+}, or Ba^{2+} cause noncompetitive inhibition on the RNase P reaction, indicating that RNase P possesses a second binding site responsible for the inhibitory effect of Ca^{2+}, Sr^{2+}, and Ba^{2+}. Both activator and inhibitory sites can be occupied by Ca^{2+}, Sr^{2+}, or Ba^{2+} at the same time.

Ribonuclease P (RNase P) is the endonuclease responsible for maturation of 5′-termini of tRNA precursors. In all organisms so far investigated, this enzyme is composed of both RNA and protein (*1*). In bacteria, the RNA subunit alone suffices for activity; the protein component although essential for the enzyme activity in vivo, can be substituted by high salt concentrations in vitro (*2*). Similar behavior has not yet been demonstrated in RNase P enzymes examined from archaea or eukarya. Bacterial RNase P RNA is the only known trans-acting ribozyme in nature. All ribozymes (cis or trans-acting) have an absolute dependence on metal ions, and they are considered as an important new class of metalloenzymes (*3*).

Metal ions are essential for efficient chemical catalysis by RNase P. They are required for the stabilization of RNase P RNA structure. They can perform the electrostatic shielding function in the absence of the protein subunit and preserve the structural properties necessary to keep the substrate and the enzyme in appropriate conformations for reaction (*4*). The processing of tRNA precursors by RNase P holoenzymes or/and bacterial RNA subunits is Mg^{2+} dependent. It has been proposed that the 2′-hydroxyl group of the ribose at the cleavage site of precursor tRNAs is important for efficient RNase P cleavage. This 2′-OH functions as a ligand for Mg^{2+} and may be involved in the actual mechanism of cleavage (*5*, *6*). Likewise, there is data suggesting that the true substrate for RNase P is a precursor which harbors a Mg^{2+} coordinated in the vicinity of the cleavage site (*7*). It has also been reported that a Mg^{2+} ion is involved in the stabilizaton of the Watson−Crick interaction between *Escherichia coli* RNase P RNA (M1 RNA) and its substrate (*8*). Furthermore, the formation of *E. coli* RNase P holoenzyme is efficiently promoted by the presence of specific monovalent and divalent cations. M1 RNA adopts the specific three-dimensional structure required for recognition by the C5 protein in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+} (*9*).

It has been proposed that the Mg^{2+} ion(s) bound closely to A258 and A295 of M1 RNA play a significant role during catalysis (*8*, *10*). Kinetic analysis showed that the catalytic mechanism of *E. coli* RNase P RNA has a cooperative dependence upon Mg^{2+} concentration, and at least three Mg^{2+} ions are required for optimal activity, suggesting a multiple metal ion mechanism (*6*). Precursor tRNA substrates carrying a single Rp or Sp-phosporothioate modification at the RNase P cleavage site are hydrolyzed at much slower rates than the natural substrate by RNase P (*11*). However, processing of the Rp-diastoreomeric substrate was rescued by the addition of Cd^{2+} or Mn^{2+} as the sole cofactor, demonstrating the direct metal coordination to the *pro*-Rp oxygen. In the case of Cd^{2+}, Hill plot analysis suggested that two metal ions participate in the reaction. For the reaction catalyzed by *E. coli* RNase P RNA, direct metal ion coordination to the *pro*-Rp oxygen was further supported by Mn^{2+} rescue experiments using a different precursor tRNA substrate with an Rp-phosphorothioate modification at the RNase P cleavage site (*12*).

Can other divalent cations substitute for Mg^{2+}? Surrat et al. (1990) (*13*) reported that Sr^{2+}, Ca^{2+}, and Ba^{2+} ions support *E. coli* RNase P mediated tRNA processing in the presence of Mg^{2+} concentrations otherwise insufficient to permit processing. Under certain conditions, Mn^{2+} and Ca^{2+} can each replace Mg^{2+} in the processing of precursor tRNAs by M1 RNA and P RNA subunit of RNase P from *Bacillus subtilis* (*4*, *14*−*16*). Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} ions are all effective inhibitors of the M1 RNA catalyzed cleavage reaction (*4*, *15*). Recently, it was reported that the rate of processing by *E. coli* RNase P RNA, using tRNA substrates

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carrying a single Rp-phosphorothioate modification at the RNase P cleavage site, was reduced at least 1000-fold. Processing of the Rp-diasteromeric substrate was largely restored in the presence of "thiophilic" Cd^{2+} as the only divalent metal ion (*11*).

The present paper deals with studies on the metal ion requirements of RNase P from the slime mold *Dictyostelium discoideum*. This is the first report on metal ion requirements of a eukaryotic RNase P enzyme. Data are presented which indicate that the alkaline earth cations Ca^{2+}, Sr^{2+}, and Ba^{2+} exhibit a bimodal action at the kinetic phase of the reaction. Also, under appropriate conditions, Ca^{2+}, Sr^{2+}, and Ba^{2+} can substitute to some extent for Mg^{2+}. Finally, the transition metal ions Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} are efficient inhibitors of the enzyme activity.

EXPERIMENTAL PROCEDURES

Assay for RNase P Activity. The substrate for RNase P assays was an in vitro labeled transcript of the *Schizosaccharomyces pombe* $tRNA^{ser}$ gene *supS1*. The transcription reaction with SP6 RNA polymerase was carried out as previously described (*17*). Enzyme assays were carried out at 37 °C in 20 μL buffer D (50 mM Tris/HCl, pH 7.6, 10 mM NH_4Cl, 5 mM $MgCl_2$, and 5 mM dithiothreitol) containing 2−5 fmol of RNA substrate and 3.5 μg of protein from the RNase P fraction. The enzyme was extensively dialyzed in buffer D without $MgCl_2$ containing 0.1 mM EDTA and used in assays carried out to detect the effect of divalent metal ions on RNase P activity. The reactions were stopped by addition of 5 μL of stop dye (80% formamide, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). Reaction products were resolved on a denaturing 10% polyacrylamide/8 M urea gel and visualized either by autoradiography or by phosphorimager (prosporimager SI-Molecular Dynamics) without drying. Activity was quantified by Cerenkov counting of excised gel slices, or by measuring the X-ray film bands in an image analysis system.

Enzyme Purification. Growth of Cells. Dictyostelium discoideum cells (strain AX2 wild type) were grown in 10 l HL-5 medium (14.3 g/L peptone, 7.1 g/L yeast extract, 0.49 g/L KH_2PO_4, 0.64 g/L $Na_2HPO_4 \times 2H_2O$, 1% glucose, supplemented with 250 mg/L ampicillin, 250 mg/L streptomycin, and 15 mg/L tetracyclin) at 22 °C to a density of $(6-8) \times 10^6$ cells/mL. The cells were harvested by centrifugation, washed twice with equal volumes of 16.7 mM potassium phosphate, pH 6, and stored at −70 °C. All subsequent steps in the enzyme purification were carried out at 4 °C.

Cell Breakage. Frozen cells (35 g) were resuspended in 50 mL of buffer A (50 mL of Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 10% by vol. glycerol, 10 mM 2-mercaptoethanol, 1 mM phenylmeth and sulfonyl fluoride, 2 mM benzamidine, 1 mM EDTA, and 0.5 mM EGTA) containing 50 mM KCl (buffer AK 50). The cell resuspension was mixed with 100 g of cooled (−20 °C) acid-washed glass beads (0.5 mm) and broken in a bead-beater cell disrupter with 30 s bursts, allowing 2 min cooling periods. The broken cell resuspension was centrifuged for 10 min at 8000*g*. The supernatant was removed and spun at 100000*g* for 1 h, yielding a 15 mL S-100 fraction.

DEAE−Cellulose Chromatography. Gel Filtration. The S-100 fraction was loaded onto a DE 52 column (40 mL; 2.1 cm × 24 cm) which had been equilibrated with AK 50 buffer. The column was washed with the same buffer until the A_{280} dropped almost to zero. RNase P was then eluted with a 120 mL linear gradient of 50 to 300 mM KCl in buffer A at a flow rate of 2 mL/min. Activity was eluted at 130−150 mM KCl. The active fractions were pooled and dialyzed overnight against 4 L of AK 50 buffer. The RNase P fraction was loaded onto a second DE 52 column (20 mL; 2.1 × 24 cm) and eluted as described above. The active fractions were pooled and dialyzed against buffer D. A final gel filtration column was added to improve the overall purification of the RNase P. Purified RNase P (0.6 mL) was loaded onto a Sephacryl S-300 column (24 mL; 1 × 32 cm) and eluted with 40 mL of buffer D at a flow rate of 0.4 mL/min. Activity elutes with the void volume of the column. The active fractions were pooled, concentrated in dialysis bags with PEG 20.000, dialyzed against buffer D, and stored at −20° C in the presence of 50% glycerol. The overall purification was estimated to be 820-fold over the S-100 fraction.

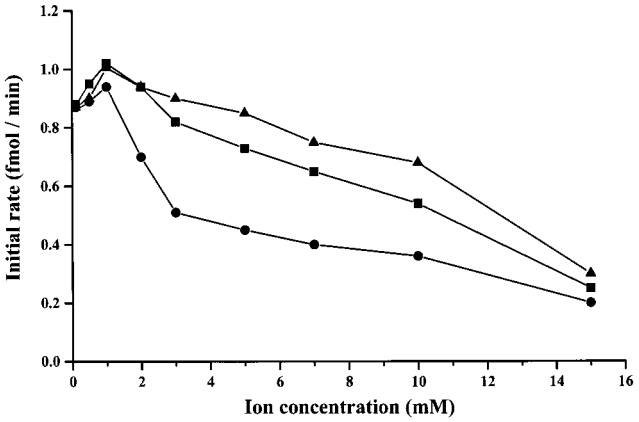

FIGURE 1: Effect of increasing concentrations of Ca^{2+}, Sr^{2+}, and Ba^{2+} on *D. discoideum* RNase P activity in the presence of 5 mM $MgCl_2$. All metal ions where preincubated with the enzyme for 5 min at 37 °C before the addition of the substrate. Initial rates were calculated from the corresponding time plots. Ca^{2+} (●), Sr^{2+} (▲), Ba^{2+} (■).

RESULTS

Figure 1 shows the effect of Ca^{2+}, Sr^{2+}, and Ba^{2+} on *D. discoideum* RNase P activity in the presence of 5 mM $MgCl_2$. It is of interest that, Ca^{2+}, Sr^{2+}, and Ba^{2+} exhibit a bimodal action at the kinetic phase of RNase P reaction and both activation as well as inhibition of tRNA maturation are observed, depending on metal ion concentration. At relatively low concentrations of Ca^{2+}, Sr^{2+}, or Ba^{2+}, the rate of cleavage is stimulated. The maximum stimulation is observed at 1 mM of Ca^{2+}, Sr^{2+}, or Ba^{2+}. Further addition of the above metal ions has an inhibitory effect on RNase P activity (Figure 1). These results show that, apart of the Mg^{2+} binding sites on the RNase P holoenzyme, there must be activation and inactivation binding sites for Ca^{2+}, Sr^{2+}, and Ba^{2+}.

Under the standard assay conditions (37 °C, 30 min), Ca^{2+}, Sr^{2+}, and Ba^{2+} at optimum concentrations (1 mM) do not support any detectable enzyme activity in the absence of Mg^{2+} (Figure 2). Increasing the Mg^{2+} concentration restored the enzyme activity, exhibiting the maximum stimulation at

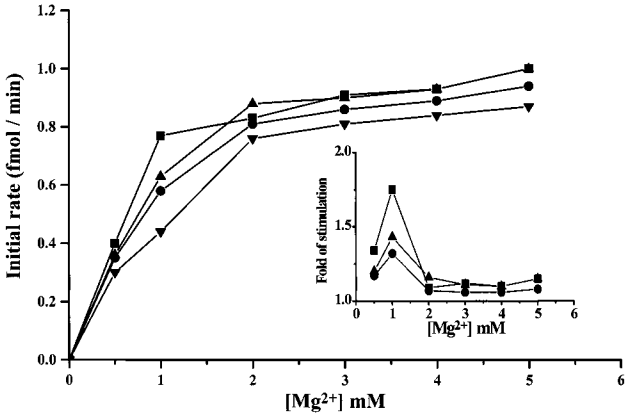

FIGURE 2: Effect of increasing Mg^{2+} concentrations in the presence of 1 mM Ca^{2+} (●), Sr^{2+} (▲) or Ba^{2+} (■). Mg^{2+} alone (▼). Both, Mg^{2+} and the second metal ion where preincubated with the enzyme for 5 min at 37 °C before the addition of the substrate. Initial rates were calculated from the corresponding time plots. (Inset) Fold of stimulation as function of Mg^{2+} concentration. Ca^{2+} (●), Sr^{2+} (▲), Ba^{2+} (■).

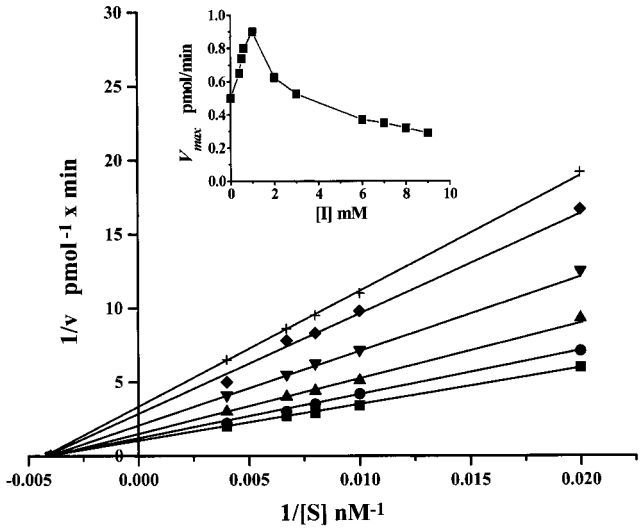

FIGURE 3: Double reciprocal plot ($1/v$ versus 1/[pre-tRNA]) for RNase P reaction in the presence of Ba^{2+}. The reaction was carried out at each one of the indicated concentrations in the presence or in the absence of Ba^{2+}. All reactions were carried out at 37 °C in 20 μL buffer D. The initial velocity in the presence or absence of Ba^{2+} was determined from the initial slopes of time plots. (▼) without inhibitor; with Ba^{2+} at (▲) 0.4 mM, (●) 0.6 mM, (■) 1.0 mM, (◆) 6 mM, (+) 8 mM. (Inset) $V_{max(app)}$ values as a function of Ba^{2+} concentration. The data are obtained from the double reciprocal plots shown in the figure and analogous plots.

1 mM $MgCl_2$ (Figure 2, inset). It is obvious that the presence of Ca^{2+}, Sr^{2+}, or Ba^{2+} lowers the optimum concentration of Mg^{2+} from 5 to 1 mM. Detailed kinetic analysis of the effect of Ba^{2+} in the presence of 1 mM Mg^{2+} showed that, at relatively low concentrations of Ba^{2+} (<1 mM), the $V_{max(app)}$ value increases, while the K_m remains constant (Figure 3). The maximum stimulation is observed at 1 mM Ba^{2+}. Further addition of Ba^{2+} decreases the $V_{max(app)}$ value while the K_m value remains constant (Figure 3). The insert of Figure 3 provides a better representation of the dependence of $V_{max(app)}$ on Ba^{2+} concentration. Analogous results were taken with Ca^{2+} and Sr^{2+} (not shown). Further kinetic analysis of the activation phase ([Ba^{2+}] < 1 mM) was made by plotting the $V_{max(app)}/(V_{max(app)} - V_{max})$ values, calculated at each

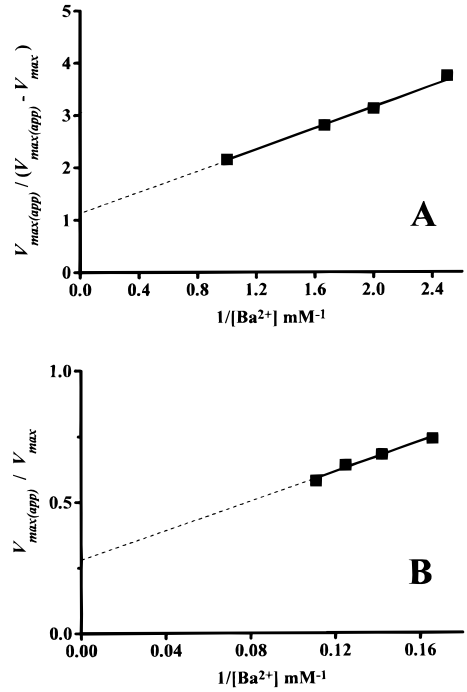

FIGURE 4: Variation of $V_{max(app)}/[V_{max(app)} - V_{max}]$ (A) or $V_{max(app)}/V_{max}$ (B) as a function of $1/[Ba^{2+}]$. The V_{max} and $V_{max(app)}$ values are calculated from the corresponding double reciprocal plots (Figure 3).

concentration of pre-tRNA as a function of $1/[Ba^{2+}]$ (Figure 4A). The linearity of this plot supports that a single molecule of Ba^{2+} is involved in the mechanism of activation (Appendix, eq 4). The number of Ba^{2+} molecules, which participate in the inhibition phase ([Ba^{2+}] > 1 mM), was evaluated by calculating the $V_{max(app)}/V_{max}$ ratio and plotting it as a function of $1/[Ba^{2+}]$ (Figure 4B). The linearity of this plot suggests that only one molecule of Ba^{2+} is kinetically involved in the mechanism of inhibition (Appendix, eq 6). Analogous plots were taken for Ca^{2+} and Sr^{2+} (not shown). Since Ca^{2+}, Sr^{2+}, and Ba^{2+} stimulate the enzyme activity even in the presence of optimum Mg^{2+} concentration, the binding sites(s) of these metals at the catalytic center of the enzyme must be different. In competition experiments, the first metal ion added (Ca^{2+}, Sr^{2+} or Ba^{2+}), is present for 10 min prior the addition of Mg^{2+} and then the reaction was carried out for a further 20 min. During this time, a recovery of the enzyme activity could be observed (Figure 5). These results show that effects directed by Ca^{2+}, Sr^{2+}, and Ba^{2+} are reversible.

Ca^{2+}, Sr^{2+}, and Ba^{2+} Support the Cleavage Reaction. From all divalent cations tested, only Ca^{2+}, Sr^{2+}, and Ba^{2+} support RNase P activity in the absence of Mg^{2+}, but in a considerably reduced rate. Enzyme samples have been extensively dialyzed in the presence of 0.1 mM EDTA. Assays have been carried out in the standard buffer at 37 °C for 3 h. Figure 6 shows the results of the effect of Ca^{2+}, Sr^{2+}, and Ba^{2+} on RNase P activity in the absence of Mg^{2+}. In these assays, the quantity of the enzyme used was 20-fold more of that used in the control assay (Figure 6; lanes 5, 7, 9, and 1, respectively). Under these conditions, Ca^{2+}, Sr^{2+}, and Ba^{2+} in the absence of enzyme do not induce any cleavage of pre-tRNA (Figure 6, lanes 4, 6, and 8). Because of the precautions taken against contamination of the reaction with Mg^{2+}, we conclude that the enzyme activity supported

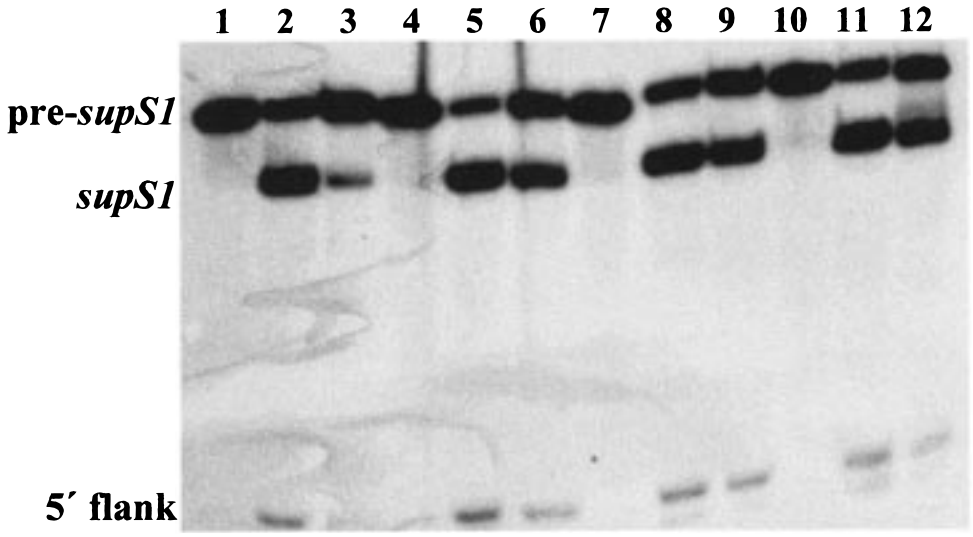

FIGURE 5: Competition experiments. Dialyzed enzyme was incubated in the presence of *supS1* with Ca^{2+}, Sr^{2+}, or Ba^{2+} for 10 min prior the addition of Mg^{2+} following a further 20 min incubation. All reactions were carried out for 30 min at 37 °C. Lanes: 1, *supS1* alone; 2, control; 3, RNase P incubated for 10 min prior the addition of *supS1* following a further 20 min incubation; 4, dialyzed RNase P in the presence of 1 mM Ba^{2+}; 5, dialyzed RNase P in the presence of 5 mM Mg^{2+} and 1 mM Ba^{2+}; 6, dialyzed RNase P incubated for 10 min in the presence of 1 mM Ba^{2+} prior the addition of 5 mM Mg^{2+} following a further 20 min incubation; lanes 7, 8, and 9, same as lanes 4, 5, and 6 but with 1mM Ca^{2+}; lanes 10, 11, and 12, same as lanes 4, 5, and 6 but with 1 mM Sr^{2+}.

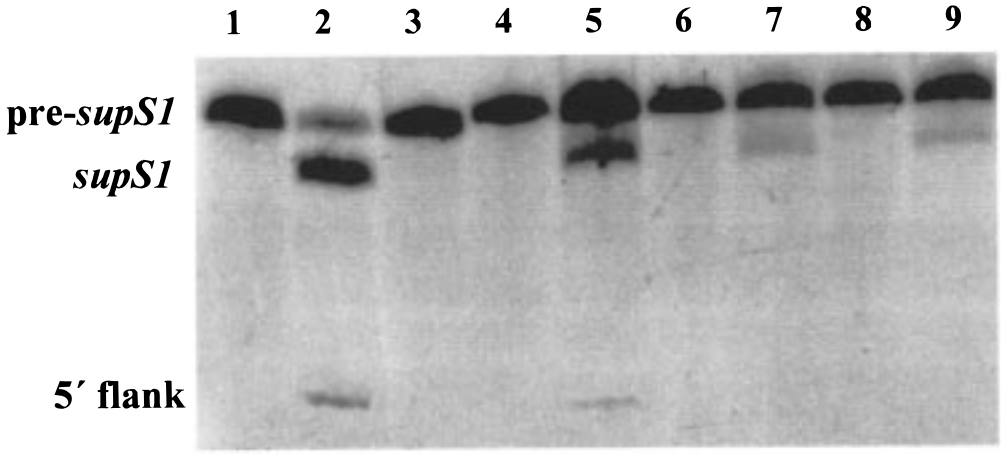

FIGURE 6: Effect of Ca^{2+}, Sr^{2+}, and Ba^{2+} on *D. discoidem* RNase P activity in the absence of Mg^{2+}. All reactions were carried out for 3 h at 37 °C. Lanes: 1, *supS1* alone; 2, control; 3, dialyzed RNase P; 4, *supS1* alone in the presence of 1 mM Ba^{2+}; 5, dialyzed RNase P in the presence of 1 mM Ba^{2+}; 6, same as lane 4 but with 1 mM Ca^{2+}; 7, same as lane 5 but with 1 mM Ca^{2+}; 8, same as lane 4 but with 1 mM Sr^{2+}; 9, same as lane 5 but with 1 mM Sr^{2+}.

by Ca^{2+}, Sr^{2+}, and Ba^{2+} is genuine. The slower rate of Ca^{2+}, Sr^{2+}, and Ba^{2+} reaction can be attributed to the greater ionic radius of these metals.

In the absence of Mg^{2+}, the transition metals Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+}, do not support *D. discoideum* RNase P activity. Co^{2+}, Ni^{2+}, and Cd^{2+} at 0.5 mM are inhibitory in the presence of 5 mM Mg^{2+}, while Mn^{2+} has the same effect at 6 mM showing that this metal ion behaves as a weaker inhibitor (Figure 7).

DISCUSSION

D. discoideum RNase P requires both protein and RNA components for activity and displays an absolute requirement for NH_4^+ and Mg^{2+} ions (*18, 19*). In the presence of Mg^{2+}, the alkaline earth metals Ca^{2+}, Sr^{2+}, and Ba^{2+} exhibit a bimodal action at the kinetic phase of RNase P reaction; at low concentrations, these metals lower Mg^{2+} requirement, while at higher concentrations, they inhibit the RNase P reaction. It is likely that this complexity is due to a multisite interaction of Ca^{2+}, Sr^{2+} and Ba^{2+} with RNase P. According to this assumption, a complex kinetic scheme (Figure 8) is needed in order to interpret the bimodal action of Ca^{2+}, Sr^{2+}, and Ba^{2+}. This kinetic scheme is based on the following.

Ca^{2+}, Sr^{2+}, and Ba^{2+} act as nonessential activators, because ES (in the presence of 1 mM Mg^{2+}) is catalytically active.

Ca^{2+}, Sr^{2+}, and Ba^{2+} have no effect on the K_m value (Figure 3). This implies that the affinity of each of E, $EI_{(A)}$, and $EI_{(A)}I$ complexes for pre-tRNA is the same (K_m = constant). Therefore, the binding of pre-tRNA on $EI_{(A)}$ and $EI_{(A)}I$ complexes has no effect on their dissociation constants.

The experimental plot of V_{max} versus Ba^{2+} concentration (Figure 3, inset) is similar to that predicted by eq 1 in the Appendix.

The shape of the plot presented in Figure 3, inset, indicates that the activator and the inhibitor sites are not mutually exclusive. If the activator-metal ion [$I_{(A)}$] and the inhibitor-

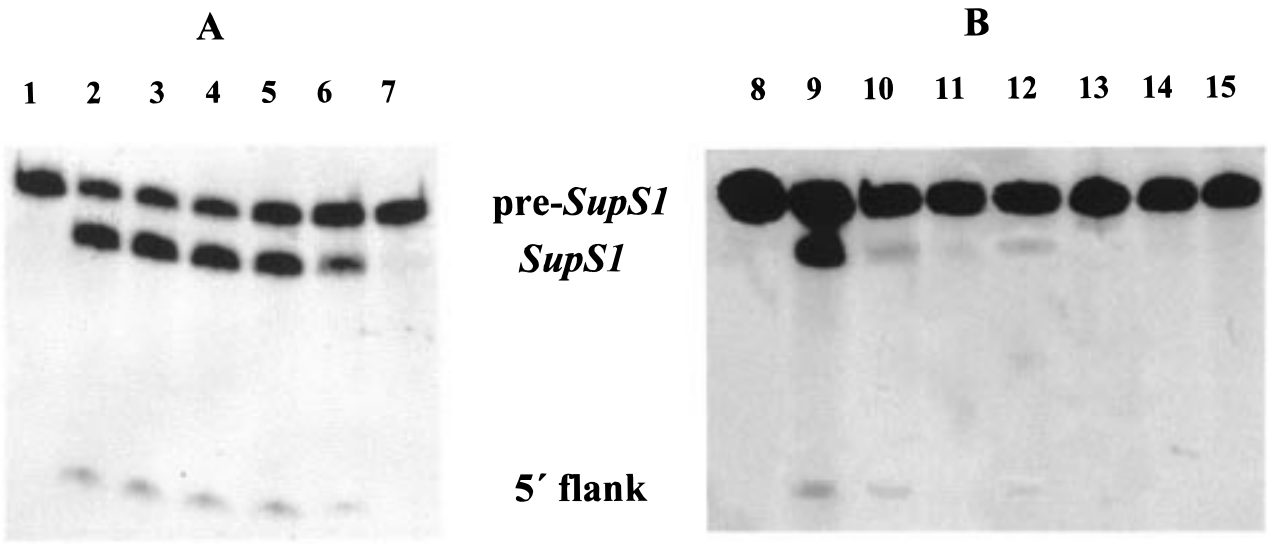

FIGURE 7: Effect of Mn^{2+} (A) and Co^{2+}, Ni^{2+} and Cd^{2+} (B) on *D. discoideum* RNase P activity in the presence of 5 mM $MgCl_2$. All metal ions where preincubated with the enzyme for 5 min at 37 °C before the addition of the substrate. After the substrate was added the mixture was incubated for further 20 min at 37 °C. (A) Lanes: 1, *supS1* alone; 2, control; 3−7, Mn^{2+}, 0.25, 0.5, 1, 3, 6 mM, respectively. (B) Lanes: 8, *supS1* alone; 9, control; 10, 11, Ni^{2+}, 0.25, 0.5 mM, respectively; 12, 13, Co^{2+}, 0.25, 0.5 mM, respectively; 13, 14, Cd^{2+}, 0.25, 0.5 mM, respectively.

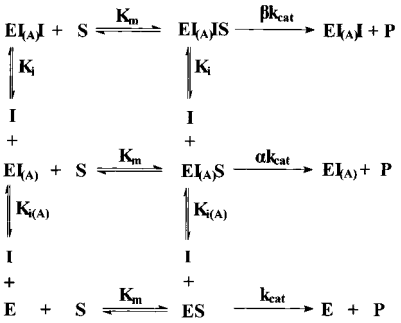

FIGURE 8: Kinetic model for *D. discoidem* RNase P activity in the presence of the alkaline earth cations Ca^{2+}, Sr^{2+}, or Ba^{2+}. E = RNase P−Mg^{2+} complex, S = pre-tRNA, I and $I_{(A)}$ = Ca^{2+}, Sr^{2+}, or Ba^{2+} bound to the inhibition and the activation site of RNase P, respectively.

metal ion (I) were competitive with respect to each other, the plot of V_{max} versus metal ion concentration should be a hyperbolic descending or ascending curve, dependent upon the relative values of the catalytic and equilibrium constants involved in the kinetic scheme.

The estimation of the metal ion interaction coefficient was based on the selection of two limited cases, at low and at high Ba^{2+} concentration, due to the fact that the eq 2 is very complicated. At low concentrations of Ba^{2+}, the relationship between $V_{max(app)}/[V_{max(app)} - V_{max}]$ and the reciprocal of Ba^{2+} concentration is given by eq 4. From this equation, it is obvious that the plot $V_{max(app)}/[V_{max(app)} - V_{max}]$ versus 1/[I] is linear only when one molecule of Ca^{2+}, Sr^{2+}, or Ba^{2+} participates noncooperatively in the kinetic mechanism of activation. Indeed this seems to be the case (Figure 4A). At high concentrations of Ba^{2+}, the plot of Figure 4B $V_{max(app)}/V_{max}$ versus 1/[I] is linear, as predicted by eq 6.

It is interesting to note that similar kinetic analysis on the Mg^{2+} dependence of *D. discoideum* RNase P cleavage reaction revealed that one Mg^{2+} ion participates in the cleavage mechanism. These kinetic data support the notion that one of the affore-mentioned metal ions is simultaneously coordinated with Mg^{2+} ion in the active site of the enzyme through the formation of a cation-binding center near the scissile bond in the enzyme−substrate complex. Due to the fact that the stoichiometry was deduced by a kinetic curve rather than a real measurement of the amount of the bound metal ion, we cannot exclude the existence of additional metal ion binding sites on RNase P holoenzyme. We assume that these additional binding sites, if they exist, are not involved in the kinetic model of activation or inhibition, but may confer on RNase P an appropriate conformation. We could speculate that these results are in agreement with the general two-metal ion mechanism for catalytic RNAs proposed by Steitz and Steitz (*20*). In this mechanism, deduced from the crystal structure, one Mg^{2+} activates the attacking water or sugar hydroxyl and a second acts to stabilize the oxyanion leaving group. Furthermore, Warnecke et al. (*11*), based on a Hill coeficient of 1.8 obtained for Cd^{2+}-dependent cleavage of a precursor tRNA carrying a single Rp-phosphorothioate modification by M1 RNA, proposed that two Cd^{2+} ions directly coordinate to the Rp-distereomeric sulfur, in agreement with the two metal ion mechanism. Recently, Pontius et al. (*21*) proposed a two metal ion mechanism for the hammerhead ribozymes. According to this model, a Mg^{2+} ion is directly coordinated to the 5′-oxygen of the leaving group and stabilizes the negative charge buildup on the 5′-oxygen in the transition state, and a Mg^{2+} ion is directly coordinated to the 2′-oxygen and increases the acidity of the 2′-OH proton. Also, at least one of these Mg^{2+} ions interacts with the *pro*-Rp oxygen at the cleavage site. Alternatively, on the basis of the results that Ca^{2+}, Sr^{2+}, and Ba^{2+} ions reduce the Mg^{2+} requirements of *D. discoideum* RNase P reaction, we could speculate that there is a dual function for divalent cations under these reaction conditions. Ca^{2+}, Sr^{2+}, and Ba^{2+} could satisfy a structural role for the reaction and Mg^{2+} is required for catalytic function. The idea that divalent cations make two different contributions to catalysis, like structural contribution by a variety of divalent cations and catalytic contribution,

by Mg^{2+} has been supported previously. Guerrier-Takada et al., (*4*) reported that Mg^{2+} and, to a lesser extent, Mn^{2+} ions can function at the catalytic center of M1 RNA, while other ions such as Ca^{2+} or Sr^{2+} function as structural counterions. Furthermore, it has been reported that Ca^{2+}, Sr^{2+}, Ba^{2+}, and polyamines, although they do not provide measurably activity, lower the Mg^{2+}/Mn^{2+} requirement of the *Tetrahymena* ribozyme activity (*22*, *23*). On the basis of these results, the authors proposed two distinct functions for the divalent cations, the structural and the catalytic.

From all metal divalent cations tested, only Ca^{2+}, Sr^{2+}, and Ba^{2+} can support *D. discoideum* RNase P activity in the absence of Mg^{2+}, but at a reduced rate. This may be due to the greater ionic radius of Ca^{2+}, Sr^{2+}, and Ba^{2+} than that of Mg^{2+}. So, these metals cannot fit well into the catalytic site which is probably limited in size and steric properties than the structural sites. Alternatively, Mg^{2+} could play important role to the native structure of the RNA-enzyme. The role of specific ions in the stabilization of RNA tertiary structure is well established in the case of transfer RNA. Furthermore, it has been reported that the native structure of the *Tetrahymena* ribozyme requires Mg^{2+} (*23*). Therefore, Mg^{2+} may have a novel role in the ribozyme's folded structure. It has been reported previously that Ca^{2+}, Sr^{2+}, and Ba^{2+} can act as cofactors for M1 RNA under certain conditions. At pH 9.5 and in the presence of 10% ethanol, Ca^{2+} and, to a lesser extent Sr^{2+} and Ba^{2+}, can substitute for Mg^{2+} in the processing of precursor tRNAs by M1 RNA (*15*). Also, it has been reported that precursor $tRNA^{Asp}$ processing by M1 RNA was supported by Ca^{2+} in the absence of Mg^{2+} (*16*). In contrast with previous reports (*4*, *14*−*16*) we found that Mn^{2+} cannot support activity. Mn^{2+}, has also been reported to substitute for Mg^{2+} in the processing of precursor tRNAs, carrying a single Rp-phosphorothioate modification at the RNase P cleavage site, by M1 RNA (*11*). In our system, all transition metal ions tested, like Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+}, act as inhibitors of RNase P activity. Co^{2+}, Ni^{2+}, and Cd^{2+} at 0.5 mM completely suppress RNase P activity in the presence of 5 mM Mg^{2+}, while Mn^{2+} has the same effect at 6 mM, showing that Mn^{2+} is a weaker inhibitor than the other transition metals. It has been previously reported that Co^{2+}, Ni^{2+}, and Cd^{2+} are very effective inhibitors of M1 RNA (*4*, *15*). The finding that Mn^{2+} cannot replace Mg^{2+} and acts only as inhibitor, as the rest of the transition metals, can be attributed to the contribution of protein(s) of the holoenzyme, in the structure of potential metal binding sites or to a denaturation effect. Mn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} are all more polarizible acids than Mg^{2+}, and they can bind to phosphate oxygen and to nitrogen lone electron pairs of nucleotides (*24*), thus distorting the structural sites in *D. discoideum* RNase P RNA.

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APPENDIX

The differential rate low, corresponding to the kinetic scheme of Figure 7 involving one activation site and one inhibition site, is given by the equation

$$v_0 = \frac{V_{max}[S]\left(\frac{\beta[I]^2}{K_{i(A)}K_i} + \frac{\alpha[I]}{K_{i(A)}} + 1\right)}{(K_m + S)\left(1 + \frac{[I]}{K_{i(A)}} + \frac{[I]^2}{K_{i(A)}K_i}\right)} \quad (1)$$

Therefore, the apparent maximum velocity is given by the equation

$$V_{max(app)} = V_{max}\frac{\left(\frac{\beta[I]^2}{K_{i(A)}K_i} + \frac{\alpha[I]}{K_{i(A)}} + 1\right)}{\left(1 + \frac{[I]}{K_{i(A)}} + \frac{[I]^2}{K_{i(A)}K_i}\right)} \quad (2)$$

At low concentrations of metals ([I] < 1 mM), the $\beta[I]^2/K_{i(A)}K_i$ in the numerator and the $[I]^2/K_{i(A)}K_i$ in the denominator may be ignored and eq 2 reduces to

$$V_{max(app)} = V_{max}\frac{\left(\frac{\alpha[I]}{K_{i(A)}} + 1\right)}{\left(1 + \frac{[I]}{K_{i(A)}}\right)} \quad (3)$$

From eq 3, it follows that

$$\frac{V_{max(app)}}{V_{max(app)} - V_{max}} = \frac{\alpha}{\alpha - 1} + \frac{1}{[I]}\frac{K_{i(A)}}{\alpha - 1} \quad (4)$$

Alternatively, at high concentrations of metals ([I] > 1 mM), eq 2 can be simplified to

$$V_{max(app)} = V_{max}\frac{\left(\frac{\beta[I]^2}{K_{i(A)}K_i} + \frac{\alpha[I]}{K_{i(A)}}\right)}{\frac{[I]^2}{K_{i(A)}K_i}} \quad (5)$$

Therefore, it follows that

$$\frac{V_{max(app)}}{V_{max}} = \beta + \frac{1}{[I]}\alpha K_i \quad (6)$$

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