

The RNA-Cleaving Bipartite DNAzyme Is a Distinctive Metalloenzyme

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Much interest has focused on the mechanisms of the five naturally occurring self-cleaving ribozymes, which, in spite of catalyzing the same reaction, adopt divergent strategies. These ribozymes, with the exception of the recently described *glmS* ribozyme, do not absolutely require divalent metal ions for their catalytic chemistries *in vitro*. A mechanistic investigation of an *in vitro*-selected, RNA-cleaving DNA enzyme, the bipartite, which catalyzes the same chemistry as the five natural self-cleaving ribozymes, found a mechanism of significant complexity. The DNAzyme showed a bell-shaped pH profile. A dissection of metal usage indicated the involvement of two catalytically relevant magnesium

ions for optimal activity. The DNAzyme was able to utilize manganese(II) as well as magnesium; however, with manganese it appeared to function complexed to either one or two of those cations. Titration with hexaamminecobalt(III) chloride inhibited the activity of the bipartite; this suggests that it is a metalloenzyme that utilizes metal hydroxide as a general base for activation of its nucleophile. Overall, the bipartite DNAzyme appeared to be kinetically distinct not only from the self-cleaving ribozymes but also from other *in vitro*-selected, RNA-cleaving deoxyribozymes, such as the 8–17, 10–23, and 614.

Introduction

Four of the five naturally occurring small, self-cleaving ribozymes, the Hammerhead, the Hairpin, the Hepatitis Delta Virus (HDV), and the Varkud Satellite (VS), share many properties, including a common catalyzed chemistry (reviewed by Doudna and Cech in ref. [1]). However, they show significant differences in the details of their mechanisms, for instance in their utilization (or not) of metal ions as cofactors for their catalysis. Very recently, a metabolite-responsive ribozyme, the *glmS*, has been discovered in different gram-positive bacteria.^[2] While it has not been shown to be an obligate metalloenzyme, it appears to require the binding of four magnesium ions for catalytic activity. In addition to the naturally occurring ribozymes, a series of RNA-cleaving DNA enzymes that catalyze the same overall chemistry with comparable efficiency (reviewed in ref. [3]), including the 8–17, 10–23^[4] and bipartite DNAzymes,^[5] have been reported from *in vitro*-selection experiments. While numerous mechanistic studies (including investigation of the roles of metal ions) have been carried out on the four classic self-cleaving ribozymes (reviewed in ref. [1]), investigation of the mechanisms of the DNA enzymes is at a relatively preliminary stage.^[6–10] As with the naturally occurring ribozymes, *in vitro*-selected DNA enzymes have been found to either require^[4,5,11–13] or not require^[14,15] divalent metal-ion cofactors for their optimal function.

Divalent cations are able to aid ribozyme/DNAzyme function in numerous ways. One key role is structural: these metal ions assist the negatively charged nucleic-acid enzyme to fold (reviewed in ref. [16]). Divalent cations have also been postulated to participate directly in the cleavage chemistry in several ways, including by providing metal-bound hydroxyl ions as general base for deprotonation of the 2'-OH nucleophile; or, as a Lewis acid, coordinating directly with the oxygen atom of

the nucleophile or leaving group (reviewed in ref. [17]). The discovery that four of the five naturally occurring self-cleaving ribozymes are able to function *in vitro* in the absence of divalent cations and in the presence of high concentrations of monovalent cations^[18–20] has brought up interesting questions about the precise role(s) of divalent cations in the chemistry of these ribozymes.

The discovery that DNA molecules are able to catalyze reactions has led to the *in vitro* selection of DNA enzymes (deoxyribozymes or DNAzymes) as catalysts analogous to ribozymes (reviewed in ref. [3]). Deoxyribozymes have been shown to be able to catalyze a variety of different reactions, one of which is RNA phosphodiester cleavage.^[4,5,11–15]

We have recently reported the *in vitro* selection from a random-sequence DNA library of a general RNA-cleaving enzyme, the "bipartite" DNAzyme,^[5] with catalytic rates comparable to those of the naturally occurring ribozymes.^[17] The bipartite DNAzyme showed a preference for cleavage at unpaired A↓A ribonucleotides, followed, optimally, by three putatively unpaired substrate ribonucleotides. Figure 1 shows a typical sequence, "E1", of this DNAzyme (lower strand), bound to an RNA substrate, "S9" (upper strand). Our initial studies on this DNAzyme^[5] showed that it requires divalent cations for catalytic activity. The enzyme's initial intramolecular form ("bipartite I"), incorporating an appended DNA substrate contain-

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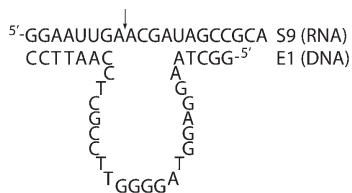


Figure 1. Substrate S9 with enzyme E1. The arrow indicates the cleavage site.

ing a single internal target ribonucleotide, utilizes magnesium, calcium, manganese, zinc, or cobalt; whereas the DNAzyme's intermolecular form ("bipartite II"), which uses an all-RNA substrate, could not use zinc or cobalt. This difference in metal usage was thought to be connected to differences in active-site metal-coordination sites within the all-RNA substrate and the single ribonucleotide within a DNA substrate.^[5] The intramolecular DNAzyme, ("bipartite I") showed optimal catalysis in the presence of ~30 mM magnesium chloride, with a measured Hill coefficient of 2.01 ± 0.39 for magnesium usage.^[5] The log of observed rate constants of bipartite II (at 30 mM magnesium), plotted against pH, yielded a bell-shaped dependence.^[5]

The above preliminary studies provided tantalizing evidence for a unique, metal-utilizing mechanism for the bipartite deoxyribozyme. Here, we report a comprehensive investigation into the bipartite DNAzyme's mechanism by making detailed studies on the usage of magnesium and manganese cations by the DNAzyme; pH dependence of catalysis in the presence of these cations; deuterium kinetic solvent isotope effects; and examining the effect of titration of hexaamminecobalt(III) cations on the manganese-supported catalysis of the bipartite DNAzyme.

Results and Discussion

Magnesium dependence of the catalysis of DNAzyme construct E1 with substrate S9

All experiments described in this paper were carried out on the *intermolecular* combination of the DNAzyme construct E1 acting upon the RNA substrate S9 (both shown in Figure 1). As summarized in the Introduction, our earlier study on the magnesium requirement of the *intramolecular* form of the DNAzyme had given a Hill coefficient for magnesium usage of 2.01 ± 0.39 . However, notable differences in metal usage^[5] between the intra- and the intermolecular forms of the DNAzyme (*vide infra*) necessitated an independent measurement of the magnesium requirements of the intermolecular E1–S9 system.

Figure 2 plots the dependence of the observed rate constant (k_{obs}) on $\log[\text{Mg}^{2+}]$ for the bipartite-catalyzed intermolecular reaction, measured at pH 7.46. Observed rate constants (k_{obs}) from reactions carried out at different MgCl_2 concentrations were fit to the equation below:

$$k_{\text{obs}}(\log[\text{Mg}]) = k_{\text{obs min}} + \frac{k_{\text{obs max}} - k_{\text{obs min}}}{1 + 10^{n(\log K_d - \log[\text{Mg}])}} \quad (1)$$

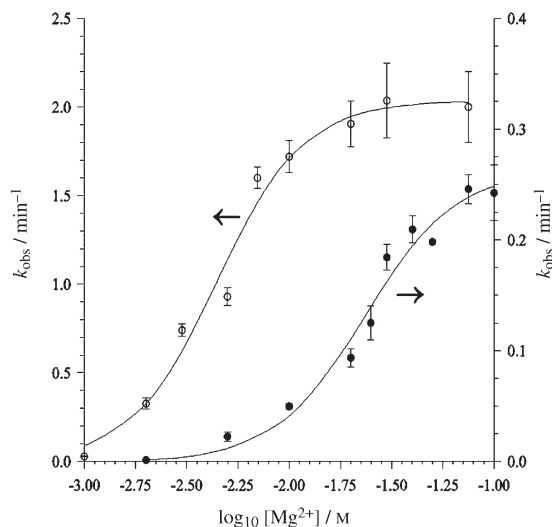


Figure 2. Plot of k_{obs} versus $\log[\text{Mg}^{2+}]$ for the intramolecular (\circ data taken from ref. [5]) and intermolecular (\bullet) bipartite DNAzymes. The two solid lines are the best nonlinear least-squares fits to the Hill equation (Equation (2), see text for full details).

Here, $k_{\text{obs min}}$ and $k_{\text{obs max}}$ are the k_{obs} values at the lower and upper rate plateaux, respectively; $K_{\text{d(app)}}$ is the apparent dissociation constant for Mg^{2+} ions; and n is the Hill slope, a measure of the number of Mg^{2+} involved in the rate-limiting step. The nonlinear least squares fit to this equation produced a value for $k_{\text{obs min}}$ that was indistinguishable, within experimental error, from zero. Therefore, $k_{\text{obs min}}$ was set equal to zero, and the calculated Hill coefficient for the E1–S9 system was found to be 2.0 ± 0.4 ; this suggests the involvement of *two* magnesium ions in the transition state of the RNA-cleavage reaction. These data are fully consistent with the earlier data on the intramolecular form of the DNAzyme, which had a Hill coefficient of 2.01 ± 0.39 ^[5] (the intramolecular data are also plotted in Figure 2—these data were obtained at pH 7.40^[5]). Clearly, the intramolecular case can be activated by a lower magnesium concentration because entropic factors (substrate and enzyme are physically linked) permit the system to fold easily; whereas the intermolecular system may require a higher magnesium concentration, in part to effect the association of the distinct enzyme and substrate molecules.

pH dependence of E1-mediated S9 cleavage in the presence of MgCl_2

Having implicated two magnesium ions in bipartite catalysis, we wished to estimate the number of protons being exchanged in the rate-determining step. Figure 3A shows two sets of $\log k_{\text{obs}}$ versus pH data, measured between pH 4.5 and 8.0 in the presence of 30 and 10 mM magnesium chloride, respectively. To determine the stoichiometry of catalytically relevant protonation/deprotonation events within this pH regime, the data were framed within the reaction scheme.

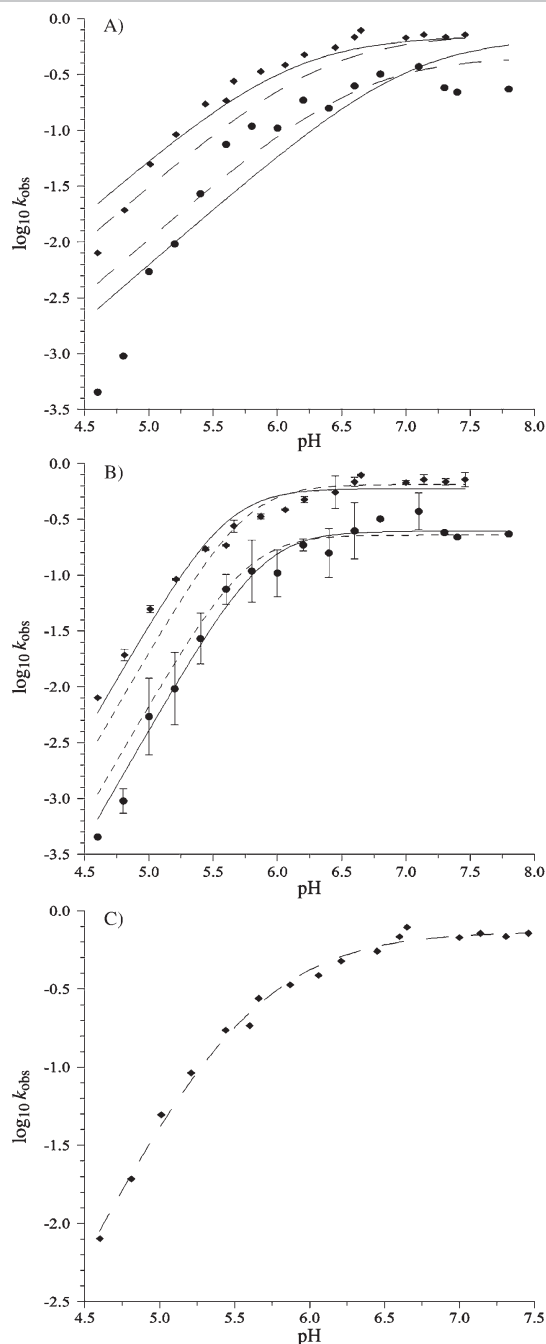
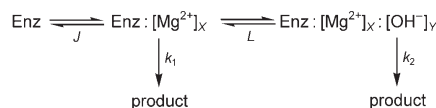


Figure 3. $\log k_{\text{obs}}$ versus pH profiles in the presence of 30 (\blacklozenge) and 10 mM (\bullet) MgCl_2 , respectively, a) fitted to the formalism from Equation (2), presuming a single deprotonation event and either a single (---) or two (—) catalytically relevant magnesium ion(s); b) fitted to formalism of Equation (2), which postulates two catalytically relevant magnesium ions and a single catalytically relevant deprotonation event (shown as broken lines); and, the formalism of Equation (2), postulating two catalytically relevant magnesium ions as well as two deprotonation events (—); c) $\log k_{\text{obs}}$ of cleavage of S9 by excess E1, in the presence of 30 mM MgCl_2 , plotted against pH by using the formalism of Equation (3). Two closely spaced $\text{p}K_{\text{a}}$ values, at 5.20 ± 0.11 and 5.82 ± 0.08 were obtained.



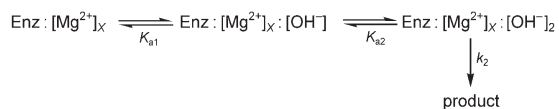
For the lower pH data, measured in the presence of 30 and 10 mM magnesium, respectively, each fit that included a k_1 kinetic term resulted in that rate constant being indistinguishable from zero. Therefore, in all fits to the kinetic data the value of k_1 was set to zero, and the data were fit with a formalism derived, by using standard methods,^[21] from the kinetic scheme:

$$\log k_{\text{obs}} = \log \frac{k_2 [\text{Mg}^{2+}]^X}{(J \times L \times [\text{H}^+]^Y) + L + [\text{Mg}^{2+}]^X} \quad (2)$$

Here, the J and L terms incorporate the various equilibrium constants for the ionization of H^+ and the binding of Mg^{2+} ions (the values and units of J and L depend on the integer values of X , the number of relevant magnesium ions, and Y , the number of relevant deprotonations). This equation and others, below, are of this form.

Initially, very poor fits were obtained with both sets of data when a single deprotonation event ($Y=1$) and either one ($X=1$: plots shown as broken lines in Figure 3A) or two ($X=2$: plots shown as solid lines in Figure 3A) catalytically relevant magnesium ions were presumed. Progressively better fits were obtained for two catalytically relevant deprotonation events with one magnesium ion [Equation (2): $Y=2$ and $X=1$: plots shown as broken lines in Figure 3B]; and, two deprotonation events linked to two catalytically relevant magnesiums [Equation (2): $Y=2$ and $X=2$: plots shown as solid lines in Figure 3B]. The latter ($Y=2$ and $X=2$), in particular, gave excellent fits to both the 10 and 30 mM magnesium data.

To estimate the $\text{p}K_{\text{a}}$ values of the two deprotonation events proposed for catalytic activity, the $\log k_{\text{obs}}$ versus pH plot for bipartite catalysis at 30 mM magnesium was taken to adhere to the following simplified kinetic scheme.



Equation (3) was derived from this scheme and used to fit the k_{obs} versus pH data shown in Figure 3C.

$$\log k_{\text{obs}} = \log \frac{k_2 \times K_{a1} \times K_{a2}}{(K_{a1} \times K_{a2}) + (K_{a1} \times [\text{H}^+]) + [\text{H}^+]^2} \quad (3)$$

In this equation, K_{a1} and K_{a2} represent the K_{a} values for the two deprotonation events. Two closely spaced $\text{p}K_{\text{a}}$ values, 5.20 ± 0.11 and 5.82 ± 0.08 , were derived from the plot shown in Figure 3C. Fits that included a reactive monodeprotonated spe-

cies suggested that the catalytic activity of such a species was indistinguishable from zero.

What physical picture of the DNAzyme's active site is consistent with these data? Our finding that two magnesium ions as well as two deprotonation events are optimal for catalysis suggests a role for magnesium hydroxide, likely acting as a general base in this DNAzyme's chemistry (we cannot, however, rule out at this stage the involvement of DNAzyme nucleobase(s) as general base). The involvement of more than one magnesium ion in catalysis by other ribozymes has been reported—up to three magnesiums, for instance, are thought to participate in Group I ribozymes.^[1] In the case of the bipartite, however, the requirement for two magnesium ions as well as two deprotonations suggests that a hydroxide-bridged bimetallic system, involving two magnesium ions, might be involved. In such a scenario, the two implicated magnesium ions are located in close proximity within the active site. The first of the two required deprotonations occurs from a water molecule bound to a magnesium ion; however, this hydroxide serves to bridge the two magnesium ions and is unavailable as general base for the catalysis. A *second* deprotonation of a magnesium-bound water (facilitated by the first) can then generate a metal hydroxide capable of acting as a general base in the DNAzyme's catalysis. Bimetallic centres such as that proposed above have been reported in a crystal structure of an *E. coli* 5S RNA domain.^[22] Moreover, cases of bimetallic catalysts and the necessity of removing *two* protons from them to generate an active species have been reported in other systems.^[23]

The falling part^[5] of the bell-shaped pH profile (at pH > 8.0) can be comfortably fit to a single ionization event [$pK_{a3} = 8.93 \pm 0.07$, obtained by using Equation (4)].

$$k_{\text{obs}} = \log \frac{k_2 \times [\text{H}^+]}{[\text{H}^+] + K_{a3}} \quad (4)$$

The fall-off in catalytic activity with this third deprotonation suggests that the triply deprotonated species is either inactive or only weakly active. This third deprotonation may involve an active-site nucleobase required for maintaining the catalytically relevant folded structure of the DNAzyme. It is also possible to conceptualize such a loss of activity in terms of an accumulation of excess negative charge on the catalytically relevant magnesium ions.

The manganese reaction: pH dependence of E1-mediated S9 cleavage

Given that the E1 DNAzyme's activity was supported comparably by manganese and by magnesium, we investigated the stoichiometry of metal usage and deprotonations in the manganese-supported catalysis.

Figure 4A shows the dependences of $\log k_{\text{obs}}$ on pH for catalysis by the bipartite DNAzyme in the presence of manganese(II) chloride concentrations of 1, 4, and 30 mM, respectively. The formalism used for magnesium was also used to fit these data, and the best fits were obtained (Figure 4A) with the postulated participation of one manganese cation and two de-

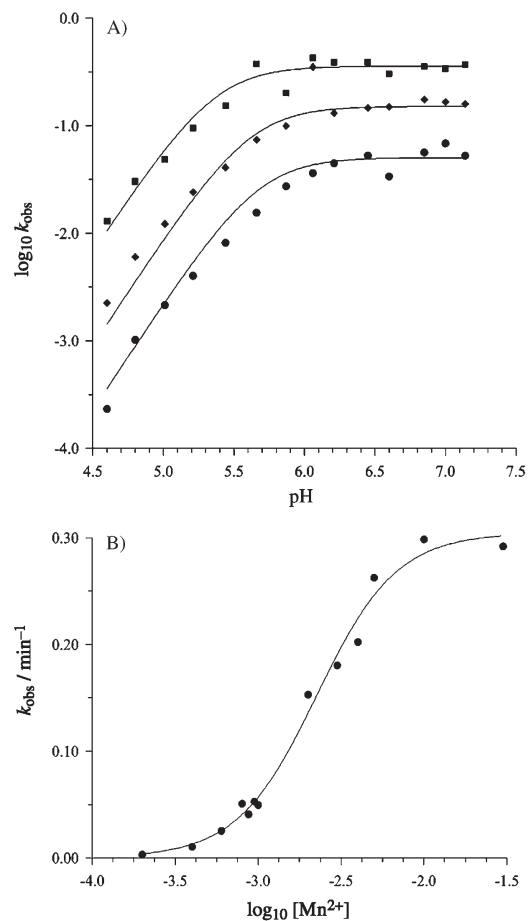


Figure 4. a) $\log k_{\text{obs}}$ of cleavage of S9 by excess E1 in the presence of manganese, plotted against pH. The manganese concentrations used were 1 (●), 4 (◆), and 30 mM (▲), respectively; b) $\log k_{\text{obs}}$ of cleavage of S9 by excess E1 plotted against $\log [\text{Mn}^{2+}]$.

protonation events. Even so, the fit of the data points in the rising parts of the curves appears less optimal, especially for the low manganese concentrations, than in the case of magnesium (Figure 3B); when the manganese data were fit by using a formalism which incorporated a variable number of deprotonations (first reaction scheme, X not an integer), the best fits gave values for X of 1.56 ± 0.10 ; 1.71 ± 0.19 ; and 1.65 ± 0.18 , respectively, for the three manganese concentrations. Figure 4B shows a Hill plot for manganese usage (based on rate constants measured with different manganese concentrations from the "plateau" region (pH 7.46) of the pH plots shown in Figure 4A) and this gave a calculated Hill coefficient of 1.80 ± 0.15 , an observation that is consistent with the involvement of two manganese ions in catalysis. However, the noted best fits for rate versus pH data implicated a single manganese ion. Cumulatively, these data emphasized the complexity of the mechanism, that DNAzyme complexed to *either* one or two manganese cations might have detectable catalytic activity, and

that the same might also hold for true singly deprotonated and doubly deprotonated forms of the manganese-utilizing DNAzyme.

Monovalent cations do not support the catalytic activity of the bipartite

In the absence of added divalent cations the bipartite was found to be totally inactive, even in the presence of very high concentrations (4 M) of KCl or NaCl (data not shown). A very slight activity, $\sim 10^4$ -fold lower than measured in millimolar concentrations of magnesium, was detectable in 4 M LiCl. In its nonresponsiveness to high concentrations of monovalent cations alone, the bipartite differs from the naturally occurring self-cleaving ribozymes.^[18] This lack of catalysis by monovalent cations also supports the likelihood that divalent metal-bound hydroxide might be a key player in the mechanism of the bipartite DNAzyme.

Competition with hexaamminecobalt cations

The kinetically inert hexaamminecobalt(III) cation has been used to investigate the role of metal-bound water and hydroxide molecules in the catalytic mechanisms of ribozymes.^[24] Since the hexaamminecobalt(III) cation is substitution-inert with respect to its ammonia ligands (and given that the metal-bound ammonia ligands are incapable of the acid–base chemistry characteristic of metal-bound water or hydroxide ligands), competition experiments involving titration with hexaamminecobalt(III) salts into reaction solutions containing a fixed Mg^{2+} (or Mn^{2+}) concentration (and vice versa) are able to reveal the catalytic necessity—or not—of Mg^{2+} or Mn^{2+} .

Our competition experiments, carried out on the manganese-utilizing DNAzyme, revealed that the hexaamminecobalt(III) cation inhibited the RNA cleavage reaction. That suggested that there was at least one outer-sphere metal-binding site on the DNAzyme. Figure 5 shows a Hill plot for the inhibition of the Mn-DNAzyme by hexaamminecobalt(III), in which each reaction had a fixed Mn^{2+} concentration of 4 mM and was titrated with hexaamminecobalt³⁺ from the $1\text{ }\mu\text{M}$ to 10 mM range. The slope of the Hill plot was -1.44 ± 0.30 . Given that a hexaamminecobalt(III) cation is able to substitute one-to-one for a structurally isomorphous Mg^{2+} or Mn^{2+} ion,^[24] this inhibition slope is consistent with both mono- and dicoordinated manganese ion complexes being catalytically active. The reverse titration was also carried out, in which in the presence of a fixed hexaamminecobalt concentration of 0.5 mM k_{obs} values were measured with progressive titrations of manganese (data not shown). A Hill plot for manganese usage in those experiments gave a Hill coefficient of 1.54 ± 0.15 . Overall, these various competition experiments and the monovalent cation data strongly suggest the likelihood that at least one of the two catalytically relevant deprotonation events noted in both the magnesium- and manganese-DNAzymes refer to a metal-bound water molecule, and that the second deprotonation might also be from metal-bound water or from a nucleobase.

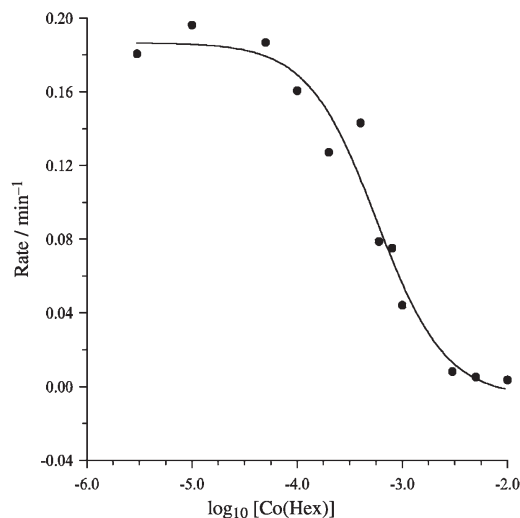


Figure 5. The rate of 59 cleavage by excess DNAzyme E1 plotted against the log of concentration of hexaamminecobalt(III) cations (Co(Hex)). All reactions were carried out in the presence of a fixed manganese(II) cation concentration of 4.0 mM .

Kinetic solvent isotope effect experiments

To explore further the role of deprotonation in the catalytic step of the bipartite DNAzyme, kinetic solvent isotope effects (KSIE) were measured for bipartite-catalyzed RNA cleavage. Whereas, solvent isotope effects usually arise from proton transfers between electronegative atoms (such as O or N) accompanying the bond-making and -breaking steps in the transition state,^[25,26] an observed KSIE can also result from the difference in concentration of the chemically active species in the two solvents,^[9] H_2O and D_2O . To differentiate between the two possibilities it is necessary to analyze the solvent isotope effect in the “plateau” region of the pL profile (in which pL refers to pH or pD). Figure 6 plots measured $\log k_{\text{obs}}$ values for the bipartite DNAzyme in H_2O and D_2O solutions, respectively, in the presence of 30 mM MgCl_2 , within the pL (pH or pD) range of 5.0–8.0. It was found that k_{obs} was lowered in D_2O through the entire pL profile, with a KSIE in the pL-independent region of 2.11 ± 0.22 , consistent with one or more proton transfers in the rate-determining step.

It is notable once again that the data from the D_2O solution show that the reaction rate is dependent on a doubly ionized species (the data were fit to Equation (3)). In other words, the active species, by analogy with catalysis in H_2O , is presumably $\text{DNA}:[\text{Mg}^{2+}]_2:[\text{OD}^-]_2$.

The KSIE in the presence of 4 mM MnCl_2 , carried out at a single pL value of 7.47, was determined to be 2.06 ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$). This number was very similar to the KSIE observed in MgCl_2 ; this suggests, qualitatively, a similar conclusion for the two cases—that proton transfers were occurring in the rate-determining steps. Here too, however, we cannot formally rule out a role of conformational changes^[27] or, in such a complex

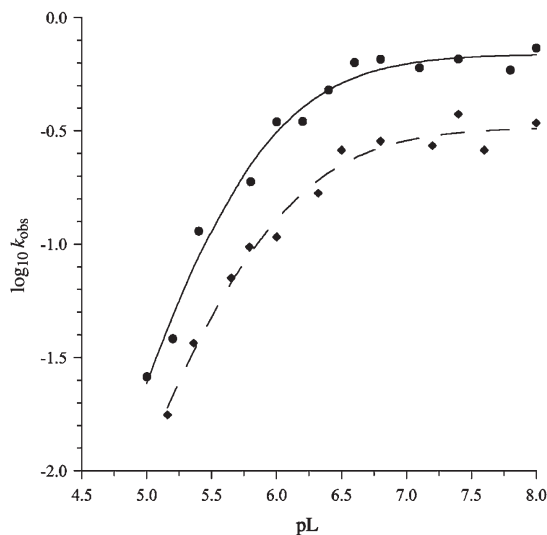


Figure 6. $\log k_{\text{obs}}$ of S9 cleavage by excess DNAzyme E1 plotted against pL (pH or pD) for S9 catalysis monitored in the presence of 30 mM MgCl_2 . Diamonds represent the D_2O containing buffer, while circles represent the H_2O experiments. The rate constant in the plateau region of the profile was 0.6914 and 0.3279 min^{-1} in H_2O and D_2O , respectively. The KSIE ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$) was therefore 2.11 ± 0.22 .

system, of a multiplicity of small isotope effects^[28] adding up to give the relatively large cumulative number.

Conclusion

In summary, we have found the bipartite DNAzyme to be an obligate metalloenzyme, requiring magnesium or manganese cations to support its catalysis. Competition for metal-binding sites within the DNAzyme by hexaamminecobalt cations led to a loss of catalytic activity. In both magnesium and manganese solutions, the DNAzyme showed approximately bell-shaped log rate constant versus pH curves. Hill analysis of metal usage indicated the requirement of two magnesium or manganese ions (although the DNAzyme utilizing just one manganese cation appeared to have some catalytic activity). Furthermore, two deprotonation events appeared to be required for the magnesium-utilizing DNAzyme (and to be optimal for the manganese-utilizing DNAzyme). Two major conclusions can thus be drawn: that bipartite is an obligate metalloenzyme that works best with two catalytically active divalent metal ions and requires two deprotonations; and that magnesium- and manganese-dependent catalyses, while similar overall, present some differences.

The proposed drop in $\text{p}K_{\text{a}}$ by 5–6 log units of magnesium-bound water molecules within the DNAzyme (from a typical unperturbed $\text{p}K_{\text{a}}$ for $\text{Mg}(\text{H}_2\text{O})_6^{2+}$ of ~ 11.8) is conceivable in a situation in which the two required magnesium ions are localized very close to each other, as we have proposed. To reduce charge repulsion between them, they could release protons, giving rise to the observed strong acidification. Nevertheless,

as discussed earlier, the possibility that one, or less likely both, of the observed deprotonation(s) originates in nucleobases or metal-bound nucleobases within the DNAzyme cannot be ruled out at this time.

Like the bipartite, the larger naturally occurring ribozymes—RNase P and the Group I and II self-splicing ribozymes appear to be obligate metalloenzymes, absolutely requiring divalent metal ions for activity (reviewed in refs. [29,30]). By contrast, four of the five small, self-cleaving ribozymes—the hammerhead, hairpin, HDV, and VS ribozymes—have been shown, at least in vitro, not to definitively require divalent metal ions for catalysis. Thus, the hammerhead and VS ribozymes have been found to be as catalytically active in 4M solutions of alkali metal chlorides as they are in low-to-physiological concentrations of divalent metal chlorides. High-resolution crystal structures,^[31,32] as well as functional studies on the hairpin ribozymes, have indicated that this ribozyme utilizes magnesium only in passive, noncatalytic ways. Although the crystal structures for the hairpin have failed to reveal metal ions coordinated at the active site, a recent crystal structure of the uncleaved form of the HDV ribozyme has shown a catalytically relevant magnesium ion at the active site.^[33] In the HDV ribozyme, RNA functionalities proximal to the substrate have also been implicated in general acid–base catalysis.^[34–38] Despite this, these four small ribozymes have been described as “nonobligate” metalloenzymes that might not *strictly* require divalent cations for catalysis, but whose activity is undoubtedly maximized by the presence of divalent cations (as would indeed be the case in vivo).

The properties of the two small RNA-cleaving DNA enzymes 10–23 and 8–17, both obtained, like the bipartite, by in vitro selection, have also been studied. Unlike the self-cleaving ribozymes, these DNA enzymes are obligate metalloenzymes that require magnesium, manganese, or calcium coordination. Otherwise, the properties of the 10–23 resemble those of the hammerhead ribozyme^[39] in key ways—for instance, the dependence of the log of the observed rate constants on pH is approximately linear, with a slope of ~ 1 ; this suggests a single deprotonation event relevant to catalysis in both enzymes.^[6] A definitive attribution of this deprotonation to metal-coordinated water, however, is problematic for a number of reasons, including the observation that magnesium and calcium produce comparable catalytic rates (although $\Delta\text{p}K_{\text{a}}$ of water molecules bound to the two metal ions is ~ 1.5). In recent studies of the 8–17 DNAzyme,^[17,40] it was found that a single magnesium ion was required for catalysis (however, two zinc ions were needed with a different 8–17 construct^[7]). Rate versus pH studies indicated, as with the 10–23, the relevance of a single deprotonation for catalysis.^[40]

It is thus apparent that the bipartite DNAzyme is kinetically (and, likely, mechanistically) distinct from the small, self-cleaving ribozymes as well as from the 8–17 and 10–23 DNAzymes. The differences noted for bipartite catalysis in magnesium versus manganese solutions might reflect small differences in properties of the water molecules bound to the respective metal cations and to the general complexity of the mechanism, as observed.

Experimental Section

Reagents and chemicals: RNA and DNA oligonucleotides were prepared and purified as described previously.^[5] The RNA substrate S9 was purchased from Dharmacon Research (Boulder, CO), and the DNA enzyme E1 was obtained from UCDNA, University of Calgary. Buffers and salts used were of the highest grade available and purchased from Sigma.

Kinetic analyses: RNA-cleavage reaction rates were measured under single-turnover conditions, under which relatively high concentrations of DNAzyme (2.25 μM) were used with trace concentrations (~ 30 nM) of 5' end-labeled RNA substrate. Substrate S9 and DNAzyme E1 were heated together in Na-HEPES (50 mM, pH 7.46) at 90 °C for 1 min, spun briefly in a microcentrifuge, and equilibrated at 23 °C for 10 min. A "null" aliquot was quenched at time zero with excess EDTA in 95% formamide. To start the reaction, of MgCl_2 of the appropriate concentration (2 μL) was added, such that the final reaction volume was 20 μL . Aliquots were collected at different time points and quenched with excess EDTA in 95% formamide. A control experiment was carried out in which substrate and DNAzyme were heated separately, equilibrated at 23 °C with MgCl_2 , and mixed together to initiate the reaction. The results obtained were indistinguishable from protocols in which magnesium was added last to mixtures of enzyme and substrate. Cleavage was followed to completion (the endpoint was typically when $\sim 80 \pm 10\%$ substrate had reacted), and the resulting time points fit to the first-order rate formalism: $y = x(1 - e^{-kt})$, where y is the fraction reacted at time t , x is the fraction reacted at the endpoint, and k the observed rate constant.

Rate versus pH profiles in the presence of magnesium and manganese: Individual rate constants at different pH values were obtained under single-turnover conditions using saturating DNAzyme concentration (2.25 μM). For the magnesium measurements, reaction rate constants were measured in the presence of 10 and 30 mM MgCl_2 . A range of buffers were used to span the pH range studied: NaOAc/AcOH (pH 4.6–5.6), NaOH/MES (pH 5.6–6.6), NaOH/MOPS (pH 6.6–7.4), NaOH/HEPES (pH 7.4–7.8), Tris.HCl (pH 8.0), NaOH/EPPS (pH 8.1–9.0), $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 9.0–10.0), NaOH/TAPS (pH 8.68–9.21), and NaOH/CAPS (pH 9.21–10.19). Several of the pH points were measured in different buffer solutions, to exclude buffer-specific effects. MES = 4-morpholine-ethanesulfonic acid; MOPS = 4-morpholine-propanesulfonic acid; EPPS = N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; CAPS = 3-(cyclohexylamino)-1-propanesulfonic acid.

Manganese experiments were carried out with the same DNAzyme concentration (2.25 μM), and rate constants were measured in the presence of 1, 4 and 30 mM MnCl_2 , respectively. The buffers used included: NaOAc/AcOH (pH 4.6–5.6), NaOH/MES (pH 5.6–6.6), NaOH/MOPS (pH 6.6–7.4), and NaOH/HEPES (pH 7.4–7.8).

Data were analyzed and fitted by using nonlinear least-squares routines from PRISM Version 4 (Graphpad Software).

Kinetic solvent isotope effect (KSIE) experiments: All solutions and buffers were prepared in D_2O (99.9%, CDN Isotopes). Buffers were prepared as described by Schowen and Schowen.^[25] The true pD value was obtained by adding 0.4 to the measured pH-meter reading, as described.^[25] The buffers used to span the pH range were: NaOD/MES (pH 5.1–6.7), NaOD/MOPS (pH 6.8–7.2), NaOD/HEPES (pH 7.4–7.6), and NaOD/EPPS (pH 8.0). Buffers were titrated with sodium deuterioxide (99 atom% deuterium, from Sigma).

For the KSIE experiments, trace amounts of labeled substrate S9 (~ 30 nM) were combined with enzyme E1 (2.25 μM) in water and

lyophilized together under vacuum. The dried pellet was redissolved in D_2O (99.9%) containing buffering salts (vide infra) at concentrations of 50 mM. A "null" aliquot was taken for time zero. Kinetics were performed under single-turnover conditions, as described earlier, except that the reaction was initiated by the addition of MgCl_2 (30 mM) in D_2O (100%; for the magnesium reactions) and MnCl_2 in D_2O (100%) to a final concentration of 4 mM MnCl_2 (for the manganese reaction). KSIE for the manganese reaction was measured only at a single pD (7.47).

Hexaamminecobalt inhibition in the presence of manganese as cofactor: The individual rate constants were obtained by varying the concentrations of hexaamminecobalt(III) chloride relative to a fixed concentration of MnCl_2 . Trace concentrations of ^{32}P -end labeled S9 were combined with E1 (2.25 μM) in NaOH/HEPES (50 mM, pH 7.46). The samples were heated to 90 °C for 1 min and equilibrated at 23 °C for 10 min. A "null" aliquot was taken for time zero. Hexaamminecobalt chloride (Sigma) was then added and the samples were incubated at 23 °C for 15 min. Adding MnCl_2 to a final concentration of 4 mM initiated each reaction.

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