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Metal ions play a passive role in the hairpin ribozyme catalysed reaction

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ABSTRACT

The hairpin ribozyme is an example of a small catalytic RNA which catalyses the endonucleolytic transesterification of RNA in a highly sequence-specific manner. The hairpin ribozyme, in common with all other small ribozymes such as the hammerhead, requires the presence of a divalent metal ion co-factor (typically magnesium) for the reaction to take place. To investigate the role of magnesium ions in the hairpin catalysed reaction we have synthesised two epimeric modified substrates in which a phosphorothioate replaces the scissile phosphodiester bond. Previously, Burke and co-workers have reported that no thio-effect is observed with the Rp-phosphorothioate isomer. We observe the absence of a thio-effect with both diastereomeric phosphorothioate hairpin substrates. Furthermore we report that inert cobalt (III) complexes are capable of supporting the hairpin ribozyme reaction, with a similar efficiency to Mg^{2+}, even in the presence of EDTA. Variation of the net charge on the inert cobalt complex does not change the observed rate of reaction. These results suggest that metal ions play a passive role in the hairpin ribozyme catalysed reaction and are probably required for structural purposes only. This places the hairpin ribozyme in a different mechanistic class to other small ribozymes such as the hammerhead.

INTRODUCTION

The hairpin ribozyme is the minimal self-cleavage domain of the negative strand of the satellite RNA of tobacco ringspot virus (1–3). The sequence requirements of the hairpin ribozyme have been elegantly determined by *in vitro* selection experiments and confirmed by mutagenesis (4–9). The secondary structure prediction of the ribozyme is illustrated in Figure 1 and consists of four helical regions interrupted by two internal loops. The essential nucleotides are situated in the looped regions (1 and 2) and there is evidence to suggest that catalysis requires the interaction of these two looped areas (10–13).

The hairpin ribozyme is a member of a family of small catalytic RNA molecules which also includes the hammerhead and hepatitis delta ribozymes (14,15). This group of small ribozymes all catalyse the endonucleolytic cleavage of RNA molecules via a similar mechanism which involves attack of a 2′-hydroxyl group on its neighbouring 3′-phosphodiester bond (Fig. 2). The products of the reaction terminate in a 2′,3′-cyclic phosphate and a 5′-hydroxyl group. The reaction proceeds with inversion of configuration at phosphorus suggesting a direct in-line attack with development of a pentacoordinate transition state or intermediate (16–19). Despite the ability to catalyse the same reaction, the primary and secondary structures of the respective ribozymes are quite distinct. There has been much interest in the chemotherapeutic applications of small ribozymes as minimal sequence requirements in the substrate allow the engineering of ribozymes to cleave almost any desired RNA molecule. Similarly, there is widespread interest in ribozyme mimetics as gene-specific cleaving reagents.

In view of the use of ribozymes as chemotherapeutic agents, and the desire to construct designer ribozyme mimetics, there has been great interest in understanding how naturally occurring ribozymes accelerate reaction rates. The cleavage reaction mediated by the hairpin and other small ribozymes requires the presence of a divalent metal ion co-factor (typically magnesium). There has been much speculation about the role of metal ions in ribozyme catalysis. It is frequently claimed that all ribozymes are metalloenzymes and that the burden of catalysis is borne by specifically positioned metal ions (20–22). By far the best studied small ribozyme is the hammerhead. Several modes of metal ion catalysis have been proposed for this enzyme. These include general base catalysis (Fig. 3A) (23), Lewis acid (electrophilic) catalysis (Fig. 3B) (16,18), activation of the leaving group (Fig. 3C) (24) or activation of the nucleophile (Fig. 3D) (25). We now report a study of the role of metal ions in the hairpin ribozyme catalysed reaction, which rules out the modes of catalysis above and suggests that metal ions are not intimately involved at the reaction centre of this catalytic RNA.

MATERIALS AND METHODS

Preparation of oligoribonucleotides

Oligoribonucleotides were synthesised on a 1 µmol scale using 2′-*O*-*t*-butyldimethylsilylnucleoside 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite monomers having a phenoxyacetyl group for protection of the amino function of A and G and benzoyl protection for C (Glen Research via Cambio). The syntheses were carried out using standard RNA synthesis procedures (49). The

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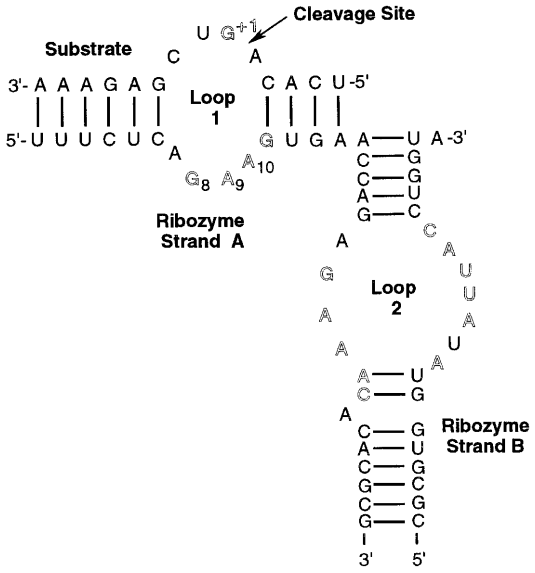

Figure 1. Secondary structure of the hairpin ribozyme showing the four regions of Watson–Crick base pairing and the internal loops 1 and 2. The cleavage site is indicated by an arrow and the outlined residues represent the essential nucleobases.

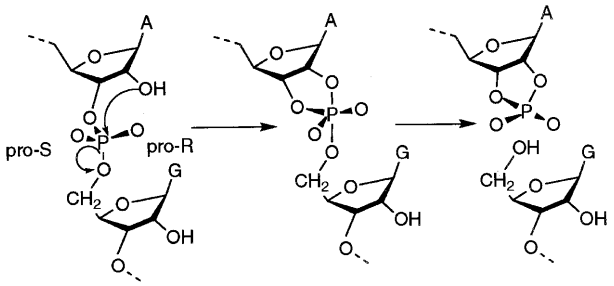

Figure 2. The mechanism of the hairpin (and other small ribozymes) consistent with the stereochemical course of the reaction. Note that for other ribozymes the identity of the two nucleobases will vary.

oligoribonucleotides containing a phosphorothioate internucleoside linkage were synthesised by modifying the standard procedure. The synthesis cycle was interrupted during the addition of the protected guanosine phosphoramidite. The oxidation step was replaced with a sulfurisation step by treating the intermediate phosphite triester with 0.05 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in acetonitrile (50,51). The sulfurising solution was allowed to flow to the column for 30 s and then the cycle was paused for 300 s. The synthesis was resumed after the column was washed with acetonitrile.

All oligonucleotides were deprotected by suspending the controlled pore glass in methanolic ammonia, at room temperature overnight. The resultant product was then treated with TBAF (1 ml of 1 M solution in THF) for 20–24 h at room temperature, in the dark, to remove the silyl protecting groups. The solution was desalted using a Sephadex NAP10 column.

Purification of oligoribonucleotides

The substrate sequences were purified by ion exchange HPLC on a semi-preparative Partisil 10-SAX column. The oligoribonucleotides were eluted under denaturing conditions using buffer A (1 mM potassium phosphate, pH 6.3, in 60% formamide) and buffer B (300 mM potassium phosphate, pH 6.3, in 60% formamide) as follows: flow rate = 2.5 ml/min; 20% B, 0 min; 20% B, 5 min; 20–90% B, 35 min; 90% B, 40 min; 90–20% B, 45 min; retention time = 30.5 min. The purified substrates were desalted by extensive dialysis against water.

The phosphorothioate containing substrate was purified further to separate the R_p and S_p epimers. This separation was achieved by using reversed phase HPLC on a µ-Bondapak C18 column (Waters) with a gradient of acetonitrile in triethylammonium acetate (pH 6.3). Buffer A (100 mM triethylammonium acetate, pH 6.3) and buffer B (100 mM triethylammonium acetate, pH 6.3, 50% acetonitrile) were used as follows: flow rate = 1.0 ml/min; 20% B, 0 min; 20% B, 5 min; 28% B, 45 min; 28% B, 50 min; 20% B, 55 min; retention times: *R*p = 34.7 min; *S*p = 36.1 min. The separated epimers were desalted again, by dialysis.

The ribozyme strands (both A and B) were purified by polyacrylamide gel electrophoresis (PAGE) on a 15% denaturing gel containing 7 M urea. The oligonucleotides were visualised by UV shadowing and the band excised from the gel and eluted using buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 6.5, 0.5% SDS). The oligonucleotide was then desalted by butanol extraction, followed by filtration using a Sephadex NAP10 column.

The purity of all the oligonucleotide solutions was checked by electrophoresis on a 15% denaturing gel (7 M urea). The oligoribonucleotides were visualised with toluidine blue (0.5%). All oligomers were found to be pure.

Characterisation of modified oligoribonucleotides

To a solution of modified oligonucleotide (≈0.6 ODs) in 80 µl of water, 2 µl of nuclease P1 (1 mg/ml) was added and the mixture was incubated at 30°C for 1 h. An aliquot of 20 µl of the reaction mixture was added to 20 µl of buffer (200 mM Tris–HCl, pH 7.5, 200 mM NaCl, 20 mM $MgCl_2$) and 1 µl alkaline phosphatase and the mixtures were incubated for a further 5 min. The ribonucleosides produced were analysed by reverse phased HPLC on a µ-Bondapak C18 column using buffer A (100 mM triethylammonium acetate, pH 6.5) and buffer B (100 mM triethylammonium acetate, pH 6.5, in 50% acetonitrile) with the gradient as follows: t = 0 min, 0% B; t = 35 min, 35% B; t = 45 min, 60% B; t = 50 min, 100% B; t = 55 min, 100% B; t = 60 min, 0% B; at a flow rate of 1 ml/min. The identity of the ribonucleosides was confirmed by co-injection of standard C, U, A and GMPS. Assignment of GpsA was tentative as no standard was available. Retention times: C = 6.2 min; U = 7.8 min; GMPS = 13.1 min; A = 18.4 min; GpsA = 34.1 min.

Determination of Michaelis–Menten parameters

An equimolar stock solution (50 nM) of the ribozyme strands, A and B in 40 mM Tris–HCl (pH 7.5) was prepared, as well as a stock solution of ^{32}P-labelled substrate (500 nM or 1 µM). The two solutions were incubated at 90°C for 1 min, and were then allowed to cool to 37°C for 10 min. The concentration of $MgCl_2$ in the ribozyme stock solution was adjusted to 10 mM and the solution was further incubated at 37°C for 15 min, alongside the substrate stock solution.

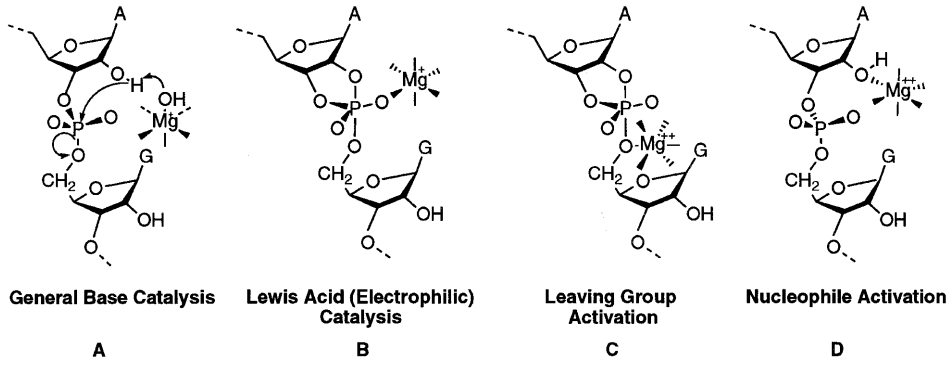

Figure 3. The possible roles of magnesium in small ribozyme catalysis of phosphodiester cleavage.

Reaction mixtures containing $MgCl_2$ and Tris–HCl (pH 7.5) were prepared and pre-incubated for 15 min at 37°C. Appropriate quantities of ribozyme solution were added to the reaction mixtures before initiating the reaction with the 5′-^{32}P-labelled substrate solution (final volume, 100 µl; 10 mM $MgCl_2$, 40 mM Tris–HCl and pH 7.5) and brief vortexing. The final concentrations of the substrate strand were 10–400 nM and for the ribozyme strand, 1–20 nM. The progress of the reactions were monitored by taking 10 µl aliquots from the reaction mixtures at six appropriate time intervals. The individual samples were quenched by immediate addition to 20 µl of denaturing 'stop' mix (7 M urea, 50 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were analysed by PAGE on 20% denaturing gels and the radioactivity quantified by phosphorimaging.

Initial rates of the reaction at various substrate concentrations were determined. This allowed the kinetic parameters to be calculated by fitting the data to the Michaelis–Menten equation:

$$\frac{v}{[E]} = \frac{k_{cat}[S]}{K_m + [S]}$$

where v, initial rate; [S], substrate concentration; [E], total enzyme concentration.

Reactions under single turnover conditions

The reactions were carried out in the same manner as the multiple turnover experiments, however the concentrations of the stock solutions were altered such that the concentrations in the reaction mixtures were 1 nM 5′-^{32}P-labelled substrate (either WT, *R*p or *S*p), 1 µM ribozyme, 40 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$. The reactions were monitored over a 12 min period and 10 µl aliquots were removed at 18 separate time intervals and quenched as before. The amount of product formed was quantified and the initial rate of cleavage was determined.

Cobalt hexaammine experiments

Stock solutions of the wild type 5′-^{32}P-labelled unmodified substrate (5 µM) and ribozyme strands (250 nM w.r.t. each strand) were prepared. Reaction mixtures were prepared with the following final concentrations where all metal complexes were used as the chloride salts: (i) 10 mM $[Mg(H_2O)_6]^{2+}$, 40 mM Tris–HCl (pH 7.5); (ii) 10 mM $[Co(NH_3)_6]^{3+}$, 40 mM Tris–HCl (pH 7.5), 1 mM EDTA; (iii) 10 mM $[Co(NH_3)_5Cl]^{2+}$, 40 mM Tris–HCl (pH 7.5), 1 mM EDTA.

Reactions were initiated as described previously to give final concentrations of 500 nM substrate and 25 nM ribozyme. Control experiments were also carried out in the absence of any metal ions. One contained 40 mM Tris–HCl (pH 7.5), while the other contained 40 mM Tris–HCl (pH 7.5), 1 mM EDTA.

RESULTS AND DISCUSSION

Lewis acid catalysis?

Substitution of one of the non-bridging diastereotopic oxygens of a phosphodiester bond in an RNA molecule with sulfur results in the formation of two epimers with differing chirality at phosphorus. Replacement of the scissile phosphodiester bond in the hammerhead ribozyme with a phosphorothioate linkage decreases the rate of the hammerhead catalysed reaction by a factor of 50–500 when the sulfur is located in the *pro-R* position (16–18). The magnitude of the rate decrease is dependent on the primary structure of the hammerhead ribozyme. No such effects are observed with the sulfur in the *pro-S* position and these substrates are cleaved with a slightly enhanced rate compared to the wild type (18). Dahm and Uhlenbeck (16) have studied the magnesium dependence of the rate of the hammerhead reaction with wild type and R_p phosphorothioate substrates and found that the apparent magnesium dissociation constant was 100-fold higher in the case of the sulfur containing substrate. It was therefore suggested that magnesium binds to the *pro-R* oxygen of the scissile phosphodiester bond and stabilises the pentacoordinate transition state of the reaction by acting as a Lewis acid (electrophilic) catalyst. Changing the divalent metal ion cofactor to manganese (II) enhanced the rate of cleavage of the R_p phosphorothioate substrate to the rate of wild type substrates (16,18). This was suggested as further evidence for the direct coordination of the *pro-R* oxygen, invoking the explanation that Mn^{2+}, a softer metal, is better able to coordinate to the softer sulfur than Mg^{2+}. However, the interpretation of this experiment has been questioned by Taira and co-workers (26), who have demonstrated that with Mn^{2+} as a divalent metal ion cofactor, the rate of cleavage of both phosphorothioate isomers are enhanced by similar amounts. Recently, however, Klug and co-workers have observed a magnesium ion within 2 Å of the *pro-R* oxygen of the scissile phosphodiester bond in a 'freeze trapped' crystal structure of the hammerhead ribozyme (27). Decreases in the rate of reaction upon phosphorothioate substitution have also been observed with other ribozymes including the hepatitis delta catalytic RNA (28).

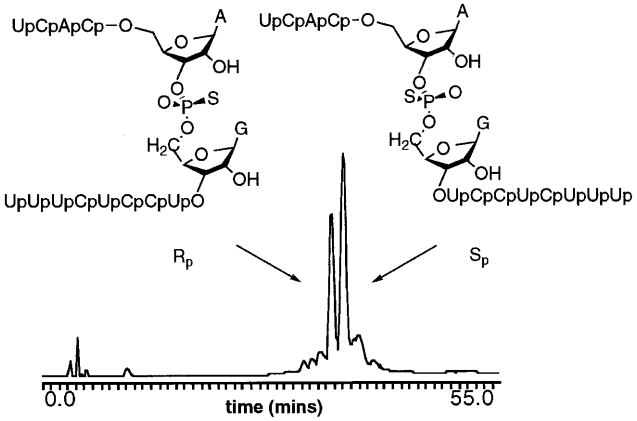

Figure 4. Separation of the two epimers of the hairpin ribozyme substrate generated by sulfurisation of the scissile phosphodiester bond as visualised by HPLC. Gradient conditions are given in Materials and Methods under purification of oligoribonucleotides.

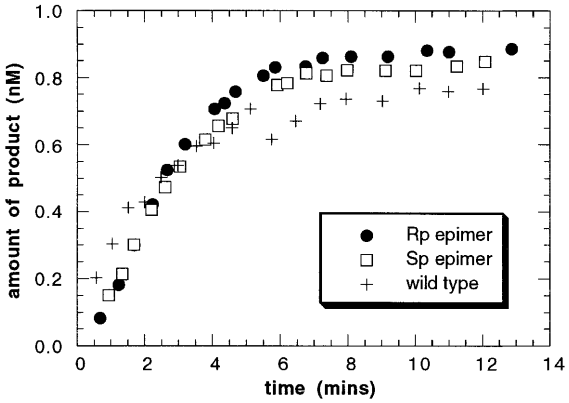

Figure 5. The rate of hairpin ribozyme catalysed reaction of the unmodified substrate and the phosphorothioate epimers measured under single turnover conditions. For full conditions of the reactions, see Materials and Methods under 'Reactions under single turnover conditions'.

In order to investigate the possibility of Lewis acid catalysis in hairpin ribozyme action, we synthesised both isomers of the hairpin substrate in which a phosphorothioate replaces the scissile phosphodiester bond (Fig. 4). This was achieved by replacing the oxidising solution usually used in RNA synthesis with the sulfurising solution, Beaucage reagent in acetonitrile, at the required internucleoside linkage. The resultant epimers were separated by reversed phase HPLC (Fig. 4) and the stereochemistry of the isomers assigned by digestion analysis with enzymes of known stereochemical preference. Nuclease P1 cleaves S_p phosphorothioate linkages at a much higher rate than their R_p counterparts (29). Alkaline phosphatase was added to the enzyme digestions to convert the nucleoside-5′-monophosphates to their respective nucleosides. Alkaline phosphatase does not catalyse the hydrolysis of nucleoside-5′-monophosphorothioates (30). Thus digestion of the fast eluting hplc peak yielded R_p (ApsG), U, C and A in a 1:6:5:1 ratio with nuclease P1. Similar experiments with the slow eluting isomer yielded GMPS (guanosine-5′-monophosphorothioate), U, C and A in a 1:6:5:2 ratio confirming this to have S_p stereochemistry. The assignment of r(ApsG) is tentative as we do not possess this standard. An identical reversed phase elution pattern (R_p fast, S_p slow) has been observed previously for RNA and DNA molecules containing phosphorothioate linkages (18,31).

The structure of the hairpin ribozyme used in our studies is illustrated in Figure 1. The ribozyme is assembled from three oligonucleotides, a substrate strand and two enzyme strands, A and B. The wild type enzyme has a K_m of 49.4 nM and k_{cat} of 0.19/min (Tris–HCl pH 7.5, 10 mM $MgCl_2$, 37°C) which is in good agreement with catalytic parameters reported earlier for an identical sequence (32). Catalytic parameters of the wild type, R_p and S_p phosphorothioate substrates were determined using standard techniques and are shown in Table 1. The observed thio effect is very small for both the S_p and R_p epimers and represents enhancements rather than decreases in the rate of reaction upon phosphorothioate substitution. Although these effects are very small the S_p isomer produces a larger thio effect than the R_p due to a decreased K_m. We have no explanation for the rate increases observed upon sulphur substitution or for the apparently tighter binding of the S_p substrate although these results are reproducible. The lack of thio effect with the R_p diastereoisomer is in good agreement with Burke and co-workers (5). In this previous study the phosphorothioate substrate was generated by transcription and so only afforded a single R_p isomer. The lack of thio effect observed here also parallels a similar negligible effect produced in the non-enzymatic base catalysed cleavage of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate (33).

It is possible that the lack of thio effect is observed due to some other step rather than the chemistry being rate limiting in this enzyme reaction. To eliminate this possibility the rate of cleavage of the substrates was measured under single turnover conditions with ribozyme in vast excess (Fig. 5). The observed first order rate constants for the normal R_p and S_p phosphorothioates are all similar under these conditions. Moreover, the first order rate constants are identical to k_{cat} measured in a multiple turnover reaction. We can therefore rule out the possibility that product release limits the rate of the reactions.

Table 1. Michaelis–Menten parameters of the unmodified and phosphorothioate epimers of the hairpin ribozyme substrate determined under multiple turnover conditions

Substrate	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}min^{-1}$)	Thio effect[a]
Wild type	49.4 ± 5.1	0.191 ± 0.006	3.87×10^6	1.00
*R*p epimer	40.0 ± 12.3	0.259 ± 0.024	6.48×10^6	1.67
*S*p epimer	18.4 ± 6.1	0.236 ± 0.015	1.28×10^7	3.31

[a]Thio effect = [k_{cat}/K_m for modified substrate]/[k_{cat}/K_m for wild type substrate].
For full conditions of the experiment, see Materials and Methods under 'Determination of Michaelis–Menten parameters'.

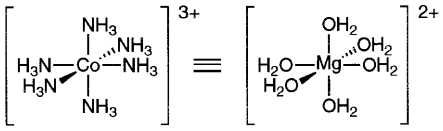

Figure 6. Cobalt (III) hexaammine and magnesium (II) hexahydrate.

General base, attacking/leaving group activation?

In the case of the hammerhead ribozyme there is some correlation between the pK_a of the hexahydrated divalent metal ion complex and the ability to support catalysis (23). Thus when manganese (II) is the divalent metal ion cofactor (with a pK_a of 10.6 compared to $[Mg(H_2O)_6]^{2+}$ which has a pK_a 11.4) the rate of the hammerhead reaction is enhanced ~6-fold. Similar rate enhancements are also observed for other metal ions such as Co^{2+} and Cd^{2+} (pK_as 10.2 and 9.0 respectively) whilst decreases are observed for divalent metal ion cofactors with higher pK_as such as Ca^{2+} (pK_a 12.8). These observations have been used to the support the hypothesis that a metal bound hydroxide deprotonates the attacking 2′-hydroxyl group acting as a general base catalyst. A metal bound hydroxide is also the accepted mechanism for lead (II) cleavage of transfer RNA for which a crystal structure of reactants and products exists (34).

However, the kinetic isotope effect observed on changing the solvent from water to D_2O for the hammerhead reaction is not as large as expected by Taira and co-workers for a general base catalysed reaction involving a metal bound hydroxide (25). Thus, they have suggested that the metal ion instead binds directly to the 2′-oxygen, thus activating the nucleophile by lowering its pK_a.

A further role for metal ions, in which the metal co-ordinates to the leaving group, has been suggested and is supported by theoretical calculations. However, attempts to obtain evidence for this role of metal ions in catalysis of reactions by the hammerhead ribozyme involving replacement of the 5′-leaving group oxygen with sulfur have proved elusive and controversial (35,36). The sulfur substitution changes the rate limiting step of the reaction to attack of the 2′-hydroxyl on the phosphorus centre (37).

All the previous modes of catalysis by metal ions require that the metal either binds to an oxygen of the RNA molecule, or is able to provide a source of hydroxide ions at neutral pH. Cowan and co-workers have recently used cobalt (III) hexaammine to probe the role of divalent metal ions in enzyme reactions (38). The cobalt (III) hexaammine complex is substitutionally inert under the reaction conditions but is of similar size and geometry to magnesium (II) hexahydrate (Fig. 6). The complex has a pK_a of ~16 (39) and thus would be unable to provide a high concentration of anion or bind to RNA forming an inner sphere complex.

We therefore tested the ability of cobalt (III) hexaammine to support hairpin ribozyme catalysis. Experiments were carried out under conditions of excess substrate, and at a concentration much greater than K_m, thus measuring the effect of the co-factor only on turnover number (k_{cat}). The hairpin ribozyme reaction proceeds with similar efficiency with $[Co(NH_3)_6]^{3+}$ as the metal ion co-factor as it does with $[Mg(H_2O)_6]^{2+}$, even in the presence of EDTA (Table 2). EDTA is added to these reaction mixtures to exclude any divalent metal ions which may be present due to purification procedures.

Table 2. The rates of reaction of the hairpin ribozyme catalysed reaction with different divalent metal ion cofactors with unmodified substrate in excess

Metal complex	ν/[E] (min^{-1})	R^2 factor
$[Mg(H_2O)_6]^{2+}$	0.244	0.9636
$[Co(NH_3)_6]^{3+}$	0.198	0.9913
$[Co(NH_3)_5Cl]^{2+}$	0.268	0.9966

Experiments with the cobalt (III) complexes were conducted in the presence of EDTA. Full details of the reaction conditions are given in Materials and Methods under ‘Cobalt hexaamine experiments’.

Other mechanisms?

Two alternative mechanisms for rate enhancement have been proposed to operate specifically for the hairpin ribozyme. Based on the observation that substitution of the essential guanosine in the substrate (G^{+1}) with inosine creates a completely inactive ribozyme, it has been suggested that the exocyclic amino group of guanosine is important in the catalytic reaction (40). It has been proposed that the amino group acts as a general base accepting the proton from the 2′-hydroxyl group or that the amino group hydrogen bonds to one of the phosphoryl oxygens of the scissile linkage. However, the former suggestion appears unlikely as the sites of protonation of guanosine are N7 and N3 whilst a pK_a for the exocyclic amino group cannot be measured and so it must be assumed to be a very poor base. In the latter case, we would expect to see changes in the rates of reaction upon phosphorothioate substitution if interaction with either of the diastereotopic oxygens was taking place.

A recent NMR structure of loop 1 of the hairpin ribozyme offers an alternative explanation for the role of the exocyclic amino group of G^{+1} (41). In this structure G^{+1} is observed to form a sheared base pair with A^9 in which the 2-amino group and N3 of G form hydrogen bonding interactions with the N7 and 6-amino group of A^9. It therefore appears unlikely that the exocyclic amino group of G^{+1} participates directly in catalysis as previously suggested.

Electrostatic catalysis? Structural metal ions?

With a combination of the phosphorothioate and $[Co(NH_3)_6]^{3+}$ experiments we are able to exclude any of the previously proposed mechanisms for ribozyme reactions for the hairpin ribozyme. There thus remains the question of the role of the metal ions in the reaction. One possibility is that the metal ions act as an electrostatic catalyst via an outer sphere mechanism. In order to test this hypothesis we varied the charge on the cobalt complex by altering the ligand sphere. With $[Co(NH_3)_5Cl]^{2+}$ the rate of the hairpin catalysed reaction is similar to that with magnesium and $[Co(NH_3)_6]^{3+}$ as co-factor (Table 2). This suggests that the charge on the complex, which would presumably moderate the ability to catalyse the reaction electrostatically, is not significant in the +2 to +3 range and appears to rule out the possibility of significant electrostatic catalysis.

An alternative explanation for the role of the metal ion cofactor in the reaction catalysed by the hairpin ribozyme is that it is required for structural purposes. It is well documented that structural divalent metal ions are required to stabilise a number of RNA molecules including tRNAs (42,43). This is an attractive proposal for the hairpin ribozyme as it is accepted that catalysis requires the association of loops 1 and 2. The association of the

looped areas of the RNA molecule must overcome the charge repulsion of the phosphodiester backbone; metal ions could assist in facilitating this.

Burke and co-workers have previously studied the ionic requirements of the hairpin ribozyme reaction (44). Mg^{2+}, Sr^{2+} and Ca^{2+} are all capable of acting as the co-factor in the hairpin ribozyme reaction although the fastest reaction rates are observed with Mg^{2+}. However other divalent metal ions such as Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+} and Ba^{2+} are incapable of supporting catalysis although reaction is observed with Mn^{2+} and Co^{2+} in the presence of spermidine. Furthermore, Mn^{2+} was found to inhibit catalysis in the presence of magnesium but the absence of spermidine. Monovalent ions such as Na^{+}, K^{+}, NH_4^{+}, Cs^{+}, Rb^{+} and Li^{+} are also inhibitors of the reaction in the presence of Mg^{2+}. One explanation for these metal ion dependence results is that only Mg^{2+}, Sr^{2+} and Ca^{2+} are capable of folding the RNA into a catalytically competent structure although this is possible in the presence of spermidine with Mn^{2+} and Co^{2+}. Other divalent metal ions, monovalent metal ions and Mn^{2+} and Co^{2+} in the absence of spermidine could force the structure into a catalytically incompetent conformation which does not permit association of the two loops 1 and 2. It should be possible to verify this hypothesis experimentally by chemically mapping the hairpin RNA in the presence of different metal ions or by studying the folding of the RNA in various buffers by fluorescence.

Limitations of our experiments

A possible explanation for the small thio effect and the negligible changes in the rate of reaction with cobalt complexes is that there could be large changes in the rates of the chemical step of the reaction but these are masked because this step may not be rate limiting. Thus one criticism of our experiments is that we have been unable to determine the rate limiting step in the hairpin ribozyme catalysed reaction. We have been able to show that the chemistry, or a step preceding this, must limit the rate of reaction by measuring the rates of reaction under single turnover conditions. Since the hairpin reaction requires loops 1 and 2 to associate it is possible that a conformational change may be slower than the chemical reaction. The hairpin ribozyme has a turnover number some 7-fold lower than an optimal hammerhead ribozyme in which the chemistry has been unequivocally demonstrated to be rate limiting (45). Thus for conformational rearrangement to be severely rate limiting it would be necessary for the chemical step of the hairpin reaction to be very much faster than that of the hammerhead. From an Arrhenius plot of results obtained at higher temperatures we estimate the half-life for transesterification of an internucleoside phosphodiester bond in RNA to be ~10 years at 37°C and pH 7.5 (46). Thus, the hairpin ribozyme provides at least a million-fold rate enhancement of RNA cleavage. In these experiments we seek to provide an understanding of this rate enhancement and are trying to identify significant contributing factors. In experiments with phosphorothioates the ability of the metal ion to coordinate to the oxygen of the cleaved bond is disabled. In the case of the cobalt complex experiments the possibility of any direct interaction with the RNA or proton transfer processes involving the ligand sphere of the metal are completely removed. Therefore, if a particular mode of catalysis contributed significantly we would expect its exclusion to make the chemistry rate limiting even if it is not in the unaltered reaction. It is therefore reasonable to argue that our conclusions are valid even if factors other than chemistry limit the rate of the reaction. It should also be noted that both the pH rate profile and a pre-steady state analysis of the hairpin catalysed reaction have not revealed any rate limiting conformational change (47,48).

Catalysis by the hairpin ribozyme

So what is responsible for the rate enhancements observed with the hairpin ribozyme? We have previously measured the rates of various mutant ribozymes which contain a nucleoside analogue in place of one of the essential nucleosides (32). The analogues represent functional group deletions or modifications of their natural counterparts, and allow us to quantify the role of functional groups of the nucleobases in catalysis. In many cases the catalytic efficiency of reaction is decreased by a factor of 50–100-fold. These changes in catalytic efficiency are mainly manifested in k_{cat} and not K_m suggesting that these functional groups may be involved in binding interactions which are being realised to lower the energy of the transition state. Thus a lowering of the energy of the transition state by a complex hydrogen bonding network which is stabilised by the presence of metal ions could offer an explanation for the rate enhancements of the hairpin ribozyme.

Conclusions

Our results suggest that metal ions play a passive role in the hairpin ribozyme catalysed reaction and enable us to discount previous mechanistic proposals. The hairpin ribozyme is thus the first example of a new class of ribozymes which although metal dependent, could be termed non-metalloenzymes in the sense that metal ions play a passive role in catalysis. This result is particularly intriguing in view of the fact that both the hammerhead and the hairpin are derived from the same RNA satellite virus; the hammerhead being responsible for cleavage of the positive strand of the satellite RNA of tobacco ringspot virus, the hairpin the negative strand.

These results have far reaching implications for the design of ribozyme mimetics. Current strategies for the synthesis of these molecules concentrate on the provision of catalytic moieties such as metal ions or general acid/base catalysts close to the target phosphodiester bond. Most mimetics fail to achieve turnover and accelerate rates by factors that are much smaller than naturally occurring ribozymes. Our results suggest that an alternative approach to the design of mimetics could concentrate on inducing local deformations in the RNA structures capable of lowering the activation energy of the reaction.

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