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Mg^{2+}-independent hairpin ribozyme catalysis in hydrated RNA films

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ABSTRACT

The hairpin ribozyme catalyzes RNA cleavage in partially hydrated RNA films in the absence of added divalent cations. This reaction exhibits the characteristics associated with the RNA cleavage reaction observed under standard conditions in solution. Catalysis is a site-specific intramolecular transesterification reaction, requires the 2′-hydroxyl group of substrate nucleotide A_{-1}, and generates 2′,3′-cyclic phosphate and 5′-hydroxyl termini. Mutations in both ribozyme and substrate abolish catalysis in hydrated films. The reaction is accelerated by cations that may enhance binding, conformational stability, and catalytic activity, and is inhibited by Tb^{3+}. The reaction has an apparent temperature optimum of 4 °C. At this temperature, cleavage is slow (k_{obs}: 2 d^{-1}) and progressive, with accumulation of cleavage products to an extent of 40%. The use of synthetic RNAs, chelators, and analysis of all reaction components by inductively coupled plasma-optical spectrophotometry (ICPOES) effectively rules out the possibility of contaminating divalent metals in the reactions. Catalysis is minimal under conditions of extreme dehydration, indicating that the reaction requires hydration of RNA by atmospheric water. Our results provide a further caution for those studying the biochemical activity of ribozymes in vitro and in cells, as unanticipated catalysis could occur during RNA manipulation and lead to misinterpretation of data.

Keywords: metal ions; ribozyme; RNA catalysis; RNA films

INTRODUCTION

Ribozymes promote site-specific transesterification cleavage of the phosphodiester backbone of RNA in the absence of protein cofactors (Long & Uhlenbeck, 1993; Burke, 1994). Although hammerhead and hairpin ribozymes catalyze analogous reactions and are found in the opposite strands of the satellite RNA of tobacco ringspot virus (sTRSV; Symons, 1994), they have distinct structures and catalytic requirements. The hairpin ribozyme (Burke et al., 1996; Earnshaw & Gait, 1997) catalyzes reversible, site-specific cleavage of RNA and the reaction is strongly stimulated by physiological concentrations of divalent cations (Chowrira et al., 1993).

Ribozyme activity requires formation of a catalytically proficient structure that positions functional groups and/or metal ions in the proper orientation with respect to the scissile phosphodiester bond. The metal ions may promote proper folding of the RNA or directly participate in chemical catalysis (Cech, 1990; Dahm & Uhlenbeck, 1991; Pyle, 1993). Ribozymes have generally been considered to be metalloenzymes, but it has proven difficult to distinguish structural from catalytic roles for the metal ions. Recent evidence suggests that the hairpin ribozyme's requirement for cations may be different from that of the other ribozymes, in that (1) a slow spermidine-dependent reaction is observed in the absence of divalent cations (Forster et al., 1987; Chowrira et al., 1993; Dahm et al., 1993) and (2) a robust reaction is supported by cobalt (III) hexammine, indicating that inner-sphere coordination of metal ions is not essential for cleavage (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997).

In dimeric (+) sTRSV RNA, hammerhead self-cleavage reactions occurred in spermidine, spermine, or EDTA to extents comparable to those observed in 5–20 mM Mg^{2+} (Prody et al., 1986). Recent results have shown that hairpin, hammerhead, and Neurospora VS ribozymes do not require divalent metal ions for either folding or catalysis; molar concentrations of monovalent cations (Na^+, Li^+, NH_4^+) were shown to be sufficient for efficient catalysis (Murray et al., 1998;

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Nesbitt et al., 1999). Divalent metal-independent nucleic acid enzymes have also been isolated from random sequence pools (Geyer & Sen, 1997; Suga et al., 1998). Thus, catalysis by metal ions is not an essential feature of ribozyme catalysis.

In the course of studies to analyze self-cleavage activity of hairpin ribozymes in mammalian cells, we made the paradoxical observation that cleavage was strongly stimulated by the addition of EDTA during RNA preparation after cellular lysis (Seyhan et al., 1998). This led us to examine the properties of RNA cleavage under conditions where RNA and solutes are concentrated and dehydrated. Here we report the characterization of divalent metal-independent hairpin ribozyme reactions under unusual conditions, in hydrated RNA films. Analogous reactions have also been reported by Kazakov et al. (1998).

RESULTS

Postlysis ribozyme activity is strongly stimulated by EDTA

Our attempts to demonstrate the intracellular cleavage activity of a self-cleaving hairpin ribozyme (Seyhan et al., 1998) led us to design control experiments to rule out ribozyme activity following cell lysis. To determine if cleavage could take place during the RNA isolation process, an excess of *trans*-acting hairpin ribozyme (10 nM; Fig. 1A) and 5′-^{32}P-labeled substrate (~0.1 nM) were added to 1 mL cell lysate and then carried through the RNA preparation procedures as described (Seyhan et al., 1998). Aliquots were used for cleavage reactions at 37 °C for 1 h with and without the addition of $MgCl_2$ to a final concentration of 12 mM. A third aliquot was carried through mock primer-extension assay without primers as described (Seyhan et al., 1998). No cleavage was observed when ribozyme and substrate containing cell lysate was carried through the RNA preparation (Fig. 1B). However, when 12 mM Mg^{2+} were included, ~40% of the substrate was cleaved (Fig. 1B). Primer-extension reactions did not support significant ribozyme activity (~0.23%, Fig. 1B), compared to ≥75% cleavage under standard assay conditions. These results show, first, that ribozyme catalysis can be obtained following cell lysis if a suitable ionic environment is provided and, second, that the protocol was sufficient to prevent cleavage from occurring after cellular lysis.

Paradoxically, significant cleavage (~70%) occurred when the cell lysate was supplemented with 50 mM sodium EDTA in all three control aliquots (Fig. 1B). This unexpected observation led us to further investigate the reaction under the conditions of RNA preparation and sample handling following concentration. Subsequent analysis indicated that similar extents of cleavage were observed following ethanol precipitation (−80 °C and −20 °C, ≥16 h; data not shown) or lyophilization, suggesting that high salt concentrations strongly enhance cleavage under these conditions. The EDTA-stimulated reaction required precipitation of RNA and/or partial drying of the pellet before it was analyzed by gel electrophoresis. Analogous studies indicated that the hammerhead ribozyme can also cleave its substrate under similar conditions (data not shown).

Cleavage occurs after concentration of RNA molecules and solutes

These results led us to hypothesize that concentration of RNA and trace amounts of solutes may be required for the ribozyme's unusual cleavage reaction. To test this hypothesis, we examined RNA cleavage following lyophilization of RNA samples from water without further manipulation. Elimination of salt in the reactions reduced, but did not eliminate, ribozyme activity (Fig. 1C). We conclude that these reactions require extensive concentration of the RNA-containing solution. However, this experiment does not establish if cleavage occurs during concentration or during subsequent sample handling. Analysis of the RNA products indicates that cleavage occurs at the normal site, 5′ of G_{+1}, generating products with 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Fig. 1C). These products are identical to those obtained in normal hairpin ribozyme cleavage reactions in solution.

Catalysis requires a partially hydrated RNA film

Differences in the extent of substrate cleavage following prolonged incubations prompted us to test the effect of hydration of RNA. Results show that catalysis takes place at the hydration levels provided by atmospheric water (Fig. 2). Reactions deprived of atmospheric water by maintaining them under vacuum after lyophilization showed a much smaller extent of cleavage (hairpin: ≤6.5%; hammerhead: ~0%) compared to reactions maintained under atmospheric pressure (hairpin: ≥90%; hammerhead: ~2.8%) throughout prolonged incubations (hairpin: 16 h; hammerhead: 24 h) at 24 °C. A further increase in water content, to the point of dissolution, inhibits catalysis (Fig. 2).

Catalysis proceeds in the absence of Mg^{2+}

To determine if catalysis is dependent on chelatable metal ion contaminants, 50 mM EDTA or EGTA were added and reactions were subsequently heat denatured (2 min, 90 °C), and the solutions were desalted using size-exclusion columns (Centri-Sep, Princeton Separations). This treatment was observed to have no effect on cleavage in RNA films (data not shown). To further rule out the presence of metal ions carried over

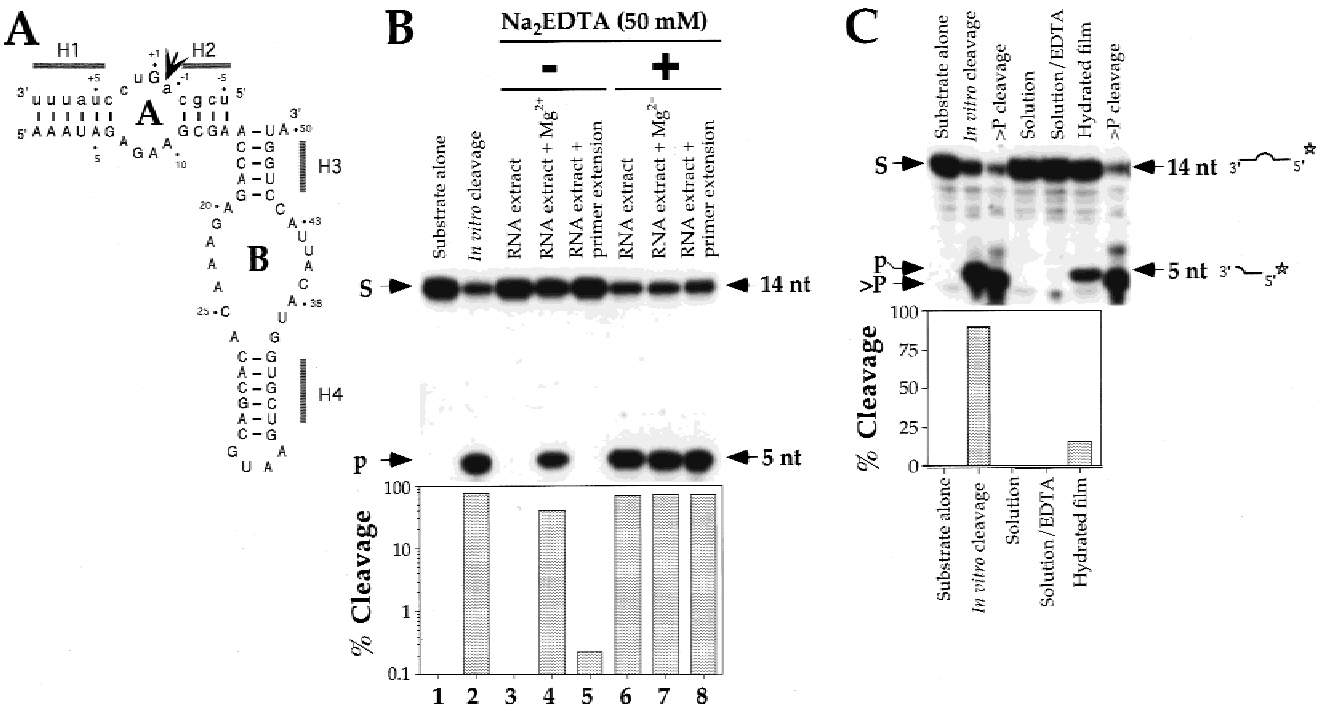

FIGURE 1. **A**: Secondary structure of the hairpin ribozyme and its cognate substrate (Esteban et al., 1997). Upper case: ribozyme sequences; lower case: substrate sequences. Dashes denote Watson–Crick base pairing. Arrow indicates substrate cleavage site. The four known helical segments (H1–H4) and two internal loops (A and B) are indicated. **B**: Ribozyme catalysis does not occur during cell lysis and RNA analysis under standard conditions; however, the reaction can be stimulated by sodium EDTA. Cleavage assays and mock primer extensions were conducted on untransfected mouse L cell lysates as described in Materials and Methods. Substrate alone (lane 1) and a standard in vitro solution cleavage control (lane 2) (50 mM Tris-HCl, pH 8, and 12 mM $MgCl_2$ for 1 h at 37 °C) were examined alongside the postlysis cleavage reaction mixes. An aliquot of purified RNA was incubated for 1 h at 37 °C and loaded on the gel without further manipulation (lane 3) or supplemented with 12 mM $MgCl_2$ and incubated for 1 h at 37 °C (lane 4). For the primer extension assay (lane 5), an aliquot of RNA was carried through a mock primer extension (Seyhan et al., 1998), without primers for 30 min at 48 °C. Lanes 6–8 are the same as lanes 3–5 except that 50 mM sodium EDTA was included in each reaction to chelate divalent cations present in the cell lysate. Note the strong stimulation of the reaction by sodium EDTA. The bands were quantified and data was plotted as the percent of the 5′ cleavage product relative to the full-length unprocessed RNA as described (Seyhan et al., 1998). **C**: Ribozyme cleavage in partially hydrated RNA films generates 5′-hydroxyl and 2′,3′-cyclic phosphate termini. Reactions were prepared in 10 μL water under conditions of ribozyme excess (25 nM ribozyme and ~0.6 nM substrate) dehydrated in a Speedvac, followed by prolonged incubation (24 h) at 24 °C. Samples were analyzed and quantified as described in Results. Substrate alone: substrate RNA; in vitro cleavage: standard cleavage reaction in ribozyme excess in standard cleavage buffer (50 mM Tris-HCl, pH 8, and 12 mM $MgCl_2$) at 37 °C for 1 h; >P cleavage, 2′,3′-cyclic phosphate breakage of a ribozyme-cleaved RNA in 0.01 N HCl at 37 °C for ≥16 h; solution: ribozyme-substrate RNAs maintained in water at 24 °C for 24 h; solution/EDTA: solution reaction containing 50 mM EDTA incubated as above; hydrated RNA film: cleavage reaction was prepared in water, lyophilized, and analyzed as described above; S: substrate RNA; p: 5′-cleavage product; >P: 2′,3′-cyclic phosphate breakage product. ☆ denotes 5′-^{32}P-end-label.

from transcription reactions and gel purification, we employed a hairpin ribozyme generated by solid-phase synthesis and purified by reverse-phase HPLC (Esteban et al., 1998); again, reactions were unaffected. These reactions were specific and blocked by noncleavable substrate containing a deoxyadenosine at position −1 (SdA_{-1}). Addition of monovalent cations (100 mM NaCl) further stimulated these reactions to the levels of standard cleavage reactions.

Trace metal ion analysis

In this work, we used ultra pure water and either transcribed or synthetic HPLC purified RNAs. However, we were concerned that trace metal ion contaminants could conceivably lead to cleavage when RNA and solutes were concentrated by dehydration. To address this issue, we performed ICPOES analyses on water, RNA, and other reaction components. Analysis of water and 25 mM RNA solutions gave identical results with the concentrations of all metal ions tested. Water and RNA analysis showed that solutions contained undetectable levels of Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+} (<0.012 ppm), and Ca^{2+} (<0.024 ppm, Table 1). We therefore calculated upper limits for divalent metal ion concentrations in the 25 nM RNA solutions, and determined that maximum Ca^{2+} and Mg^{2+} concentrations were 0.6 and 0.5 μM, respectively.

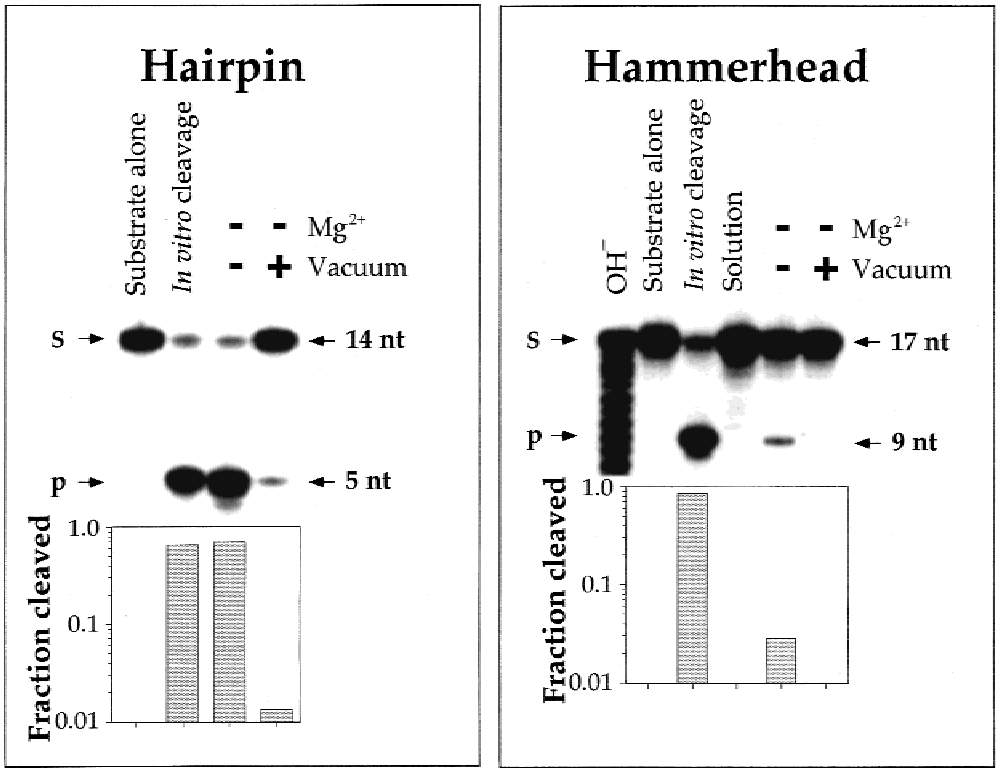

FIGURE 2. Ribozyme catalysis requires partial hydration of RNA films. The effect of hydration of RNA films on ribozyme catalysis was examined. Reactions were prepared in duplicate and RNA films were formed as described. Both reactions were incubated at 24 °C for 24 h either under vacuum (+) or under atmospheric pressure (−). Samples were quenched and analyzed as described in Results. Substrate alone: RNA substrate alone; in vitro cleavage: cleavage in standard cleavage buffer (hairpin ribozyme: 50 mM Tris-HCl, pH 8, and 12 mM $MgCl_2$, and HH16.1 (Clouet-d'Orval & Uhlenbeck, 1997): 50 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$) for 1 h at 37 °C; solution: ribozyme-substrate RNAs were maintained in water. An aliquot of substrate was used to generate an alkaline ladder in 50 mM Na_3PO_4, pH 12 at 65 °C for 10 min; S: substrate RNA; p: 5′-cleavage product. Samples were analyzed and data was plotted as described.

Inorganic and organic cations stimulate catalysis in RNA films

Reactions in RNA films prepared from 10 μL RNA solutions (25 nM ribozyme and ~0.6 nM substrate) containing 2 mM polyvalent cations (Mn^{2+}, Co^{2+}, and NH_4^+, spermidine, and spermine) or 100 mM monovalent cations (100 mM Na^+, Fig. 3, or 100 mM K^+, not shown) showed that cleavage was strongly stimulated under these conditions (Fig. 3). With one exception, these ionic conditions do not promote cleavage in solution. A very slow reaction was observed in solution for combinations of spermidine and spermine with 50 mM sodium EDTA (Fig. 3). In contrast, molar concentrations of these salts have been shown to support robust cleavage reactions in solution, where extent of cleavage

TABLE 1. ICPOES analysis.

Solution	Ca ppm	Mg ppm	Mn ppm	Co ppm	Cd ppm	Pb ppm	Zn ppm
2 mM NH_4OAc	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
100 mM NaCl	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
50 mM Tris-HCl	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
50 mM sodium EDTA	0.12	<0.02	<0.02	0.02	<0.02	<0.02	<0.02
H_2O	0.022	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
25 nM hairpin ribozyme, <1 nM substrate	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002	<0.002

The concentration of contaminants detected in all of the solutions used in this work were analyzed by ICPOES. In each case the highest value found for each solution is presented after subtracting the value for water. The concentration of the cations in the reactions as used was calculated by correcting for the change in concentration plus the value of water found for each solution.

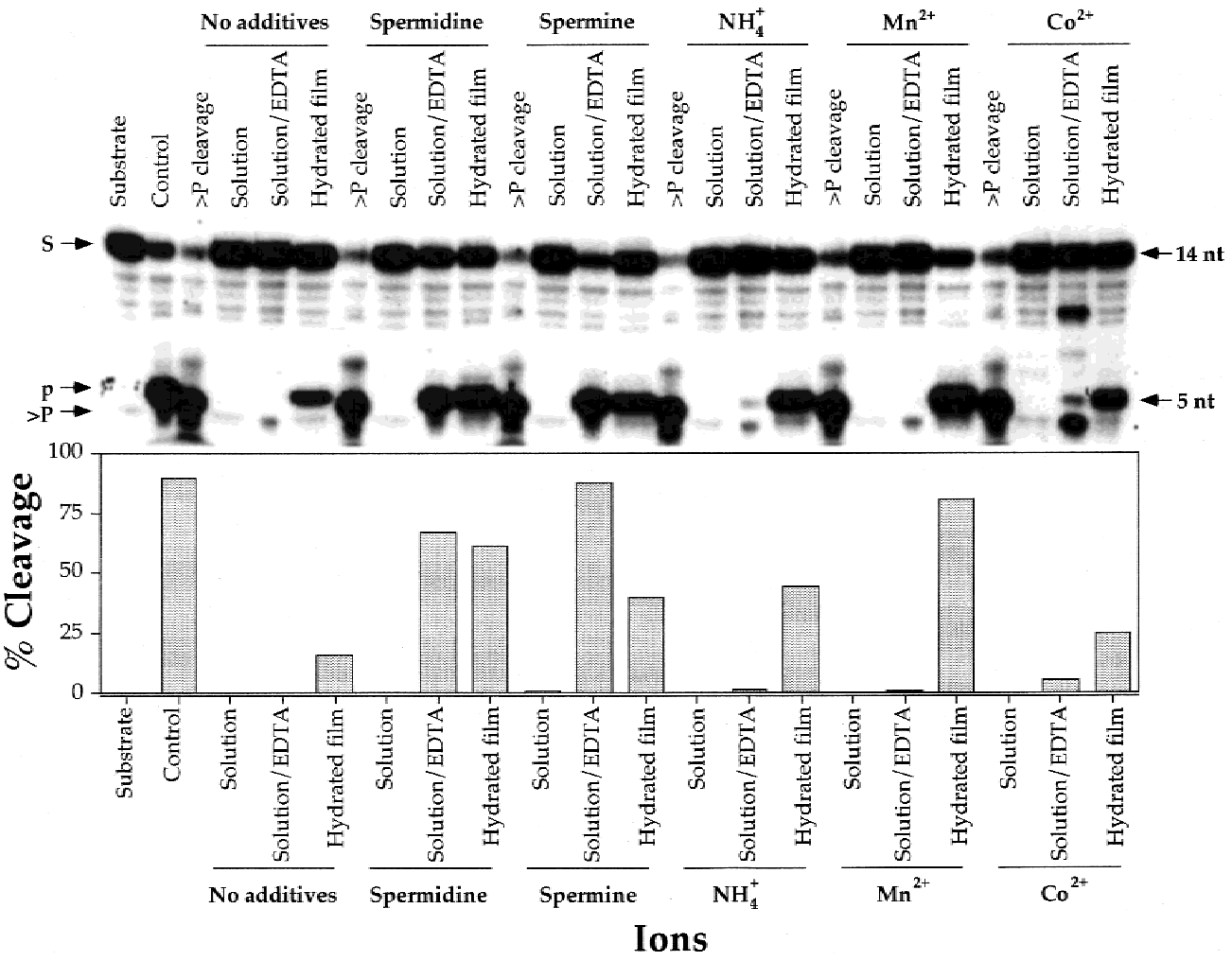

FIGURE 3. Catalysis in RNA films is stimulated by a variety of cations. A panel of cations and polyamines was used to examine their effects on catalysis in RNA films. Reactions were prepared (25 nM ribozyme, ~0.6 nM substrate) either in water alone or solutions containing various cations (2 mM final) and 50 mM EDTA, as indicated. Aliquots (10 μL) of samples were distributed into microcentrifuge tubes and either maintained as solutions covered with mineral oil to prevent evaporation or dehydrated and incubated at room temperature for 24 h as described in Materials and Methods. Samples were stopped and resolved by a 20% denaturing gel electrophoresis. Substrate: substrate alone; in vitro cleavage: cleavage in standard cleavage buffer (50 mM Tris-HCl, pH 8, and 12 mM $MgCl_2$) at 37 °C for 1 h; >P cleavage: cyclic phosphate breakage of a ribozyme-cleaved RNA in 0.01 N HCl at 37 °C for 24 h; solution: RNA was maintained as a solution; solution/EDTA: RNA solution with sodium EDTA (50 mM); hydrated RNA film: cleavage reaction was prepared in water, lyophylized, and incubated as described in Results; S: substrate RNA; p: 5′-cleavage product; >P, 2′,3′-cyclic phosphate cleavage product.

(≥85%) peaked at ~1.2 M of each salt, suggesting that this reaction resulted from a strong and apparently nonspecific stimulation by cations (Murray et al., 1998). Therefore, ribozyme catalysis in partially hydrated RNA films appears to mimic solution reactions carried out under conditions of high ionic strength.

Cleavage in RNA films proceeds over a broad range of temperature

We examined the cleavage reactions over a broad range of temperatures. Following a 24-h incubation, the maximum extent of cleavage was observed at −20 °C to 24 °C, with a smaller extent of cleavage observed at −70 °C and 37 °C (Fig. 4). In solution, the balance between cleavage and ligation shifts in favor of ligation with decreasing temperature and increasing cation concentration (Feldstein & Bruening, 1993; Nesbitt et al., 1999), suggesting that conditions that stabilize binding of the cleavage products and hairpin tertiary structure tend to favor ligation. Therefore, it is possible that the smaller extent of cleavage observed at low temperature results from a shift in equilibrium of cleavage and ligation.

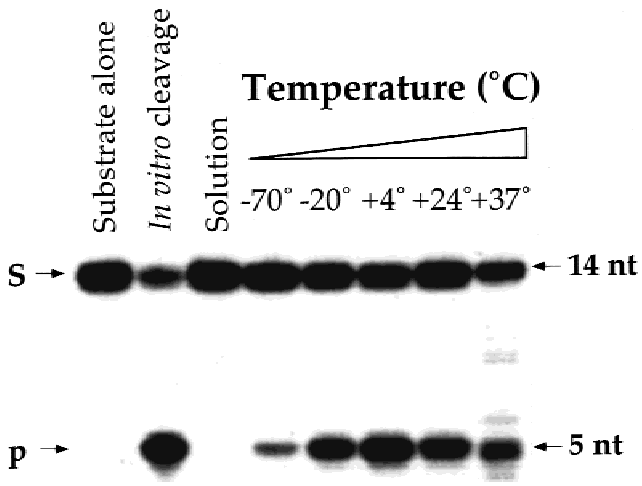

FIGURE 4. Effect of temperature on ribozyme catalysis in RNA films. To examine the temperature effect on catalysis in RNA films, RNA samples were prepared in water alone (25 nM ribozyme and ~0.6 nM substrate) and incubated at the indicated temperatures for 24 h. Samples were analyzed as described in Results. Substrate alone: in vitro cleavage control and solution controls are as described in Figure 3. S: substrate RNA; p: 5′ cleavage product.

Cleavage reaction is progressive and slow

Figure 5 shows that ribozyme cleavage in RNA films is progressive and appears to follow single exponential kinetics throughout prolonged incubations. We estimated the rate constant (2 day^{-1}) and the extent of the cleavage reaction (~40%) by curve fitting the data to a single exponential equation using nonlinear regression analysis (Esteban et al., 1997). The maximum observed extent of cleavage at ~16 h may represent the equilibrium point of the reaction.

Substrate cleavage in RNA films is a ribozyme-catalyzed reaction

Catalytically active and inactive ribozymes, noncognate ribozyme (Rz51wt; Burke, 1994), a ribozyme construct with separated domains (SBS•LB; Butcher et al., 1995) and noncleavable RNA substrate (Sd-A_{-1}; Chowrira et al., 1991) were employed for the specificity analysis (Fig. 6). Both active ribozyme and the domain-separated construct cleaved substrate in RNA films. The extent of cleavage by the active ribozyme was enhanced several-fold by the inclusion of sodium EDTA (50 mM) in the reactions. In contrast, no cleavage products were detected after prolonged incubation (16 h) with a noncleavable substrate, a catalytically inactive ribozyme (Rz-mt), or a noncognate ribozyme. These results indicate that 2′-hydroxyl of substrate nucleotide A_{-1} and sequence recognition by a catalytically active ribozyme are both essential for catalysis. The addition of EDTA did not further stimulate these latter reactions, except that of the mutant ribozyme, where some activity was observed when EDTA was present, suggesting that inhibitory effects of these mutations were partially rescued by a higher density of positive charges. These experiments show all of the essential characteristics of well-characterized solution reactions catalyzed by the hairpin ribozyme: (1) site-specificity, (2) generation of a 2′,3′-cyclic phosphate, (3) base pairing between ribozyme and substrate, and (4) requirement for a catalytically active ribozyme.

To determine if catalysis in RNA films is an intramolecular reaction requiring docking of the two domains of the ribozyme-substrate complex, a synthetic hairpin ribozyme construct in which helices 2 and 3 are fused (Porschke et al., 1999), preventing intramolecular docking of the two domains, was assayed for cleavage activity in RNA films (10 μL, 100 nM ribozyme, ~0.37 nM substrate, 0–24 h, 24 °C). Results showed a very small extent of cleavage (≤0.5% without NaCl and ~2% with 100 mM NaCl) after prolonged incubations (24 h; not shown). A significantly greater extent of cleavage was observed in solution under standard cleavage conditions with these constructs. These results suggest that productive intermolecular interactions are infrequent in the RNA films.

Tb (III) inhibits ribozyme catalysis in RNA films

Tb (III) has been shown to inhibit catalysis of both hammerhead and hairpin ribozymes by competing essential metal-binding sites (Feig et al., 1998; Walter & Burke, submitted). Tb (III) inhibits hairpin ribozyme catalysis through binding to a site within internal loop B (Walter & Burke, submitted). We asked whether Tb (III) can inhibit both catalyses in RNA films. RNA films prepared from solutions containing 2 μM and 40 μM $TbCl_3$ for hammerhead (10 μL, 100 nM ribozyme, ~1 nM substrate) and hairpin (10 μL, 70 nM ribozyme, ~0.8 nM substrate), respectively, showed that Tb (III) strongly inhibits reactions catalyzed by both ribozymes (not shown).

DISCUSSION

During the course of control experiments designed to rule out catalysis by expressed hairpin ribozymes following extraction from cultured mammalian cells, we observed that no ribozyme catalysis occurred during cell lysis, RNA extraction, and analysis (Seyhan et al., 1998). However, catalysis was strongly stimulated by the addition of sodium EDTA (50 mM). An analogous phenomenon has been identified by Kazakov et al. (1998). Additional investigation revealed that the observed reaction showed a very strong dependence on dehydration of RNA following ethanol precipitation or drying by vacuum centrifugation without added divalent cations. The apparent EDTA dependence resulted from a strong and apparently nonspecific stimulation

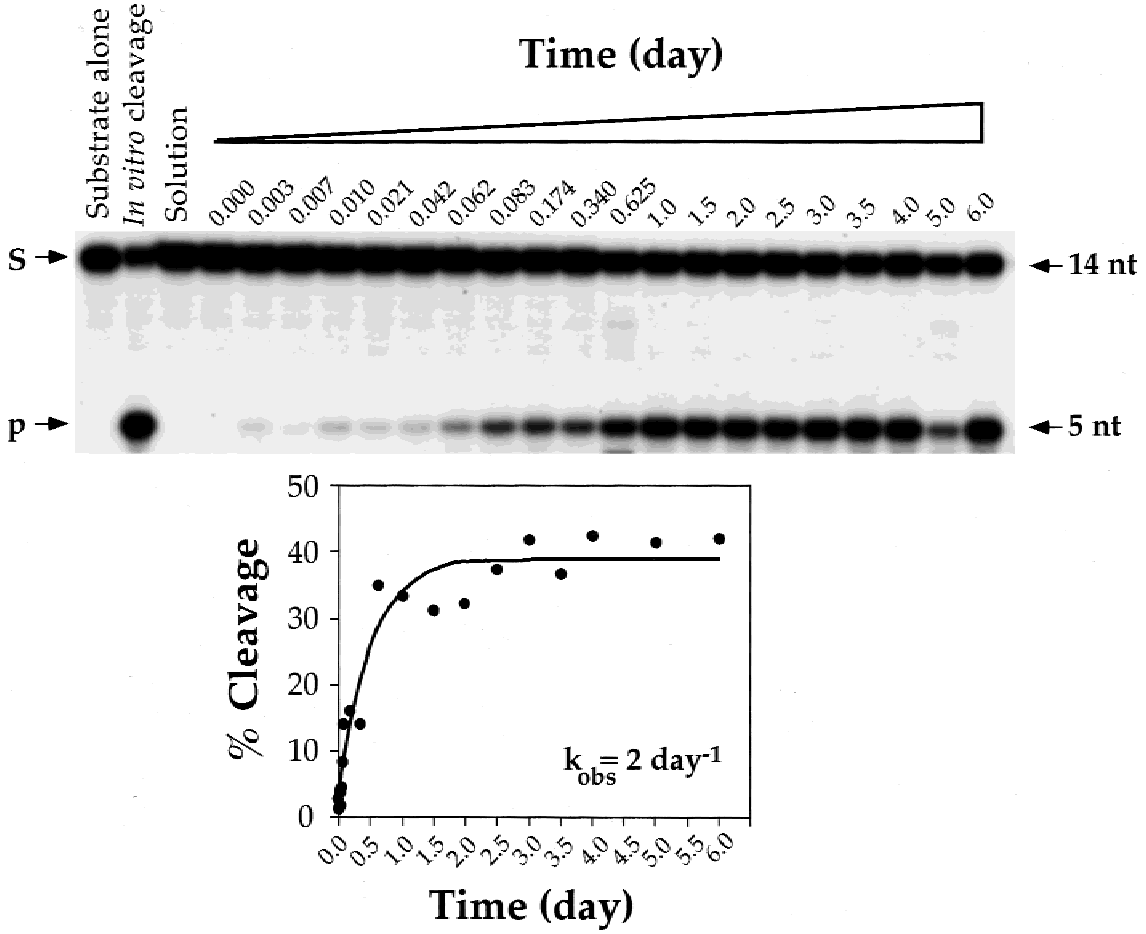

FIGURE 5. Ribozyme catalysis in RNA films is progressive. Reactions were performed under conditions of ribozyme excess (25 nM ribozyme and ~0.6 nM substrate). Briefly, the reaction was prepared in water and 10 μL aliquots were placed in microcentrifuge tubes and dehydrated in a Speedvac centrifuge. RNA films were incubated at 4 °C and were stopped at indicated time points. The fraction of substrate cleaved was plotted versus time and fit to the single exponential equation by nonlinear regression analysis as described (Esteban et al., 1997) using SigmaPlot 4.14 software (Jandel Scientific). Substrate alone and in vitro cleavage controls are as described in Figure 3. Solution: an analogous reaction was maintained in water at 4 °C for ≥36 h.

by cations, as a variety of divalent and monovalent inorganic and organic cations enhanced activity to the same degree. Elimination of added cations in the reactions containing only RNA and water resulted in slower, but still significant ribozyme-mediated catalysis upon vacuum dehydration of RNA.

Catalysis under partially dehydrated conditions shows all of the characteristics of the normal hairpin ribozyme reaction in solution. Little reactivity was observed with a ribozyme conformer incapable of intramolecular docking of its domains, suggesting that the RNA complexes are functional as monomers in RNA films, and that activity does not require packing as multimers. Tb (III) inhibits this reaction, as it does the normal ribozyme cleavage, in which it has been proposed to interfere with RNA tertiary folding, either by destabilizing the folded RNA or by inducing an alternative inactive conformer (Walter & Burke, submitted).

Although it is difficult to totally eliminate the presence of divalent cations in RNA films, several points can be used to argue against their presence: (1) analysis of all reaction components by ICPOES shows that contaminating free and bound metal ions are below the limits of detection, (2) the reaction proceeds with RNA that has been synthesized and purified in such a way that RNA has not been exposed to free divalent cations, (3) the reaction is not inhibited by EDTA or EGTA, but rather is strongly stimulated, and (4) the reaction is stimulated by low concentrations of cations that are shown to be ineffective in solution. Together, these results effectively rule out the possibility the activity is due to the presence of free or bound divalent metal ion contaminants.

Collectively, our results show that ribozymes can catalyze cleavage reactions in the absence of added metal ions in hydrated RNA films, suggesting that catalysis may result from the RNA and solute reaching a critical concentration upon dehydration, thereby increasing the probability of folding to the active tertiary structure. In addition to mass action and electrostatic effects, high

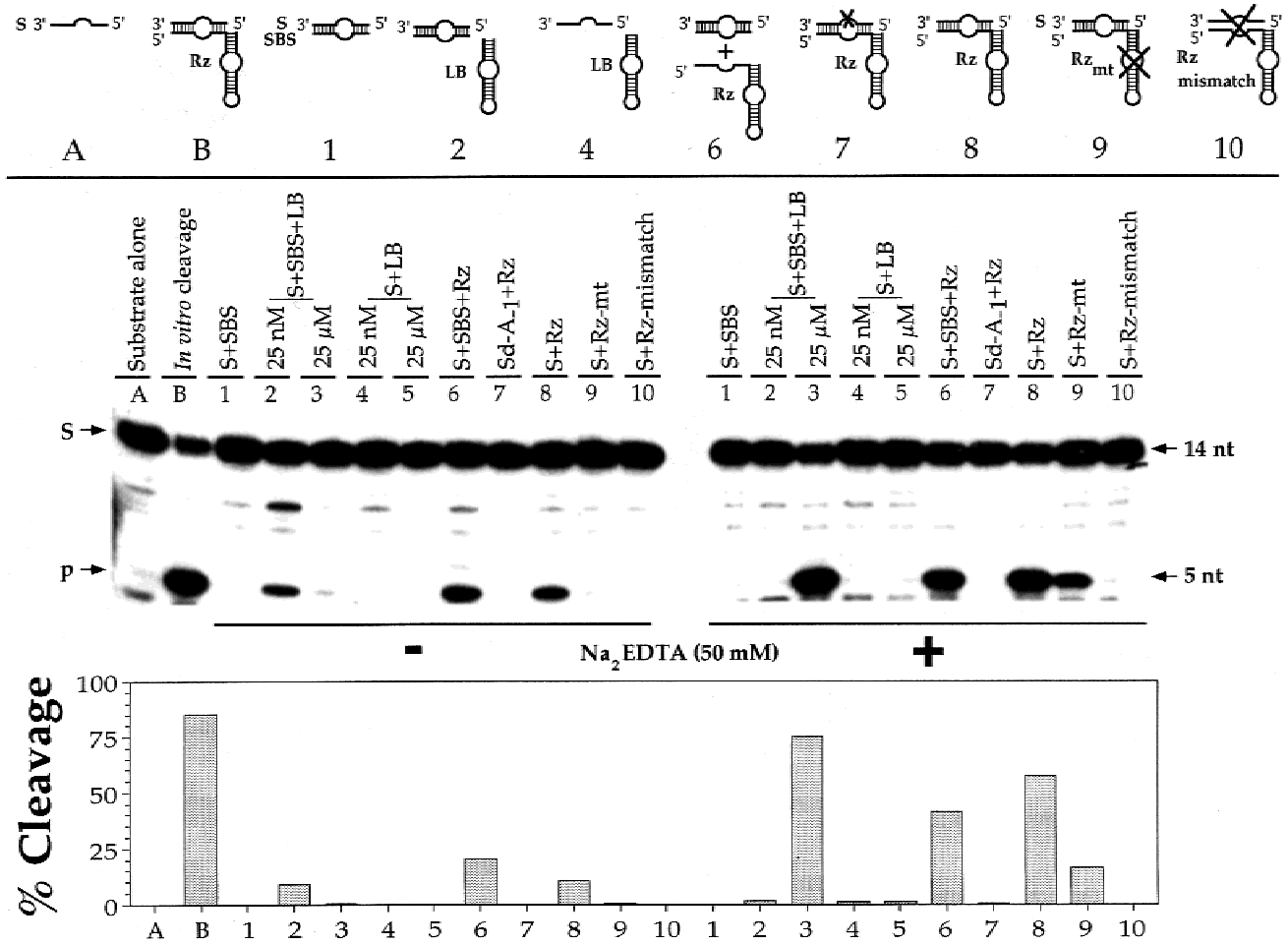

FIGURE 6. Catalysis in RNA films has the same requirements as in standard solution reaction conditions. Panel of ribozymes and substrate constructs were used to examine the specificity of the catalysis. The hairpin ribozyme-substrate RNAs were prepared as described above (25 nM ribozyme, ~0.6 nM substrate, except for the ribozyme construct with separated domains as indicated) in water. Ribozyme construct with separated domains are as described (Butcher et al., 1995). Briefly, the loop B domain (LB) was separated at the junction between helices 2 and 3, yielding a three-piece ribozyme consisting of a duplex between substrate (S) and substrate binding strand (SBS) together with the loop B domain (concentrations of separated domains are indicated while keeping substrate concentrations constant). S (dA_{-1}): noncleavable substrate containing a deoxyadenosine at position −1, 5′ to the cleavage site (Chowrira et al., 1991). Mutations within the ribozyme (Rz-mt, $A_{26}U$, $G_{36}A$, and $U_{37}G$; R.A. Banerjee, pers. comm.) and substrate (dA_{-1}) eliminates cleavage but do not affect substrate binding. Hairpin ribozyme with wild-type substrate specificity (Burke, 1994) was used as a specificity control. Reactions were prepared in two parallel sets; the first contained only RNA and water whereas the second set contained 50 mM sodium EDTA. Reactions were dehydrated in a Speedvac centrifuge and RNA films were incubated for ≥16 h at 24 °C. Samples were analyzed as described in Results. Substrate: substrate alone; in vitro cleavage: cleavage in standard cleavage buffer.

osmolarity promotes displacement of water, favoring compact RNA structures with fewer associated water molecules (Nesbitt et al., 1999, and references therein).

Details of the requirement of a small amount of water for hairpin ribozyme catalysis are unknown. The conformation of the partially hydrated RNA may well be different than that of the fully dehydrated RNA molecule; partial hydration may provide ribozyme with conformational flexibility needed for catalysis. At least one global conformational change has been shown to be part of the reaction pathway that leads to cleavage (Walter et al., 1998). The shallow pH dependence of the cleavage and ligation reactions suggests that an additional localized conformational change may be the rate-limiting step for catalysis (Nesbitt et al., 1997, 1999). Partial solvation of RNA would be expected to result in numerous changes in polar and hydrophobic interactions, supporting electrostatic contributions to catalysis (Murray et al., 1998).

Partial rescue of ribozyme-inactivating mutations by monovalent cations appears to parallel that observed for divalent metal ions (Jaeger et al., 1991; Hanna & Szostak, 1994). Monovalent cations, however, can also facilitate RNA catalysis at ≥500 mM concentrations (Murray et al., 1998). Evidence clearly indicates that the hairpin ribozyme does not require inner-sphere co-

ordination of metal ions (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997) and the cleavage and ligation reactions are supported by a variety of organic and inorganic cations (Chowrira et al., 1993; Earnshaw & Gait, 1998; Murray et al., 1998; Nesbitt et al., 1999). Catalysis could be due to a conformational change, or functional groups in the ribozyme may participate directly in catalysis via general acid–base catalysis (Hampel & Cowan, 1997; Nesbitt et al., 1997). These results indicate that all essential elements of catalytic function are provided by the folded RNA itself, with a variety of cations functioning to stabilize the active structure by electrostatic screening of charged phosphodiester backbone.

This partially dehydrated RNA reaction resembles that reported for several protein enzymes. Studies of lysozyme powders and protease α-chymotrypsin equilibrated with water in air have shown that catalysis occurs at a hydration level below monolayer water coverage (Bone & Pethig, 1982; Zaks & Klibanov, 1988; Affleck et al., 1992). NMR analysis of the hydration process indicates that the catalytic activity is a direct consequence of an increase in lysozyme's conformational flexibility by water's ability to effect dielectric screening (Affleck et al., 1992). The protease subtilisin Carlsberg in partially hydrated organic solvents was used as an alternative to hydrated enzyme powders (Affleck et al., 1992; Maksareva & Khurgin, 1995). As in hydrated powders, enzyme function in partially hydrated media is dependent on the water content of the enzyme. However, the role of water in molecular events at the enzymatic active site in a low-water environment is not well understood.

RNA catalysis under conditions of high nucleic acid concentrations and reduced water may, in some ways, be analogous to the intracellular environment where protein factor(s) may partially substitute for the function of cations. We hope that the concepts and experimental approaches developed in this study will be conducive to further exploration of enzymology of ribozymes and to provide a further caution for investigators attempting to examine the biochemical activity of engineered ribozymes in vitro and in cells. Careful attention to controls is clearly essential, as unanticipated catalysis can occur during cell lysis, RNA extraction, precipitation, drying, and/or other steps in RNA manipulation.

MATERIALS AND METHODS

DNA and RNA preparation

DNA and RNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using standard phosphoramidite chemistry, or RNA was transcribed from DNA oligonucleotides (Seyhan et al., 1998). Synthetic RNA oligonucleotides were deprotected as described (Sproat et al., 1995) and purified by denaturing PAGE and subsequent C8-reversed-phase HPLC. Synthetic RNA substrates (14 nt) were 5′-end labeled with γ-^{32}P-ATP and T4 polynucleotide kinase as described (Seyhan et al., 1998).

Cleavage reactions

Reactions (10 μL) were prepared in double-distilled deionized water in ribozyme excess (25 nM ribozyme and 0.6 nM 5′-labeled substrate) in microcentrifuge tubes. A fluorescence quenching assay showed that complex formation did not occur in water alone (Walter & Burke, 1997; N.G. Walter & J.M. Burke unpubl. results). RNA solutions were lyophilized at room temperature in a Speedvac vacuum centrifuge to form RNA films. Reactions prepared with various inorganic and organic cations were examined following drying of solutions.

To determine whether ribozyme catalysis occurred during RNA preparation and analysis after cellular lysis, cleavage assays and mock primer extensions were conducted using mouse L cell lysates as described (Seyhan et al., 1998). Hairpin ribozyme (10 nM) was preincubated with 5′-labeled substrate ($\sim$0.1 nM) for 10 min at 37 °C in water. The ribozyme and substrate solution was then added to cell lysate, and carried through RNA extraction, purification, and reactivity assays as described (Seyhan et al., 1998).

Standard in vitro solution cleavage reactions were conducted in ribozyme excess in 50 mM Tris-HCl (pH 8) and 12 mM $MgCl_2$ for 1 h at 37 °C. Reactions were quenched with 10 μL formamide loading buffer (90% formamide and 25 mM EDTA). Samples were denatured at 90 °C for 2 min and resolved by electrophoresis on 20% polyacrylamide-8 M urea gels. Gels were analyzed using a Bio-Rad GS-525 molecular imager and Molecular Analyst 2.1 software. The bands were quantified and data was plotted as the percent of the 5′ cleavage product relative to the full length unprocessed RNA as described (Seyhan et al., 1998). Decyclization of the 2′,3′-cyclic phosphate (>P) terminus generated by ribozyme was performed by incubating the ribozyme cleavage products in 0.01 N HCl for 24 h at 37 °C. Reactions containing $TbCl_3$ (40 μM) were prepared as described in water alone and processed as above.

Trace metal analysis

All reagents were ultrapure grade and purchased from Sigma, water was double-distilled deionized grade used for the experiments. RNA samples were HPLC purified except as noted and dissolved in water. Upper limits for metal-ion content were established by analyzing all reaction components by ICPOES (Perkin-Elmer 3000 DV, Table 1).

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