The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone

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Background: The catalytic activity of RNA enzymes is thought to require divalent metal ions, which are believed to facilitate RNA folding and to play a direct chemical role in the reaction.

Results: We have found that the hammerhead, hairpin and VS ribozymes do not require divalent metal ions, their mimics such as $[Co(NH_3)_6]^{3+}$, or even monovalent metal ions for efficient self-cleavage. The HDV ribozyme, however, does appear to require divalent metal ions for self-cleavage. For the hammerhead, hairpin and VS ribozymes, very high concentrations of monovalent cations support RNA-cleavage rates similar to or exceeding those observed in standard concentrations of Mg^{2+}. Analysis of all reaction components by inductively coupled plasma-optical emission spectrophotometry (ICPOES) and the use of a variety of chelating agents effectively eliminate the possibility of contaminating divalent and trivalent metal ions in the reactions. For the hairpin ribozyme, fluorescence resonance energy transfer experiments demonstrate that high concentrations of monovalent cations support folding into the catalytically proficient tertiary structure.

Conclusions: These results directly demonstrate that metal ions are not obligatory chemical participants in the reactions catalysed by the hammerhead, hairpin, and VS ribozymes. They permit us to suggest that the folded structure of the RNA itself contributes more to the catalytic function than was previously recognised, and that the presence of a relatively dense positive charge, rather than divalent metal ions, is the general fundamental requirement. Whether this charge is required for catalysis *per se* or simply for RNA folding remains to be determined.

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Introduction

We have investigated the metal-ion dependence of four ribozymes that are derived from self-cleaving sequences found within natural RNA replicons. The hammerhead and hairpin ribozymes are found in opposite strands of the satellite RNA of tobacco ringspot virus (sTRSV) and related sequences [1], whereas the HDV ribozyme [2] is necessary for replication of a unique human pathogen, the hepatitis delta virus. The VS ribozyme [3] is embedded within a circular single-stranded RNA associated with the Varkud retroplasmid of *Neurospora* mitochondria. These self-cleaving RNAs all undergo a transesterification reaction that generates 5′-hydroxyl and 2′,3′-cyclic phosphate termini.

The activity of these and all ribozymes characterised to date is strongly stimulated by the presence of moderate concentrations of divalent metal ions; typically Mg^{2+} (5–50 mM) is used for *in vitro* studies of structure and activity. It is believed that Mg^{2+} is the key ionic contributor to ribozyme activity within cells, yet cellular concentrations of available Mg^{2+} (~1 mM [4]) are lower than the *in vitro* optima. It is therefore possible that additional factors accelerate ribozyme-catalysed reactions *in vivo*. Candidates for such components include specific and nonspecific RNA-binding proteins, segments of the self-cleaving RNAs not present in the *trans*-acting derivatives, and other cationic species, including organic amines and monovalent cations.

A number of studies have been conducted that explore the role of metal ions in ribozyme folding and catalysis (reviewed in [5,6]). It is clear that Mg^{2+} and other divalent cations can function in the formation of secondary and tertiary structures, as has been well documented for a wide variety of RNA molecules. Monovalent cations, however, can also support RNA folding, including, in at least some cases, the formation of specific tertiary structures. For the hammerhead ribozyme, nuclear magnetic resonance (NMR) [7,8] and crystallographic studies [9,10] have shown that Na^+, Li^+ and NH_4^+ can support formation of tertiary structures that are similar or identical to those observed in the presence of Mg^{2+}. This has led to a

generally accepted model in which a variety of monovalent or divalent metal ions can function to stabilise ribozyme structure, whereas one or more divalent metal ions play a direct role in active-site chemistry.

In the case of the hammerhead ribozyme (reviewed in [11]), studies using phosphorothioate-modified RNA [12–19], experiments analysing the pH-rate profiles of reactions in the presence of different metal ions [16,20–22] and the crystallographic observation of Mg^{2+} in the vicinity of the *pro*-R oxygen of the scissile phosphate [10] are taken as strong evidence for the direct participation of divalent metal ions in active-site chemistry. A number of different mechanisms for hammerhead cleavage have been proposed involving one or two divalent metal ions. The divalent metal ion can participate as either a Brönsted acid/base via the first sphere of hydration [20] or a Lewis acid by inner-sphere coordination [22–24]. Unambiguous assignment of catalytic function to divalent cations bound to specific sites in the hammerhead ribozyme has, however, proven to be elusive. The function of divalent metal ions in VS and HDV ribozyme catalysis has been explored less extensively.

Data are available which hint that the active participation of metal ions in catalysis is not obligatory. Studies on the hammerhead and hairpin ribozymes have shown that, with spermidine or spermine added in place of Mg^{2+}, the cleavage reaction proceeds at rates three orders of magnitude slower than the Mg^{2+}-mediated reaction [20,25]. In dimeric (+)-sTRSV RNA, monomerisation (via a hammerhead motif) occurred in spermidine, spermine or EDTA to extents comparable to those in 5–20 mM Mg^{2+} [26]. Recently, it has been shown that cobalt (III) hexaamine [$Co(NH_3)_6^{3+}$] can replace divalent metal ions in hairpin ribozyme cleavage, despite the fact that its inert coordination shell precludes inner-sphere coordination of the RNA by the metal [27–29]. Furthermore, the rate of the reaction observed in $Co(NH_3)_6^{3+}$ can exceed that obtained in Mg^{2+}, and the apparent K_m for $Co(NH_3)_6^{3+}$ is lower than that for Mg^{2+} by a factor of ten. These results appear to rule out all extant models for the direct participation of divalent metal ions in hairpin ribozyme reaction chemistry. Further evidence suggesting that not all nucleic-acid enzymes require divalent metal ions for catalytic function comes from the isolation of divalent-metal-ion-independent DNA and RNA enzymes from random sequence pools that catalyse RNA cleavage [30,31].

Here, we describe the outcome of studies demonstrating that the hammerhead, hairpin and VS ribozymes are capable of catalysing efficient RNA-cleavage reactions in the absence of divalent cations, when high concentrations of monovalent metal ions or ammonium ions are present. We also describe a series of control experiments designed to rule out the possibility that trace metal contaminants are responsible for our observations. Direct metal-ion participation in the reaction chemistry is therefore not a requisite feature of the catalytic mechanism of these three ribozymes under conditions of high ionic strength.

Results

Conditions that promote ribozyme activity in the absence of divalent metal ions

In our laboratories, time-resolved crystallographic studies of the hammerhead ribozyme [10,32] and cellular-activity analyses of the hairpin ribozyme [33] have led us to re-examine the role of divalent metal ions in the small ribozymes.

Kinetic characterisation of the hammerhead ribozyme in solutions that mimic conditions within active crystals revealed similar cleavage rates in 2 M Li_2SO_4, whether or not divalent metal ions were added. Although we initially suspected contamination by divalent metal ions, we found that the reaction was not inhibited by the addition of 500 mM EDTA in either 2 M Li_2SO_4 or 4 M LiCl.

Our studies of hairpin ribozyme activity in mammalian cells led to a similar finding. Control experiments designed to rule out ribozyme activity following cell lysis showed that no cleavage was obtained when a standard RNA-extraction solution was used prior to precipitation of the RNA. Paradoxically, extensive cleavage took place when the RNA-extraction solution was supplemented with the sodium salt of EDTA. Subsequent analysis revealed that cleavage took place during precipitation or lyophilisation, suggesting that high salt concentration was a requirement for cleavage (A.A.S and J.M.B., unpublished observations). When a hammerhead ribozyme was handled in the same manner, identical results were obtained.

EDTA titrations as a control for multivalent metal-ion contamination

We designed an EDTA titration experiment to rule out the possibility that divalent and trivalent metal ions could be contributing to the cleavage reactions observed, and to test the generality of our initial observations. Hairpin, hammerhead, VS and HDV ribozyme reactions were initiated under standard *in vitro* conditions, containing 12, 10, 100 and 10 mM $MgCl_2$, respectively. These reactions were then titrated with EDTA at constant pH. We observed extensive cleavage when Mg^{2+} concentrations exceeded EDTA concentrations. Cleavage reactions catalysed by each of the ribozymes were completely quenched by the presence of EDTA concentrations equal to, or in modest molar excess over, the concentration of Mg^{2+}, demonstrating quantitative chelation of the Mg^{2+} and concomitant inhibition of the reaction. Further addition of EDTA showed a recovery of cleavage activity for the hairpin, hammerhead and VS ribozymes at high Na^+ concentration (0.3–1 M). To determine if EDTA or a Mg^{2+}–EDTA

complex was acting as the essential co-factor under these conditions, we repeated the above titrations with EDTA and NaCl in the absence of Mg^{2+}. As expected, no reactions were observed at low cation concentrations, but hairpin, hammerhead, and VS ribozyme activity was observed at the same Na^{+} concentrations as in the Mg^{2+}/EDTA titration experiments.

These results demonstrate that magnesium ions support catalysis of these three ribozymes at low ionic strength, are quantitatively sequestered and rendered inert by the chelating effect of EDTA, and are functionally compensated for by high concentrations of sodium ions.

The hepatitis delta ribozyme does not function in high concentrations of monovalent salts alone

The hepatitis delta ribozyme was the only one of these four catalytic motifs that showed the activity that would have been anticipated at the outset of this study: EDTA quenched the cleavage reaction, and no activity was detected at higher sodium ion concentrations. The inability of high concentrations of sodium ions to rescue activity of the hepatitis delta ribozyme suggests that there might be a specific requirement for divalent metal ions in HDV catalysis and/or folding.

Testing hammerhead, hairpin and VS cleavage activity in lithium ions

We examined the activity of the hairpin, VS, HDV and two distinct hammerhead ribozymes in single-turnover kinetic studies conducted under standard conditions (in which Mg^{2+} is present), and in the presence of high concentrations of Li^{+} with additives (Table 1). To further ensure that the reaction was not catalysed by Mg^{2+} (or a similar contaminant) that was not sequestered by EDTA under these conditions, we determined the cleavage activities in deliberately contaminated reactions (20 mM $MgCl_2$, 2 M Li_2SO_4 and 25 mM EDTA). No significant changes in cleavage activities were observed.

For the hairpin and the previously crystallised hammerhead (HH_{xtal}) ribozymes, the cleavage rate constants in the presence of 4 M Li^{+} and 25 mM EDTA are 45–70% of that observed in Mg^{2+} (0.058 cf. 0.083 min^{-1} and 0.032 cf. 0.071 min^{-1}, respectively) and are essentially unaffected by the addition of EDTA plus Mg^{2+}, or the removal of EDTA (0.058 cf. 0.059 and 0.093 min^{-1} and 0.032 cf. 0.026 and 0.025 min^{-1}, respectively). In both cases, the addition of 10 mM Mg^{2+} without EDTA restores full activity (0.1 cf. 0.083 min^{-1} and 0.059 cf. 0.071 min^{-1}, respectively).

For the optimised hammerhead ribozyme ($HH_{16.1}$), the rate of cleavage in 4 M Li^{+} and 10 mM Mg^{2+} is reduced eightfold relative to the rate under 'standard' conditions (0.65 cf. 5.5 min^{-1}). The Li^{+} mediated cleavage is only 25–32-fold slower than the cleavage in Mg^{2+} alone (0.17, 0.21 and 0.22 cf. 5.5 min^{-1}). The partial inhibition of the $HH_{16.1}$ reaction upon addition of 4 M Li^{+} provides additional compelling evidence that the observed cleavage activity does not result from a trace amount of contaminating divalent cations. Hammerhead cleavage in the presence of 4 M LiCl gave identical results, thus ruling out a role for a contamination specific to a certain salt.

Although the cleavage rate of $HH_{16.1}$ in 4 M Li^{+} plus 25 mM EDTA is less than that under standard conditions, it still exceeds that of HH_{xtal} and the hairpin ribozyme under the same conditions. We conclude that the rescue of hammerhead ribozyme activity by high concentrations of monovalent cations is not an idiosyncrasy of one particular hammerhead sequence. These rates are 10^5 to 10^6 times faster than the previously estimated background rate of cleavage [34] under standard reaction conditions and at least 100–1000-fold faster than previously reported for the hammerhead ribozyme in the absence of divalent metal ions [16].

Table 1

***Trans*-cleavage rates (single-turnover) under various conditions.**

	HH_{xtal} (min^{-1})	$HH_{16.1}$ (min^{-1})	Hairpin (min^{-1})	HDV (min^{-1})	VS (min^{-1})
4 M Li^{+}, 25 mM EDTA	0.032	0.17	0.058	4×10^{-5}	0.26
4 M Li^{+}, no EDTA	0.025	0.21	0.093	4×10^{-5}	0.13
4 M Li^{+},25 mM EDTA, 20 mM Mg^{2+}	0.026	0.22	0.059	5×10^{-5}	0.22
4 M Li^{+}, no EDTA, 10 mM Mg^{2+}	0.059	0.65	0.10	0.0023	0.28
4 M NH_4^{+}	0.0035	0.051	0.32	~1×10^{-6}	0.013
4 M Li^{+}, 25 mM CDTA	nd	0.19*	0.18†	nd	0.24†
4 M Li^{+}, 25 mM DTPA, 1mM each Me^{2+}	nd	0.21*	0.22†	nd	0.12†
4 M Li^{+}, 25 mM EDTA, 1mM each Me^{2+}	nd	0.20*	0.29†	nd	0.2†
4 M Li^{+}, 1 mM each Me^{2+}	nd	5.4*‡,	0.087†	nd	0.67†
'Standard' reaction conditions	0.071	5.5	0.083	0.043	0.0093

HH_{xtal} indicates the crystallised hammerhead sequence [10], and $HH_{16.1}$ indicates the sequence from Clouet-d'Orval and Uhlenbeck [50]. The hairpin-ribozyme sequence is SV5 EH4 from Esteban *et al.*, [47], the HDV-ribozyme sequence is CDC200 from Puttaraju *et al.*, [51], and the *Neurospora* VS sequence is from Guo and Collins [52]. Reaction conditions were 50 mM Tris-HCl pH 8 (except *Neurospora* VS pH 7.5) at 25°C and varying cation concentrations as indicated. $MgCl_2$ concentrations under standard conditions were 10 mM for HH_{xtal}, $HH_{16.1}$ and HDV, 12 mM for the hairpin ribozyme, and 20 mM for the *Neurospora* VS ribozyme. All experiments were done at pH 8, 50 mM Tris–HCl and 4 M Li^{+}. 1 mM each Me^{2+} is mixture of the divalent cations Ba, Ca, Cd, Co, Mg, Mn, Pb, Sr and Zn at 1 mM each except Ba and Sr were not used for the hairpin ribozyme. *Ribozyme and substrate strand at 3 μM and 0.1 μM, respectively, identical results were obtained for ribozyme and substrate strands at 30 and 1 nM, respectively. †Ribozyme and substrate concentrations were increased to 1 μM and 0.1 μM, respectively. ‡pH 7. nd, not determined.

The VS ribozyme shows significantly enhanced activity (14–30-fold) in 4 M Li^+ relative to that under standard conditions, regardless of the presence of Mg^{2+}. In contrast, the HDV ribozyme, like $HH_{16.1}$, is inhibited by the addition of 4 M Li^+. Unlike the other three ribozymes, however, the HDV ribozyme is essentially inactive in Li^+ alone.

The hammerhead, hairpin and VS ribozymes are catalytically active in ammonium ions

Although sodium and lithium ions are poor Lewis acids, it is conceivable that they might be able to mimic the chemistry of magnesium ions [6] at the high concentrations employed in this study. We therefore measured ribozyme activity in the presence of ammonium ions (Table 1), which are non-metallic and are unable to function as Lewis acids. Our results show that the reactions of hammerhead, hairpin, and VS ribozymes can be supported by ammonium ions. The hairpin ribozyme, in particular, shows a fourfold increase in cleavage rate when magnesium ions are replaced by ammonium ions. The VS ribozyme has activity equivalent to that observed in the standard magnesium-containing buffer. Although both of the hammerhead constructs displayed significant activity in the presence of ammonium ions, the magnitude of the activity was substantially reduced relative to the activity under standard conditions, being inhibited at concentrations higher than 4 M. This reduction is particularly notable in the case of the $HH_{16.1}$ construct, whose activity was optimised in the standard magnesium-containing buffer. These results are consistent with a hypothesis in which monovalent cations stimulate ribozyme activity through a common mechanism not involving a Lewis acid.

Na^+ mimics the role of Mg^{2+} in hairpin ribozyme docking

Recently, we have developed an assay to monitor docking of the two domains of the hairpin ribozyme, using fluorescence resonance energy transfer (FRET) [35], with donor and acceptor fluorophores coupled to the ends of the two domains of the ribozyme–substrate complex. Results of the initial study indicate that docking of the two domains is required for cleavage, and is blocked by a wide variety of mutations and functional-group substitutions in the ribozyme and substrate that have previously been shown to inhibit cleavage.

We used the FRET assay to compare docking of the two domains when the ribozyme–substrate complex was incubated under a variety of ionic conditions. In 12 mM Mg^{2+}, docking is efficient and leads to a pronounced increase in relative FRET efficiency after mixing ribozyme and substrate. When $MgCl_2$ was replaced by NaCl, no increase in the FRET signal was observed at NaCl concentrations less than or equal to 1 M. At 1.5 and 2.0 M NaCl, however, a rapidly increasing, comparably small, but reproducible FRET signal was seen, providing physical evidence for interaction of the domains in high concentrations of monovalent salts. Increasing monovalent cation concentrations led to an enhanced FRET signal, indicating further stabilisation of the catalytically proficient docked tertiary structure.

Evidence against an aberrant cleavage mechanism in monovalent ions

To test whether the hammerhead ribozyme cleavage mechanism in the presence of monovalent cations is the same as that occurring with Mg^{2+} present, we substituted the attacking 2′-hydroxyl group at the scissile bond (position 17) with a methoxyl group. As with the Mg^{2+}-stimulated reaction, this modification completely blocked cleavage, indicating that the two sets of reaction conditions support the same chemical mechanism of bond cleavage (attack upon the scissile phosphate by the adjacent 2′-oxyanion). Furthermore, we tested the activity of a modified hammerhead construct with a G5→U base substitution that is known to have significantly reduced cleavage activity [36]. It catalyses cleavage with a rate of $1.3 \times 10^{-4} min^{-1}$ ($HH_{16.1}$) in 4 M Li^+, 25 mM EDTA, at pH 8, a 1500-fold reduction in cleavage activity. The hammerhead cleavage reaction in Mg^{2+} was found previously to be strongly pH-dependent [20], which is also the case for the reaction in 4 M Li^+, ($HH_{16.1}$ 0.2 min^{-1} at pH 8 and 0.0015 min^{-1} at pH 6; HH_{xtal} 0.03 min^{-1} at pH 8 and 0.0006 min^{-1} at pH 6).

In the case of the hairpin ribozyme, cleavage in the presence of high Na^+ concentrations was found to require base-pairing between ribozyme and substrate (i.e., a non-cognate substrate was not cleaved), and was blocked by ribozyme and substrate modifications known to inhibit the standard reaction, such as a triple mutant ribozyme (A26→U,G36→A,U37→G) and deletion of the attacking 2′-hydroxyl group at substrate position A_{-1} ([37]; A.R. Banerjee and J.M.B., unpublished observations).

Finally, we confirmed that the hammerhead- and hairpin-cleavage reactions in monovalent salts each proceed with formation of a 2′,3′-cyclic phosphate (data not shown). Taken together, these results indicate that the cleavage reactions catalysed by hammerhead and hairpin ribozymes in the presence of divalent cations are the same as those catalysed by the hammerhead and hairpin in the absence of divalent cations.

Trace metal ion analysis

Throughout this work, we have used ultra-pure reagents. We remained concerned, however, that low levels of divalent metal-ion contaminants might be present, and that these contaminants could potentially lead to the observed cleavage. To address this issue, we analysed all of our reagents for divalent metal-ion contamination, details of which are contained in the Materials and methods section. The highest concentration of contaminants found are Ca^{2+} and Mg^{2+} in the Li^+- and Na^+-containing buffers.

Even with a low concentration of divalent metal-ion contaminants, the possibility that monovalent cations could assist folding, leaving the divalent cations to contribute a specific chemical function, is *a priori* quite plausible. We therefore calculated the amount of free divalent metal ions in the presence of 4 M Li^+ and 25 mM EDTA from the equation:

$$Li^+ + M^{2+}\text{–EDTA}^{4-} \overset{K_{Li^+/Mg^{2+}}}{\rightleftharpoons} M^{2+} + Li^+\text{-EDTA}^{4-}$$

with

$K_{Li^+/M^{2+}} = K_{Li^+}/K_{M^{2+}}$ from [38]

$[Li^+] = 4$ M, $[EDTA]_{tot} = 25$ mM

$[EDTA]_{tot} = [Li^+\text{–EDTA}] + [M^{2+}\text{–EDTA}]$

$[M^{2+}]_{free} = [M^{2+}]_{tot} - [M^{2+}\text{–EDTA}]$

The equation for $[M^{2+}\text{–EDTA}]$ can be then derived as follows:

$0 = [M^{2+}\text{-EDTA}]^2 - ([M^{2+}]_{tot} + [EDTA]_{tot} + (K_{Li^+/M^{2+}})[Li^+])[M^{2+}\text{–EDTA}] + [M^{2+}]_{tot}[EDTA]_{tot}$

The least effectively chelated divalent cations, Ca^{2+} and Mg^{2+}, are present at the highest concentrations, below 3 ppm (7.5×10^{-5} M) and 1 ppm (4.167×10^{-5} M), respectively. The other cations, present at lower concentrations, are chelated much more strongly [39] and therefore can be neglected in determining the free divalent metal-ion concentration in the presence of 25 mM EDTA. Thus, the free Ca^{2+} and Mg^{2+} concentrations are below 1.5×10^{-10} and 8.3×10^{-9}, respectively. These low values indicate that it is extremely unlikely that the activity observed at high monovalent ion concentration is due to the presence of free divalent metal ions. Furthermore, changing the concentration of the ribozymes by 100-fold (Table 1) gave no change in the observed rates, indicating that the sub-stoichiometric amounts of divalent cations are not responsible for the observed cleavage activity.

The chelators CDTA and DTPA give the same results as EDTA

There also remained the possibility that the metal–EDTA complex, despite its net charge of 2–, was able to activate the scissile phosphate (with its net charge of 1–) thus catalysing the cleavage chemistry. We tested this hypothesis using the effective and kinetically more inert chelators *trans*-1,2,-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA) and diethylenetriaminepentaacetic acid (DTPA) [39]. CDTA is an EDTA-like compound with a 100–1000-fold higher affinity for divalent and trivalent cations. Substituting EDTA with CDTA and increasing the RNA concentration 100-fold, effectively reducing the concentration of 'free' divalent metal ions to at least 10,000 lower than the RNA concentration, gave essentially identical cleavage rates in 4 M Li^+ (Table 1). We also tested the penta-coordinated chelator DTPA as well as EDTA in the presence of 1 mM of each of several divalent and trivalent metal ions that promote cleavage. Still, no substantial change in catalytic rates was observed (Table 1). The observed activity therefore appears not to be due to metal-ion–chelator complexes of contaminating cations.

Discussion

Our results show that molar concentrations of monovalent metal (Na^+ and Li^+) and nonmetal (NH_4^+) ions can replace divalent metal ions (e.g., Mg^{2+}) in support of the catalytic activity of three small RNA enzymes, the hairpin, hammerhead and VS ribozymes. We believe that we have rigorously ruled out the possibility that contaminating divalent and trivalent metal ions are contributing to catalysis in these reactions. It is significant that the cleavage rates that we have observed in monovalent salts approach, and in some cases exceed, those observed under standard conditions where Mg^{2+} is used to facilitate the reaction, despite the fact that the presence of these monovalent salts tends to inhibit the effectiveness of Mg^{2+} when it is also present.

Metal ions are not required for catalytic function of the hairpin ribozyme

In the case of the hairpin ribozyme, recent results have shown that cobalt (III) hexaamine can replace Mg^{2+} for all folding and catalytic functions [27–29]. As the ammonia ligands are nonexchangeable, all mechanisms for metal-ion catalysis involving inner-sphere coordination (as described below) have already been ruled out. In the case of the hairpin ribozyme, the results reported in this paper indicate that the function(s) of Mg^{2+} and $Co(NH_3)_6^{3+}$ can also be replaced by molar concentrations of sodium, lithium or ammonium ions. Our observation of high cleavage rates in monovalent salts is consistent with the ability of cobalt hexaamine to support the reaction, but these high rates were unanticipated because it has been assumed [27–29] that cobalt hexaamine mimics a fully hydrated magnesium ion essential for hairpin ribozyme folding and function. Perhaps more surprising is our finding that the rates of cleavage in monovalent cations can surpass those observed in magnesium-containing buffers.

Parsimony, as well as the production of identical reaction products both in the absence and presence of divalent cations, clearly suggests that the same catalytic mechanism is employed by the hairpin ribozyme regardless of whether the cationic species is a divalent metal ion, a monovalent metal ion, $Co(NH_3)_6^{3+}$ or ammonium ions. The most plausible conclusion from these studies is that all essential elements of catalytic function are provided by the folded structure of the hairpin ribozyme–substrate

complex, with cations functioning to stabilise the active structure by electrostatic screening of the charged phosphodiester backbone. In fact, we were able to prove a significant level of tertiary structure folding of the hairpin ribozyme in the presence of high concentrations of monovalent cations using FRET.

The hammerhead ribozyme does not require divalent metal ions

A large number of experiments have analyzed the role of divalent metal ions in hammerhead ribozyme catalysis in low ionic strength conditions (reviewed in [11]), and have delineated three potential roles for the metal to play in the cleavage reaction (reviewed in [24,40]). The first of these interactions, involving base abstraction of the proton from the active site 2′-OH, can be catalyzed either by abstraction of the 2′-proton by a divalent metal-ion-bound hydroxide ion or through direct coordination of a divalent metal ion to the 2′-OH that lowers the pK_a of this functional group. The second potential interaction is between nonbridging oxygens of the scissile phosphate and a divalent metal ion. The third potential interaction is between the leaving-group 5′-oxygen of the scissile phosphate and a divalent metal ion. Again, either an inner-sphere mechanism, in which the metal ion, functioning as a Lewis acid, directly coordinates the leaving-group oxygen, or an outer-sphere mechanism, in which a divalent metal-bound water molecule donates a proton to the leaving-group oxygen as negative charge accumulates, is, in principle, possible.

There are, in principle therefore, at least three chemically distinct catalytic roles for a divalent metal ion to play in hammerhead-ribozyme catalysis, although there is no uniform consensus on the mechanisms of any of these potential interactions. Our results permit us to suggest that under appropriate conditions of high ionic strength, allowing the hammerhead ribozyme to fold correctly, there is no strict requirement for any metal ions for the cleavage reaction. Instead, all that appears to be fundamentally critical is the presence of a positive charge at high density. Whether this positive charge at high density is provided through tight binding of a divalent metal ion at low ionic strength, or from an atmosphere of generic positive charges at high ionic strength, or, *in vivo*, by an appropriately positioned functional group from a protein or other parts of the RNA genome, is apparently unimportant from the point of view of the chemical mechanism of hammerhead RNA cleavage.

Specific divalent metal-ion-binding sites in the hammerhead ribozyme have, for the most part, eluded detection by means other than those that chemically change the affinity of the RNA for metal ions through thiophosphate substitution, where changes in the charge distribution might alter metal-binding sites or chemical reactivity. The main exceptions are the divalent metal-ion-binding site found in the original crystal structure at phosphate position A-9 [9] and others identified in subsequent crystal structures [10,32,41]. Moreover, the $K_{Mg^{2+}}$ for the hammerhead-ribozyme cleavage reaction has been estimated to be between 5 and 100 mM, indicating that specific binding of the putative catalytic divalent metal ion would be relatively weak compared to analogous binding sites within protein metalloenzymes. Our findings could explain this low affinity in terms of a lack of a requirement for an essential specific divalent metal-ion-binding site.

Our results do not, by necessity, rule out divalent metal ion involvement in hammerhead-ribozyme catalysis under low ionic strength conditions. Rather, they simply suggest that the requirement for metal ions is not of a fundamental nature. Instead, what is fundamental is the requirement for a correctly folded ribozyme, as well as the presence of a high density of positive charge. Whether this positive charge plays an active role in the chemistry of catalysis, or a more passive role in merely aiding the correct folding of the hammerhead ribozyme, is unclear at present.

VS ribozyme activity is enhanced by monovalent cations

Like the hammerhead and hairpin ribozymes, the VS ribozyme shows significant catalytic activity in the absence of divalent metal ions, as long as high concentrations of monovalent salts are present. In fact, the VS ribozyme appears to be more active in high concentrations of ammonium and especially lithium ions than it is in 20 mM Mg^{2+}, the standard conditions. (Table 1). In addition, we have observed an acceleration of VS cleavage rates with increasing RNA concentrations, suggesting that at low RNA concentration and low ionic strength formation of the ribozyme–substrate complex might be rate-limiting.

The HDV ribozyme serves as an important negative control

The activity of the HDV ribozyme differs dramatically from the other three ribozymes we have examined in that it is strongly dependent on the presence of Mg^{2+} and cannot be rescued by high concentrations of monovalent cations. The significance of this latter observation is twofold. First, it suggests that divalent metal ions might play an indispensable role in folding and/or active site chemistry. Second, it provides an important control that rules out the possibility that an undefined and nonchelatable contaminant is present that functions to replace Mg^{2+}, or that the added monovalent cations are somehow replacing Mg^{2+} in one of the mechanisms for inner-sphere catalysis described in [29,40,42].

In the case of the HDV ribozyme, it could be argued that high concentrations of lithium ions could inhibit the reaction by interfering with folding. This appears unlikely for three reasons, however. First, these conditions do not interfere significantly with the activity of the other three ribozymes. Second, the HDV ribozyme is active in 4 M Li^+ plus 10 mM Mg^{2+}. Third, the HDV ribozyme remains

very active in highly denaturing conditions such as 8 M urea and 10 M formamide [43].

The apparent function of various cations in hammerhead, hairpin, and VS ribozyme catalysis

We cannot rule out the possibility that Mg^{2+} ions participate in active-site chemistry of the hammerhead, hairpin, and VS ribozymes *in vivo*. Our results do demonstrate, however, that they are not strictly required. Previously published results have hinted at this, because very slow reactions have been seen when hammerhead and hairpin ribozymes have been incubated in the presence of organic polyamines plus chelating agents. It is important to recognise that the metal-independent activities reported here are generally similar to or greater than those observed in Mg^{2+}, and are a minimum of three orders of magnitude greater than those reported in the previous studies. Indeed, if the divalent cations do participate in the cleavage chemistry under standard reaction conditions, they might serve as folding agents and might not necessarily bind specifically to the active site of the ribozyme.

For the hairpin ribozyme, similar cleavage rates are observed at optimal concentrations of $Co(NH_3)_6^{3+}$, Mg^{2+}, monovalent metal ions and ammonium ions. The concentrations required to reach that rate, as well as detectable tertiary folding, however, are very different: in the order of 0.5 mM for $Co(NH_3)_6^{3+}$, 10 mM for Mg^{2+}, and 1 M for monovalent salts. Results for the hammerhead and VS ribozymes follow the same trend, except that activities in $Co(NH_3)_6^{3+}$ have not been reported. It is significant that these trends and magnitudes precisely reflect what is seen in phase diagrams of tRNA folding, where molar concentrations of sodium ions and millimolar concentrations of magnesium ions lead to similar or identical folds [44]. We believe that it is therefore likely that divalent cations are strongly preferred over monovalent cations because of their charge density rather than because of other chemical characteristics.

A hydroxide ion bound to a divalent metal ion will have a net positive charge, enabling it to avoid the electrostatic repulsion that a free hydroxide ion will encounter upon approaching the active site of the negatively charged RNA. The superior ability of divalent metal ions to assist in folding RNA, together with the overall positive charge of a divalent metal hydroxide complex ion, might account for the preference for divalent cations in ribozyme catalysis at lower ionic strengths.

RNA folding and catalysis under physiologically relevant conditions

We have shown that divalent cations are not strictly required for catalytic cleavage by three small ribozymes if the ionic strength of their surroundings is sufficiently high to promote proper folding of the RNA. This is not to suggest that such high ionic strengths are physiologically relevant. Rather, it suggests that the natural folding agents *in vivo*, be they divalent metal or organic cations, RNA-binding proteins, or other parts of the genomic RNA, can be mimicked by high ionic strength conditions *in vitro*. The fact that divalent metal ions are not required for catalysis *per se*, along with the fact that a solution of high ionic strength provides an *in vitro* substitution for the protein components of RNase P and some group I and group II introns, where cleavage is not observed in the absence of divalent cations, permits us to suggest that specific RNA-binding proteins, or other parts of the genomic or antigenomic RNA, might assist in the correct folding of the hammerhead, hairpin and VS self-cleaving motifs in their native environments, and therefore enhance the inherent catalytic activity of these RNAs in much the same way as does a tenfold physiological excess of Mg^{2+} under standard *in vitro* reaction conditions. The recent finding that the hammerhead ribozyme derived from the newt is part of an RNA–protein complex is especially intriguing in this context [45]. The relatively poor catalytic activity of ribozymes employed as RNA inactivating reagents *in vivo* might therefore be enhanced significantly by improving folding under intracellular conditions, perhaps by providing an RNA-binding ligand that functions to stabilise the active fold.

The cumulative evidence thus provides convincing support for the absence of essential functional interactions between magnesium ions and the attacking nucleophile, the scissile phosphate, and the leaving group of the hammerhead, hairpin and VS ribozymes. Artificial nucleic-acid enzymes displaying similar ionic requirements to those described here have been isolated from random sequence pools [30,31] and could employ analogous catalytic strategies.

Significance

We have found that three naturally occurring catalytic RNAs, the hammerhead, hairpin and VS ribozymes, do not require divalent metal ions for efficient catalysis. The monovalent cations Li^+, Na^+ and NH_4^+ at 1–4 M support efficient catalysis. Thus, the cation's role appears to be charge neutralisation allowing RNA folding, and possibly enhancing catalysis, rather than playing an essential chemical role in catalysis. Although these *in vitro* conditions might substitute effectively for the presence of divalent cations *in vivo*, it is also possible that they instead (or in addition) substitute for a required protein component of the ribozyme or simply mimic the low water activity that exists in a cellular environment.

The ability of NH_4^+, a nonmetal, to support efficient catalysis suggests that extant models for ribozyme catalysis that posit an obligatory inner sphere coordination of RNA ligands by divalent metal ions require re-evaluation. These models suggest that RNA structure provides a scaffolding

that functions to place catalytic divalent metal ions at the active site. Instead, we believe a different model merits consideration, in which the fundamental role of cations is to support RNA structures that mediate catalytic function.

Materials and methods

Synthesis, purification and radiolabelling of the hammerhead and hairpin oligoribonucleotides were carried out as previously described [46–48]. The HDV enzyme strand was synthesized using the procedure described by Price *et al.* [49]. The VS ribozyme and substrate were transcribed from *Ssp*I-linearised plasmid G11wt 26 and a synthetic DNA template, respectively. Hairpin and VS ribozyme cleavage reactions were carried out under single-turnover conditions (0.1–1 μM enzyme and 0.001–0.1 μM substrate) as described [47], except for changes in the composition of the reaction buffer, as indicated in Table 1. The preparation of solutions required careful monitoring of pH as it can be changed significantly by the presence of divalent cations, chelators (EDTA, DTPA, and CDTA), and high concentrations of monovalent salt. Hammerhead and HDV reactions were carried out in a volume of 16.5 μl. A 0.5 μl aliquot of annealed hammerhead complex was added to 16 μl of the appropriate reaction buffer to initiate the reaction. The final concentration of enzyme strand was 3 μM and substrate strands was 0.3 μM and 10 nM for the HH_{xtal} and $HH_{16.1}$, respectively, unless otherwise stated. Aliquots (1 μl) were removed at various times and quenched with 20 μl of 8 M urea pH 5, 25 mM EDTA. Five microlitres of the quenched reaction were then subjected to denaturing polyacrylamide gel electrophoresis (PAGE). The radiolabelled bands were visualised and quantified using a Molecular Dynamics Phosphorimager. Experiments were performed at least twice using a minimum of ten data points. The hairpin and VS reactions were carried out in an analogous manner, except for the following changes: The pH was 7.5 for the VS reaction, the concentration of $MgCl_2$ was 12 mM for the hairpin and 20 mM for VS, the reactions were quenched by the addition of formamide loading buffer. Rate constants were obtained by nonlinear curve-fitting as described in [47].

Hairpin ribozyme-EDTA titration experiments were performed using 5′–^{32}P-labeled substrate incubated for 60 min. at 25°C in a buffer containing 12 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) [47] and the indicated concentrations of EDTA. Hammerhead and HDV reactions were the same, except that $MgCl_2$ concentration was 10 mM, and the VS ribozyme EDTA titration was performed using 100 mM $MgCl_2$ as an initial concentration. Ribozyme and substrate concentrations were 10 nM and 1 nM, respectively, except for VS ribozyme which was 100 nM ribozyme, 0.5 nM substrate at pH 7.5.

Table 2

The concentration of divalent metal-ion contaminants found the solutions used in this work.

Solution	Ca (ppm)	Mg (ppm)	Mn (ppm)	Co (ppm)	Cd (ppm)	Pb (ppm)	Zn (ppm)
2 M Li_2SO_4	2.4	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
4 M NaCl	3.2	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
4 M NH_4OAc	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
50 mM Tris–HCl	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	>0.01
25 mM sodium EDTA	0.08	<0.01	<0.01	0.01	<0.01	<0.01	<0.01
50 mM sodium CDTA	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
25 mM sodium DTPA	<0.005	<0.005	<0.005	<0.005	<0.005	0.024	<0.005

In each case the highest contaminant level detected for each solution is shown.

All reagents were purchased from Fluka of puriss or microselect grade for the hammerhead and HDV experiments. Reagents for the hairpin and VS work were purchased from Sigma at the highest grade obtainable for each reagent. We had the reagents independently analysed at the Agricultural and Environmental Testing Laboratory, University of Vermont, Burlington, VT, USA by ICPOES (Perkin-Elmer 3000 DV). Analysis with ICPOES can not be performed with high ionic strength solutions such as our reaction buffers. Therefore, the salt solutions were analyzed at 50 mM (Li^+) and 25 mM (Na^+ and NH_4^+) cation concentration. The concentration of the contaminants in the solutions as used was then calculated by correcting for the change in concentration and are shown in Table 2.

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