Ionic Requirements for RNA Binding, Cleavage, and Ligation by the Hairpin Ribozyme†

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ABSTRACT: Metal ion requirements for RNA binding, cleavage, and ligation by the hairpin ribozyme have been analyzed. RNA cleavage is observed when Mg^{2+}, Sr^{2+}, or Ca^{2+} are added to a 40 mM Tris-HCl buffer, indicating that these divalent cations were capable of supporting the reaction. No reaction was observed when other ions (Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Ba^{2+}, Na^+, K^+, Li^+, NH_4^+, Rb^+, and Cs^+) were tested. In the absence of added metal ions, spermidine can induce a very slow ribozyme-catalyzed cleavage reaction that is not quenched by chelating agents (EDTA and EGTA) that are capable of quenching the metal-dependent reaction. Addition of Mn^{2+} to a reaction containing 2 mM spermidine increases the rate of the catalytic step by at least 100-fold. Spermidine also reduces the magnesium requirement for the reaction and strongly stimulates activity at limiting Mg^{2+} concentrations. There are no special ionic requirements for formation of the initial ribozyme·substrate complex—analysis of complex formation using native gels and kinetic assays shows that the ribozyme can bind substrate in 40 mM Tris-HCl buffer. Complex formation is inhibited by both Mn^{2+} and Co^{2+}. Ionic requirements for the ribozyme-catalyzed ligation reaction are very similar to those for the cleavage reaction. We propose a model for catalysis by the hairpin ribozyme that is consistent with these findings. Formation of an initial ribozyme·substrate complex occurs without the obligatory involvement of divalent cations. Ions (e.g., Mg^{2+}) can then bind to form a catalytically proficient complex, which reacts and dissociates. Because spermidine acts to reduce the Mg^{2+} requirement but cannot itself promote an efficient reaction, we propose that two different cation-binding sites exist in the active ribozyme·substrate complex.

Catalytic RNA molecules, or ribozymes, act to cleave and/or form phosphodiester linkages in nucleic acid substrates using transesterification or hydrolytic mechanisms. Like protein enzymes that act on nucleic acids, ribozymes show strong requirements for divalent metal ions as cofactors in these reactions (Symons, 1989). For both classes of biocatalyst, this requirement is typically fulfilled by Mg^{2+}. There is strong evidence that divalent metal ions serve as essential components of the catalytic site in reactions catalyzed by both protein enzymes (Fersht, 1985) and ribozymes (Grosshans & Cech, 1989; Dahm & Uhlenbeck, 1991; Kazakov & Altman, 1991; Smith et al., 1992). In addition, ribozymes require cations to function in neutralizing the highly negative charge of the phosphate–sugar backbone to promote ribozyme folding and substrate binding in vitro (Jack et al., 1977; Celander & Cech, 1991). In vivo, this charge-shielding function can sometimes be provided by proteins that facilitate many ribozyme-catalyzed reactions (Reich et al., 1988; Gampel et al., 1989).

Ribozymes characterized to date show widely varying selectivity in their utilization of different cations for structural and catalytic purposes. For reactions catalyzed by the *Tetrahymena* group I ribozyme, a large number of divalent metal ions can satisfy the structural ion requirement (Mg^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}), while only Mg^{2+} and Mn^{2+} ions can fulfill the catalytic requirement (Grosshans & Cech, 1989). In a similar manner, the catalytic RNA subunit of RNase P can utilize a wide variety of monovalent cations to stabilize the structure required for complex formation but, as for the *Tetrahymena* ribozyme, either Mg^{2+} or Mn^{2+} is required for catalysis (Guerrier-Takada et al., 1986; Smith et al., 1992). In contrast, the hammerhead ribozyme can utilize a variety of metal ions for both structural and catalytic functions, including Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Sr^{2+}, and Ba^{2+} ions (Dahm & Uhlenbeck, 1991). Other metal ions, (Zn^{2+} and Cd^{2+}) can fulfill the catalytic ion requirement for the hammerhead ribozyme, but only if spermine is present to stabilize the active structure of the ribozyme·substrate complex (Dahm & Uhlenbeck, 1991). NMR studies on the hammerhead system have shown that the ribozyme can bind to the substrate in the absence of Mg^{2+} ions (Heus & Pardi, 1991). The biochemical separation of binding and cleavage activities in the presence of different ions provides solid evidence distinguishing structural and catalytic functions of divalent cations.

The hairpin ribozyme (Figure 1) is derived from the minus strand of tobacco ring spot virus satellite [(–)sTRSV] RNA and catalyzes a reversible site-specific RNA cleavage reaction in trans (Feldstein et al., 1989; Hampel & Tritz, 1989; Haseloff & Gerlach, 1989; Chowrira & Burke, 1991). Previous work characterizing the activity of the hairpin ribozyme has demonstrated a requirement for divalent cations, supplied in the form of Mg^{2+} (Hampel & Tritz, 1989; Feldstein et al., 1989). The commonly adopted reaction buffer used for RNA cleavage in trans contains 12 mM $MgCl_2$ and 2 mM spermidine (Hampel & Tritz, 1989; Chowrira & Burke, 1991). In examining self-cleavage of the full-length (–)sTRSV RNA, a slow reaction was observed in the absence of added metal ions when low concentrations of spermidine (1 mM) were present (Prody et al., 1986). However, it is not clear whether the observed reaction was truly metal-independent or, instead, catalyzed by low concentrations of metal ions copurified with the RNA.

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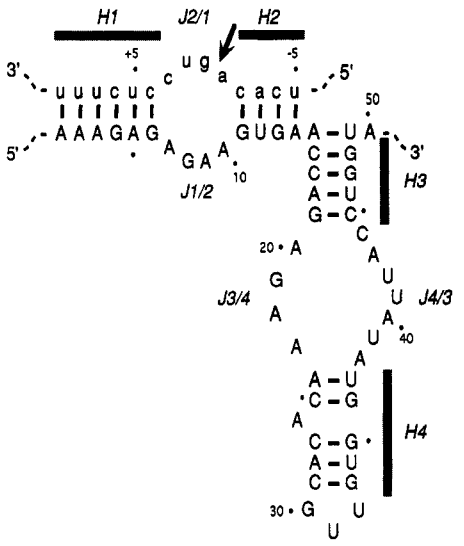


FIGURE 1: Hairpin ribozyme structure. Uppercase, ribozyme sequence; lowercase, substrate sequence. Construction and characterization of the ribozyme and substrate used in this study were as described (Chowrira & Burke, 1991). Arrow indicates substrate cleavage site. The self-cleaving construct used in the ligation studies was as described (Berzal-Herranz et al., 1992). The self-cleaving ribozyme differs from that shown in that the 3′ end of the ribozyme is joined to the 5′ end of the substrate with a pentacytidine bridge. In addition, the nucleotides at positions 4 and 13 in the ribozyme are both C, and those at positions −4 and +6 of the substrate are both G.

Here, we report a systematic characterization of the cation requirements for both the cleavage and ligation reactions catalyzed by the hairpin ribozyme. The use of a gel mobility shift assay for measuring formation of the ribozyme·substrate complex, as well as assays for cleavage and ligation reactions, have allowed us to identify those cations that provide essential functions. In addition, the identification of minimal ionic requirements for the hairpin reaction is important for structure–function studies that will lead to increased understanding of the secondary and tertiary structure of the ribozyme·substrate complex. Our results reveal important differences in the ionic requirements for the hairpin ribozyme compared to those of other well-characterized ribozymes. These results may have significant implications for the application of hairpin ribozymes as RNA-inactivating reagents.

MATERIALS AND METHODS

Synthesis of Nucleic Acids. Ribozyme and substrate RNAs were transcribed from oligonucleotide templates using bacteriophage T7 RNA polymerase as described (Milligan & Uhlenbeck, 1989; Chowrira & Burke, 1991). Mixed RNA/DNA molecules were used as substrates in the ligation assays (Berzal-Herranz et al., 1992). These ligation substrates were synthesized using solid-phase phosphoramidite chemistry, deprotected, and purified as described (Scaringe et al., 1990; Chowrira & Burke, 1991). All nucleic acids used in these studies were purified using denaturing polyacrylamide gel electrophoresis in Tris–borate–EDTA buffers, eluted by diffusion, precipitated, and stored in deionized distilled water.

Ribozyme Reactions. Metal salts used in this study were obtained from Aldrich, and all were of the highest purity available (>99.999%). $MnCl_2$ solutions were prepared and stored as described (Smith et al., 1992) to minimize oxidation. Ribozyme-catalyzed RNA cleavage reactions were performed as described (Chowira & Burke, 1991). Briefly, ribozyme and substrate were denatured and renatured under various ionic conditions in a 10-μL reaction as described (Chowrira & Burke, 1991). In most cases, reaction mixtures were assembled on ice, and reactions were initiated by incubation at 37 °C. Reactions were quenched by the addition of an equal volume of formamide loading buffer and freezing on dry ice. The samples were resolved on a 20% polyacrylamide–urea gel, and results were quantitatively analyzed by radioanalytic imaging of dried gels with a Betascan instrument (Betagen). For titration reactions with various ions, 10 nM substrate and 0.5 nM ribozyme were used.

Single-burst kinetics were performed as described (Ruffner & Uhlenbeck, 1990). Briefly, 10 nM substrate and 30 nM ribozyme were denatured at 95 °C for 2 min and then renatured at 37 °C, each in cleavage buffer containing appropriate metal ion concentrations. Reactions were initiated by mixing ribozyme and substrate. Aliquots were removed at regular time intervals, and reactions were terminated by freezing on crushed dry ice. Half-times for substrate cleavage ($t_{1/2}$) were determined from a least-squares fit of the data points from the plot of the logarithm of the uncleaved fraction versus time. First-order rate constants (k_{obs}) were obtained, using the relationship $k_{obs} = 0.693/t_{1/2}$.

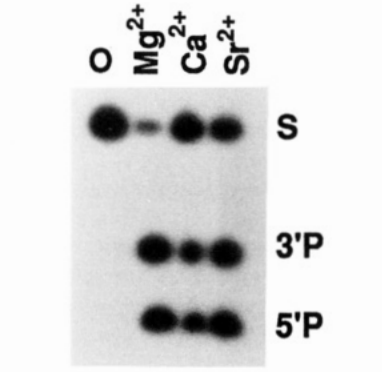
Ligation reactions were performed using a self-cleaving construct as described (Berzal-Herranz et al., 1992; see legend to Figure 1). A 60:1 ratio of ligation substrate to ribozyme was used. Conditions for ligation assays were as follows. Substrate and ribozyme were denatured under varying ionic conditions by heating to 95 °C for 2 min. The ligation reactions were initiated by incubation on ice for 30 min, followed by incubation at 4 °C for 20 min. The samples were resolved on a 10% polyacrylamide–urea gel.

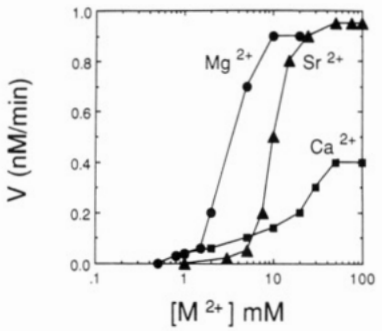
Assay of Ribozyme·Substrate Complex Formation. A noncleavable substrate analog, $G_{+1}A$, was internally labeled and used to assay formation of the ribozyme·substrate complex as described (Chowrira et al., 1991; Chowrira & Burke, 1991). Substrate binding was allowed to proceed at 4 °C under conditions sufficient to achieve steady-state binding of $G_{+1}A$ to the ribozyme in 12 mM Mg^{2+} (>2 h in standard cleavage buffer). Complex formation was monitored following native gel electrophoresis in 12 mM Mg^{2+}, in such a manner that preexisting complexes are stabilized but no additional binding occurs (see Results).

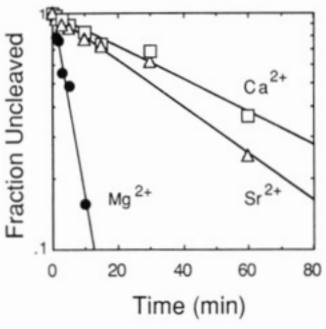
RESULTS

Mg^{2+}, Sr^{2+}, and Ca^{2+} Provide All Essential Cationic Requirements for the Hairpin Ribozyme. A variant hairpin ribozyme that cleaves a substrate containing a single G was used in these studies (Figure 1; Chowrira & Burke, 1991). Cleavage was assayed in the presence of 25 mM concentrations of 14 different cations (Mg^{2+}, Sr^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Ba^{2+}, Na^{+}, K^{+}, Li^{+}, NH_4^{+}, Rb^{+}, and Cs^{+}) in a buffer containing 40 mM Tris-HCl (pH 7.5). Cleavage activity was observed only when Mg^{2+}, Sr^{2+}, and Ca^{2+} were used (Figure 2; data not shown). In a 10-min reaction with a 5-fold molar excess of substrate, Mg^{2+} gave the greatest extent of reaction, followed by Sr^{2+} and Ca^{2+}, respectively. No site-specific cleavage products were detected when the other 11 cations were present (data not shown). Pb^{2+} and Zn^{2+} were also tested, but results were inconclusive due to extensive nonspecific RNA degradation.

Initial reaction rates as a function of ionic concentration were measured for Mg^{2+}, Sr^{2+}, and Ca^{2+}. The concentration required for the reaction to reach half-maximal cleavage rate ($K_{M^{2+}}$) was determined from a plot of initial rate as a function of ion concentration (figure 2B–D). For Mg^{2+}, maximal rates



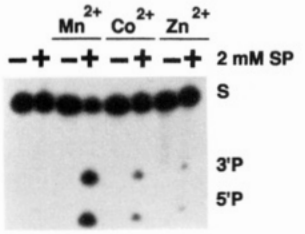




IONS	k_{rel}	$K_{[M^{2+}]}$
Mg^{2+}	1.0	3 mM
Sr^{2+}	0.4	10 mM
Ca^{2+}	0.1	20 mM

FIGURE 2: Metal ion dependence of the hairpin ribozyme. (A, top panel) Cleavage reactions. Reactions were carried out in a buffer containing metal chlorides and 40 mM Tris-HCl (pH 7.5) as described in Materials and Methods. Internally labeled substrate RNA (10 nM) was incubated with unlabeled ribozyme (2 nM) at 37 °C for 10 min under standard reaction conditions. 0, no metal ions added; Mg^{2+}, 25 $MgCl_2$; Ca^{2+}, 25 mM $CaCl_2$; Sr^{2+}, 25 mM $SrCl_2$; S, 17-nucleotide substrate RNA; 3′P and 5′P, 3′ and 5′ cleavage products. (B, second panel) Cleavage rates. Substrate RNA (10 nM) was incubated with ribozyme (0.5 nM) in the presence of indicated concentrations of metal ions. (C, third panel) Single-burst cleavage kinetics. Single-burst kinetics were performed as described in Materials and Methods. A semilog plot of fraction of uncleaved substrate vs time is shown. Data points were fitted to the lines using the least-squares method. (D, bottom panel) Kinetic parameters. k_{rel} values are relative first-order rate constants normalized to that determined in the presence of saturating $MgCl_2$ concentrations (12 mM, $k_{obs} = 0.2\ min^{-1}$). Saturating concentrations of $SrCl_2$ (25 mM) and $CaCl_2$ (50 mM) were used. $K_{[M^{2+}]}$ is the concentration of metal ions required for 50% of the cleavage rate at saturation, as determined from the data in panel B.

were observed at a concentration of 10 mM, and $K_{Mg^{2+}}$ was determined to be 3 mM. The Sr^{2+}-catalyzed reaction reached an equivalent maximal rate, but at a significantly higher concentration ($K_{Sr^{2+}} = 10$ mM). In the presence of Ca^{2+}, still higher concentrations were required to reach a maximal rate ($K_{Ca^{2+}} = 20$ mM), and only 40% of the maximal rate for Mg^{2+}



IONS	k_{rel}
Mg^{2+}	1.00
Spermidine (50 mM)	0.01
Spermidine(2 mM)	≤0.001
Mn^{2+}	0.10
Co^{2+}	0.02
Zn^{2+}	0.01

FIGURE 3: Spermidine facilitates the cleavage reaction. (A, top panel) Cleavage by transition metals requires spermidine. Cleavage reactions were carried out in the presence of transition metals alone (minus) or metals plus 2 mM spermidine (plus). $MnCl_2$ and $CoCl_2$ concentrations were 2 mM, and $ZnCl_2$ concentration was 0.5 mM. (B, bottom panel) Relative reaction rates. Relative rates of the cleavage step were determined using single-burst experiments (Materials and Methods). Rates are reported relative to that of the ribozyme in the presence of 2 mM $MgCl_2$ and 2 mM spermidine ($k_{obs} = 0.1\ min^{-1}$). Metal ion concentrations were as follows: Mn^{2+}, 2 mM; Co^{2+}, 2 mM; Zn^{2+}, 0.5 mM.

and Sr^{2+} was reached when sturating Ca^{2+} concentrations were used.

We conclude that, among the cations tested, only Mg^{2+}, Sr^{2+}, and Ca^{2+} can provide all of the essential functions required by the hairpin ribozyme. The data indicate that Mg^{2+} is the optimal cation for the ribozyme, because maximal rate enhancement is achieved at the lowest ionic concentration (Figure 2C,D).

Mn^{2+} and Co^{2+} Can Support the Reaction Only When Spermidine Is Present. Divalent metal ions that were unable to promote RNA cleavage by the hairpin ribozyme were tested in a cleavage reaction that also contained 2 mM spermidine. In the presence of 2 mM spermidine alone, no reaction is observed (Figure 3). However, when both 2 mM Mn^{2+} and 2 mM spermidine were present, a reasonably efficient cleavage reaction took place. The rate of the Mn^{2+} reaction was 10-fold lower than that of the Mg^{2+} reaction under identical conditions (2 mM M^{2+} and 2 mM spermidine; Figure 3B). The finding that Mn^{2+} and spermidine work together to stimulate a reaction, while no reaction products are detected in the presence of either cation alone, demonstrates the existence of two distinct functions for cations in this reaction. One of these functions is satisified by Mn^{2+} but not spermidine; the other is satisfied by spermidine but not Mn^{2+}.

Co^{2+} also stimulated the cleavage reaction in the presence of spermidine, although at a 5-fold lower rate than Mn^{2+} (Figure 3B). The reaction in the presence of 0.5 mM Zn^{2+} was also stimulated by spermidine. However, since substrate degradation occurred at higher Zn^{2+} concentrations, we do not know whether Zn^{2+} alone can induce a spermidine-independent reaction.

Low Concentrations of Spermidine Reduce the Mg^{2+} Requirement. The finding that spermidine and Mn^{2+} can work together to stimulate a reaction suggests that low concentrations of spermidine may reduce the requirement for Mg^{2+} in the reaction. We approached this question in two ways: first by titrating the reaction with Mg^{2+} in the presence and absence of 0.5 mM spermidine, and second by titrating

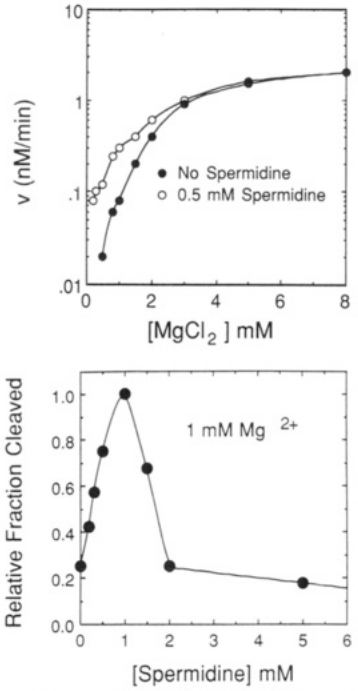


FIGURE 4: Spermidine enhances and inhibits the hairpin ribozyme reaction. (A, top panel) Spermidine decreases the magnesium requirement. Initial rates were measured in the presence and absence of 0.5 mM spermidine. $MgCl_2$ concentration was varied as indicated. Reactions (10 μL) contained 10 nM substrate and 0.5 nM ribozyme. (B, bottom panel) Enhancement and inhibition of reaction rate by spermidine. Magnesium chloride concentration was 1 mM. Spermidine concentration was varied as indicated. Reactions were for 10 min. Both ribozyme and substrate were internally labeled.

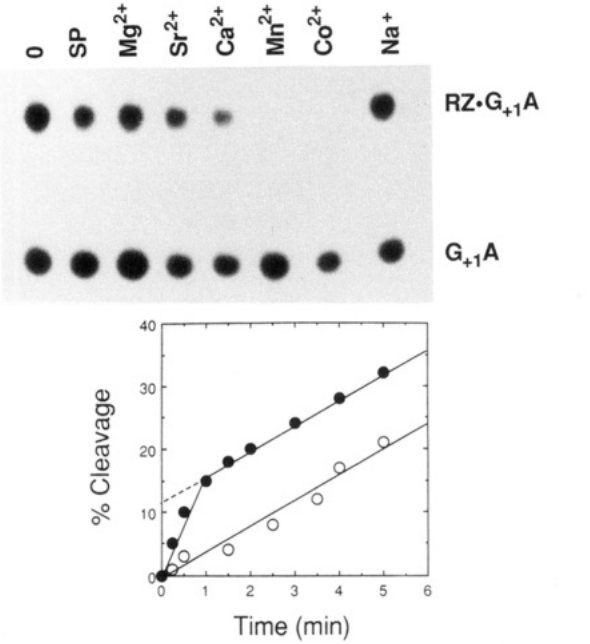


FIGURE 5: Metal ion dependence of complex formation. (A, top panel) Formation of the ribozyme·substrate complex was monitored by analysis on nondenaturing 10% polyacrylamide gels containing 12 mM magnesium acetate as described (Chowrira & Burke, 1991). A ^{32}P-labeled noncleavable substrate analog ($G_{+1}A$, 40 nM) was used (Chowrira et al., 1991). Ribozyme (20 nM) was unlabeled. Binding reactions contained 40 mM Tris-HCl (pH 7.5) plus indicated cations as chlorides at concentrations of 25 mM. O, no additions; SP, spermidine (B, bottom panel) Kinetic assay. Labeled substrate (10 nM) and unlabeled ribozyme (10 nM) were preincubated for 30 min at 37 °C in 40 mM Tris-HCl (pH 7.5). The reaction was initiated by the simultaneous addition of 12 mM $MgCl_2$ and a 5-fold excess (50 nM) of unlabeled substrate (solid circles). In the control reaction (open circles), ribozyme and a mixture of both labeled and unlabeled substrates were preincubated separately for 30 min at 37 °C in 12 mM $MgCl_2$ and 40 mM Tris-HCl (pH 7.5), and the reaction was initiated by mixing ribozyme and substrate. Time points were taken, and the rate of cleavage of radiolabeled substrate was monitored. Lines represent a least-squares fit of the data.

the reaction with spermidine in the presence of a subsaturating concentration of Mg^{2+}.

Results shown in Figure 4A clearly demonstrate that 0.5 mM spermidine reduces the Mg^{2+} requirement. Spermidine reduces $K_{Mg^{2+}}$ by a factor of 2, from 3 mM to approximately 1.5 mM. Spermidine did not affect the maximal velocity of the reaction at saturating Mg^{2+} concentrations.

Titration with spermidine in the presence of 1 mM Mg^{2+} (Figure 4B) shows an additional effect of spermidine on the hairpin reaction. As expected from the results above, low concentrations of spermidine (0.2–1.0 mM) clearly stimulate the initial rate of the reaction, with a maximal stimulation of 5-fold observed at 1 mM spermidine. An interesting effect is observed at spermidine concentrations above 1 mM, where additional spermidine inhibits the reaction. This inhibition suggests that high concentrations of spermidine may displace Mg^{2+} from sites within the ribozyme·substrate complex where spermidine cannot function efficiently, possibly a site important for catalysis (see Discussion). Under these conditions, 50% inhibition is observed at a concentration of approximately 3 mM spermidine, suggesting that the site where spermidine is competing for Mg^{2+} may have a relatively low selectivity for Mg^{2+} over the organic polyamine.

Metal Ion Dependence of Complex Formation. Formation of the ribozyme·substrate complex can be monitored under different ionic conditions. For these experiments, we first used a mobility shift assay that allows us to directly visualize binding of a radiolabeled noncleavable substrate analog ($G_{+1}A$) to unlabeled ribozyme by using nondenaturing gels containing 12 mM $MgCl_2$. In this assay, preformed complexes are stabilized in the gel, but no new complexes can form.

Results (Figure 5A) indicate that complex formation correlates with activity for those ions that support cleavage in the absence of spermidine; i.e., $Mg^{2+} > Sr^{2+} > Ca^{2+}$. Little or no complex is detected in the presence of Mn^{2+} and Co^{2+}, ions which support catalysis but require spermidine to fulfill the structural requirement. This observation supports the following two notions. First, the ribozyme cannot fold properly in the presence of only Mn^{2+} or Co^{2+} but requires spermidine for productive folding, and second, the mechanism of Mn^{2+} inhibition of ribozyme activity may involve displacement of Mg^{2+} from essential sites, with accompanying destabilization of the ribozyme·substrate complex.

Complex formation takes place under conditions where cleavage cannot take place. When only spermidine or Na^+ is present, efficient complex formation occurs. Efficient complex formation was also observed in only 40 mM Tris-HCl, without additional cations. To control for possible complex formation following the entry of molecules into the Mg^{2+}-containing gel, the solution containing ribozyme, substrate analog, and Tris-HCl was denatured by heating to 95 °C for 2 min prior to loading. Following denaturation, no complex was detectable (data not shown). Therefore, complexes observed in the native gel assay must form before loading. The observation that complexes form without the inclusion of additional cations indicates that Mn^{2+} and Co^{2+} act to interfere with complex formation.

Additional evidence demonstrating the formation of the ribozyme·substrate complex in the absence of Mg^{2+} ions is provided by the results of a kinetic experiment (Figure 5B;

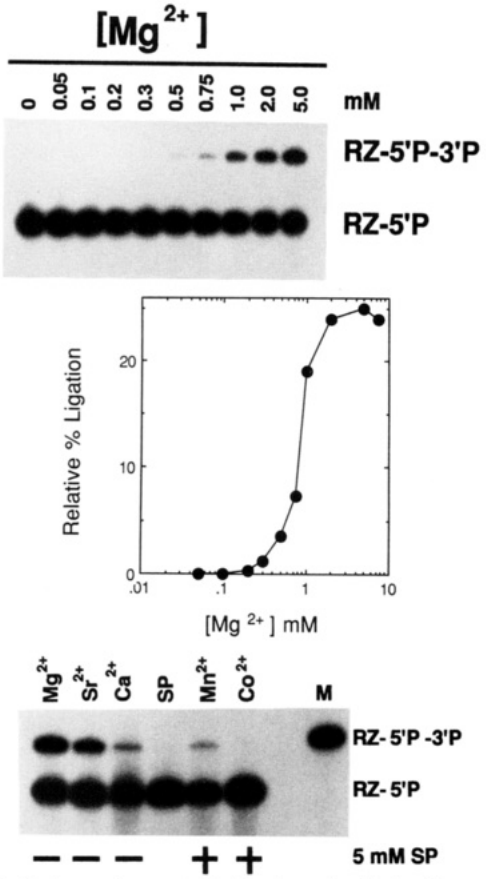


FIGURE 6: Ionic requirements for ligation. A self-cleaving construct (see legend to Figure 1) was used for ligation assays, in which a ligation substrate (unlabeled) is linked to the 3′ end of the ribozyme (labeled; Berzal-Herranz et al., 1992). (A, top panel) Magnesium dependence of ligation. Ligation reactions were carried out in a buffer containing 40 mM Tris-HCl (pH 7.5) plus indicated concentrations of $MgCl_2$. RZ-5′P, ribozyme linked to 5′ cleavage product, ending in 2′,3′-cyclic phosphate; RZ-5′P–3′P, product of ligation reaction. (B, middle panel) Plot of magnesium dependence data. Data in panel A and additional points were used. (C, bottom panel) Ionic requirements for ligation. Reactions were as described above, except that metal ions were varied. Mg^{2+}, Sr^{2+}, and Ca^{2+} concentrations were 25 mM. Mn^{2+} and Co^{2+} concentrations were 10 mM. Lane marked SP contained 25 mM spermidine and no additional metal ions. Lanes marked plus contained 5 mM spermidine in addition to metal ions indicated. No reaction was observed for Mn^{2+} and Co^{2+} in the absence of spermidine (data not shown). For the lanes marked SP and Co^{2+}, small quantities of ligation products were visible in a longer exposure of the gel shown here and were also visualized and quantitated using the radioanalytic imaging instrument.

Smith et al., 1992). In this experiment, a mixture of renatured ribozyme and labeled substrate were preincubated in the absence of Mg^{2+} for 30 min, and then the reaction was initiated by the addition of Mg^{2+} plus a 5-fold molar excess of unlabeled substrate and product formation was monitored as a function of time. Results were compared to those obtained when the preincubation step was omitted. The results show that preincubation of ribozyme and substrate in the absence of Mg^{2+} resulted in a burst of cleavage of radiolabeled substrate when Mg^{2+} was added, demonstrating cleavage of prebound substrate. We conclude that the ribozyme·substrate complex can form in the absence of Mg^{2+} and then goes on to form cleavage products following association of Mg^{2+} with the complex.

Metal Ion Requirements for Ligation. We investigated the metal ion dependence of the ligation reaction. Reversal of the cleavage reaction results in the ligation of RNA substrates containing a 5′-hydroxyl and a 2′,3′-cyclic phosphate. To increase the efficiency of ligation, we used a construct in which the 5′ cleavage product (5′P, containing the 2′,3′-cyclic phosphate) is tethered to the 3′ end of the ribozyme with a short linker sequence (Figure 1; Berzal-Herranz et al., 1992). For these experiments, the ribozyme-containing fragment (RZ-5′P) was internally labeled. A molar excess of the external substrate (i.e., the 3′ cleavage product, 3′P) is provided in trans.

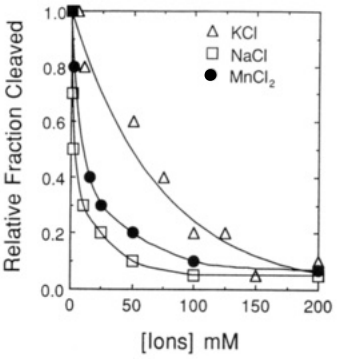


FIGURE 7: Inhibition of cleavage reaction of Mn^{2+}, Na^+, and K^+. Substrate RNA (10 nM) and ribozyme (0.5 nM) were incubated in a reaction containing 2 mM $MgCl_2$ and additional salts as indicated. No spermidine was present. Reactions were for 10 min at 37 °C. K_I values determined from these data were as follows: Mn^{2+}; Na^+, 5 mM; and K^+, 50 mM.

The ligation reaction requires somewhat lower Mg^{2+} concentrations than does the cleavage reaction (Figure 6). $K_{Mg^{2+}}$ for ligation is 1 mM, approximately 3-fold lower than for trans cleavage. The structural basis for the decreased Mg^{2+} requirement for ligation is not known. However, it is possible to speculate that fewer Mg^{2+} ions are needed to stabilize the structure of the ligation complex, since one segment of the substrate is tethered to the ribozyme, thus greatly increasing its local concentration.

In addition to Mg^{2+}, two other divalent cations (Sr^{2+} and Ca^{2+}) can support the ligation reaction in the absence of spermidine (Figure 6C). These are the same ions that function in the spermidine-independent cleavage reaction, and reactivity in the ligation reaction for the three ions followed the same order as that for cleavage: $Mg^{2+} > Sr^{2+} > Ca^{2+}$. In the presence of spermidine, Mn^{2+} was able to catalyze an inefficient reaction, while no Mn^{2+} reaction was detectable in the absence of spermidine. Only very small quantities of ligation product were obtained in the presence of 10 mM Co^{2+} plus 5 mM spermidine. All divalent metal ions that fail to activate the cleavage reaction are also incapable of promoting ligation. In the presence of 5 mM spermidine alone, no ligation products were observed. Very small quantities of ligation products were detected in the presence of 25 mM spermidine alone (data not shown).

We conclude that the cationic requirements for catalysis of the ligation reaction are essentially identical to those for cleavage. These data support a model in which ligation takes place by a simple reversal of the cleavage mechanism.

Metal Ion Inhibition of the Hairpin Ribozyme Reaction. Mn^{2+} inhibits the hairpin ribozyme reaction. In the presence of 2 mM Mg^{2+}, Mn^{2+} at all concentrations tested (1–200 mM) inhibits the reaction with an apparent K_I of 10 mM (Figure 7). This suggests that Mn^{2+} ions can compete to prevent Mg^{2+} binding to, or to induce Mg^{2+} dissociation from, essential sites within the ribozyme. The same level of inhibition was obtained when the ribozyme was renatured in the presence of Mg^{2+} prior to the addition of Mn^{2+}, suggesting that Mg^{2+}

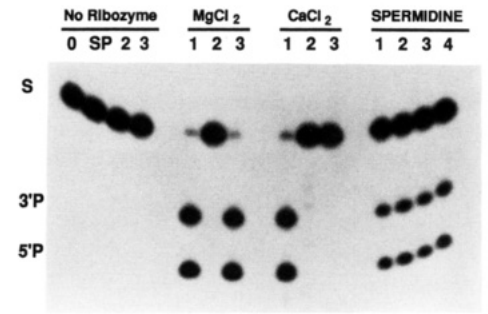


FIGURE 8: Slow cleavage reaction in the absence of added metal ions. Lanes are labeled as follows: (No ribozyme) Substrate RNA incubated in the absence of ribozyme in a buffer containing 40 mM Tris-HCl (pH 7.5) at 37 °C. (SP) Substrate incubated with spermidine alone. (0) Substrate RNA incubated in the absence of ions and chelators. (1) Substrate RNA incubated in the presence of $MgCl_2$ (12 mM), $CaCl_2$ (25 mM), or spermidine (50 mM). (2) 50 mM EDTA added. (3) 50 mM EGTA added. (4) 50 mM EDTA and 50 mM EGTA added. All reactions were for ≥15 h.

can readily exchange from structural sites essential for ribozyme activity.

None of the monovalent ions tested (Na^+, K^+, NH_4^+, Cs^+, Li^+, and Rb^+) were capable of promoting the reaction in the presence or absence of spermidine (data not shown). Since monovalent ions are capable of stabilizing RNA secondary structure (Gardiner et al., 1985; Guerrier-Takada et al., 1986), we asked if monovalent ions can provide a stimulatory function, similar to spermidine, in the hairpin ribozyme reaction. The initial rates of RNA cleavage by the hairpin ribozyme in the presence of low Mg^{2+} concentrations (2 mM) were measured in the presence of increasing concentrations of Na^+ and K^+ (Figure 7). Both Na^+ and K^+ inhibited the reaction, with apparent K_Is of 5 mM and 50 mM, respectively, at 2 mM Mg^{2+}. The apparent K_I for Na^+ increases from 5 mM at 2 mM Mg^{2+} to 40 mM at 5 mM Mg^{2+}. This observation is consistent with a competition between Mg^{2+} and Na^+. Sodium ions are significantly more effective than potassium ions at inhibiting cleavage.

A Slow Spermidine-Dependent Cleavage Reaction Is Observed in the Absence of Inorganic Divalent Cations. We wished to more closely examine reported results that processing of (–)sTRSV RNA could take place in 2 mM spermidine, without the addition of divalent metal ions (Prody et al., 1986). We were unable to detect any cleavage activity in the presence of 2 mM spermidine unless divalent metals were present (Figure 3A; data not shown). However, at concentrations greater than 5 mM, spermidine supported site-specific RNA cleavage by the ribozyme, but the reaction was very slow (Figure 8). To control for the possible presence of contaminating divalent cations in the spermidine solution, we carried out the cleavage reaction in the presence of 50 mM concentrations of the chelators EDTA and EGTA. These concentrations were sufficient to completely quench the reactions catalyzed by 12 mM Mg^{2+} and 25 mM Ca^{2+} (Figure 8). The initial rate of the reaction (k_{obs}) supported by 50 mM spermidine is 8×10^{-3} min^{-1}, approximately 1000-fold slower than observed rate of the reaction in the presence of 12 mM Mg^{2+} and 2 mM spermidine. The mechanism of the putative metal ion independent reaction is unknown.

DISCUSSION

Model for the Catalytic Cycle. Using native gel analysis and a kinetic assay, we have shown that complex formation can occur in the absence of divalent cations or in the presence of Mg^{2+}, Sr^{2+}, Ca^{2+}, Na^+, K^+, or spermidine (Figure 5). However, Mn^{2+} and Co^{2+} interfere with complex formation, unless spermidine is also present. Possible modes of inhibition by these ions are discussed below.

The association of divalent cations with the ribozyme·substrate complex is an essential prelude to the cleavage reaction. Some metal ions (e.g., Mg^{2+}) can induce cleavage alone. Others (e.g., Mn^{2+}) require the presence of spermidine for activity. In the latter case, spermidine must act to counteract the inhibition of complex formation induced by Mn^{2+} alone. Therefore, spermidine functions to stabilize the ribozyme·substrate complex, and Mn^{2+} provides a second function, possibly a direct role in active-site chemistry, as has been demonstrated for the hammerhead ribozyme (Dahm & Uhlenbeck, 1991).

Are there also multiple functions for divalent cations when Mg^{2+} is used? Grosshans and Cech (1989) found that Ca^{2+}, an ion that could not support the cleavage reaction catalyzed by the *Tetrahymena* group I ribozyme, could reduce the Mg^{2+} requirement for the reaction. From this result, they concluded that Ca^{2+} could satisfy a structural role for the reaction but that Mg^{2+} was required for catalytic function. In our work, we find that spermidine concentrations far below those needed to induce a detectable cleavage reaction (i.e., 1 mM), can strongly stimulate the reaction at subsaturating Mg^{2+} concentrations (Figure 4B) and that 0.5 mM spermidine stimulates the reaction over a range of Mg^{2+} concentrations (0.5–5 mM; Figure 4A). Thus, we infer that there are also dual functions for divalent cations under these reaction conditions.

These results are consistent with a model in which divalent cations provide two distinct functions required for catalysis. We define the function provided by Mn^{2+} in the Mn^{2+} plus spermidine reaction as catalytic and the function provided by spermidine in the same reaction as structural. We suggest that the structural function represents formation of an RNA structure essential for activity, most likely tertiary structure. This suggests a possible structural change following substrate binding to the ribozyme. We attribute the catalytic function to the possible involvement of divalent metal ions in active-site chemistry. It is important to note that we have not directly proven the involvement of a metal ion at the active site, nor have we proven that spermidine promotes the formation of a specific tertiary structure element. However, our tentative assignments of the catalytic and structural functions are consistent with our findings and with previous observations concerning the function of polyamines and divalent metal ions in other classes of catalytic RNA molecules (Grosshans & Cech, 1989; Dahm & Uhlenbeck, 1992).

Where Are the Cation Binding Sites within the Ribozyme·Substrate Complex? The identities of the cations that function at the catalytic and structural sites within the complex may provide an important clue concerning the nature of some of the functional groups that serve as metal ligands. We have found that alkaline earth cations (Mg^{2+}, Sr^{2+}, and Ca^{2+}) function at the structural sites, while transition metal ions (Mn^{2+} and Co^{2+}) do not. X-ray diffraction studies of metal-nucleoside complexes involving alkaline earth metals and transition metals have shown that there is a striking difference in the utilization of 2′-hydroxyl groups as ligands (Swaminathan & Sundaralingam, 1979; Sigel, 1979; Saenger, 1984). In these studies, alkaline earth metals were observed to coordinate 2′-hydroxyl groups but transition metals were not. If this generalization applied to the hairpin ribozyme as well, we would predict that the catalytic ion binding site(s) might involve coordination of the 2′-hydroxyl groups of one or more ribose moieties by alkaline earth metals. This model would

predict that ribozyme activity would be strongly inhibited by deoxyribonucleoside substitution at one or more such sites within the ribozyme and that high Mg^{2+} concentrations could potentially compensate for the loss of activity. We have recently identified putative Mg^{2+}-binding 2'-hydroxyl groups within the ribozyme that behave in this manner (B. Chowrira, A. Berzal-Herranz, C. Keller, and J. Burke, unpublished results).

It is likely, but as yet unproven, that the catalytic metal ion binding site lies within the active site of the ribozyme. In addition to the scissile phosphodiester linkage, known components of the active site include the 2'-hydroxyl group of A_{-1} (Chowrira & Burke, 1991) and, most likely, the 2-amino group of G_{+1} (Chowrira et al., 1991). In an elegent series of experiments, Dahm and Uhlenbeck (1991) have shown that a metal ion is coordinated to the *pro-Rp* oxygen at the scissile bond within the active site of the hammerhead ribozyme, and models have been proposed for the direct involvement of this metal ion in catalysis, perhaps in the form of a metal hydroxide. Although recent studies indicate that the analogous oxygen atom is unlikely to coordinate Mg^{2+} in the hairpin ribozyme (Chowrira & Burke, 1991, 1992), the basic features of such a mechanism may well be common to both ribozymes, since the hairpin exhibits dependencies on metal ions and pH (Hampel & Tritz, 1989) that bear several similarities to those of the hammerhead.

Cations Inhibit the Cleavage Reaction via Two Distinct Mechanisms. We have shown that Mn^{2+}, Na^+, and K^+ inhibit the Mg^{2+}-catalyzed hairpin reaction. Our results suggest that this inhibition occurs through two distinct mechanisms: (1) interference with formation of the ribozyme·substrate complex by Mn^{2+} and (2) displacement of Mg^{2+} from essential sites by Na^+ and K^+.

When Mn^{2+} is present, the substrate-binding activity of the ribozyme is greatly diminished (Figure 5A) unless spermidine is also present. Although Mn^{2+} can function at the catalytic site, it prevents formation of the ribozyme·substrate complex, possibly by stabilizing an alternative ribozyme conformation that is not capable of binding substrate.

How do monovalent ions inhibit the cleavage reaction? In the case of Mn^{2+} and Co^{2+}, the inhibition results from destabilization of the ribozyme·substrate complex (Figure 5A). The presence of 25 mM Na^+ in the binding reaction does not measurably affect complex formation as measured by the native gel assay (Figure 5A). This suggests that sodium does not alter the conformation of the ribozyme that is capable of binding the substrate. One model for monovalent ion inhibition, consistent with these observations, is that monovalent ions might compete with Mg^{2+} for occupation of the catalytic cation site within the complex. The increased inhibition of Na^+ over K^+ could be due to the fact that the ionic radius of K^+ is significantly larger than that of Na^+ or Mg^{2+}, possibly excluding K^+ from the catalytic site(s).

Ionic Requirements for Ligation Are Similar to Those for Cleavage. The ionic requirements for the ligation reaction catalyzed by the hairpin ribozyme are essentially identical to those for the cleavage reaction. The only difference that we have observed is that ligation requires a somewhat lower optimal concentration of magnesium ions. In our mutational studies, we have observed that all mutations that inhibit cleavage also inhibit ligation to a similar extent (Chowrira et al., 1991; Berzal-Herranz et al., 1992; Joesph et al., 1993). These observations support a model in which ligation occurs via a simple reversal of the RNA cleavage mechanism.

Implications for in Vivo Applications of Ribozyme Technology. Our observations concerning the inhibition of the hairpin ribozyme by monovalent cations may have important implications for in vivo applications of ribozyme technology. We find that Na^+ and K^+ inhibit the reaction with K_Is of 5 mM and 50 mM, respectively, at 2 mM Mg^{2+}. Depending on the concentration of available Mg^{2+}, typical intracellular concentrations of Na^+ and K^+ (ca. 10 mM and 160 mM; Saenger, 1984) could be significantly inhibitory to the ribozyme. We are currently evaluating ribozyme activity in cell extracts and cultured cells. If necessary, a recently developed in vitro selection system (Berzal-Herranz et al., 1992) can be used to select for variant ribozymes with increased activity in cellular extracts.

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