A unique mechanism for RNA catalysis: the role of metal cofactors in hairpin ribozyme cleavage

Arnold Hampel[1] and JA Cowan[2]

Background: Ribozymes are biological catalysts that promote the hydrolysis and transesterification of phosphate diesters of RNA. They typically require divalent magnesium ions for activation, although it has proven difficult to differentiate structural from catalytic roles for the magnesium ions and to identify the molecular mechanism of catalysis. Direct inner-sphere coordination is usually invoked in the catalytic step, although there is no evidence to support the generality of such a pathway for all ribozymes.

Results: We studied the catalytic pathway for the hairpin class of ribozyme. The substitutionally inert transition metal complex cobalt hexaammine $[Co(NH_3)_6^{3+}]$ was shown to be as active as $Mg^{2+}(aq)$ in promoting hairpin ribozyme activity, demonstrating that inner-sphere pathways are not used by this class of ribozyme. These results were confirmed by studies with R_P- and S_P-phosphorothioate substrate analogs which show a similar reactivity to that of the native substrate towards the magnesium-activated ribozyme. Monovalent cations enhance the activity of $Co(NH_3)_6^{3+}$-promoted reactions, but inhibit Mg^{2+}-activated catalysis, demonstrating a requirement for hydrated cations at several key sites in the ribozyme.

Conclusions: These results provide clear support for a model of RNA catalysis that does not involve direct coordination of magnesium to the phosphate ester, nor activation of a bound water molecule. A mechanism in which catalysis is carried out by functional groups on the RNA ribozyme itself is possible; such functional groups are likely to have pK_a values that are appropriate for carrying out this catalysis. The metal cofactor would then serve to define the architecture of the catalytic pocket and contribute to the stabilization of transient species, as has been described earlier. Hydrolytic pathways in nucleic acid reactions are apparently more diverse than was previously thought, and the hairpin ribozyme falls into a mechanistically distinct class from the *Tetrahymena* and the hammerhead ribozymes.

Addresses: [1]Departments of Biological Sciences and of Chemistry, Northern Illinois University, DeKalb, Illinois 60115, USA. [2]Evans Laboratory of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210, USA.

Correspondence: JA Cowan
E-mail: cowan@chemistry.ohio-state.edu

Key words: hairpin, magnesium, mechanism, ribozyme, RNA

Received: **15 May 1997**
Revisions requested: **13 June 1997**
Revisions received: **23 June 1997**
Accepted: **24 June 1997**

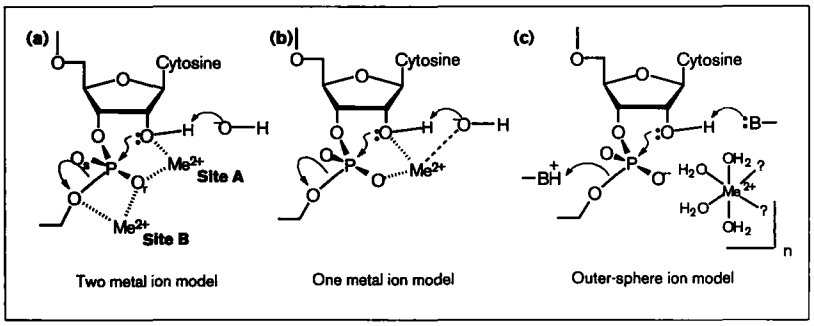
Chemistry & Biology July 1997, 4:513–517
http://biomednet.com/elecref/1074552100400513

Introduction

Ribozymes are RNA catalysts that promote the hydrolysis and transesterification of phosphate diesters of RNA [1–4]. The hammerhead ribozymes catalyze hydrolysis via internal nucleophilic attack by 2′-OH and the *Tetrahymena* ribozymes catalyze transesterification via attack by an external nucleophile [5–9]. In addition to the hammerhead ribozymes, the hairpin, hepatitis delta, and *Neurospora* ribozymes also have self-cleaving motifs that give 2′,3′ cyclic phosphate products [10]. Almost all ribozymes require divalent metal ions (typically Mg^{2+}) for activation, although it has proven difficult to differentiate structural from catalytic roles for the metal ions and to identify the molecular mechanism of catalysis. Thus far, all metal-promoted ribozyme phosphodiester hydrolysis reactions have been described by one of two general mechanistic models (Figure 1a,b) [9,11]. Both demonstrate direct inner-sphere coordination in the catalytic step, either by formation of a Lewis acid complex between the magnesium ion and the phosphodiester backbone, or by formation of an activated nucleophile by lowering the pK_a of metal-bound water.

In this paper we describe a rational strategy to distinguish inner-sphere and outer-sphere pathways in the mechanism of metal-activated ribozyme catalysis. This strategy is based on the previously demonstrated use of substitutionally inert cobalt or chromium complexes to probe for outer-sphere pathways in the mechanism of metal-mediated nuclease enzyme catalysis [12–14]. The substitutionally inert transition metal complex cobalt hexaammine, $Co(NH_3)_6^{3+}$, as well as divalent magnesium, was found to promote activity of hairpin ribozyme, indicating that neither direct binding of the cofactor to phosphate oxygen, nor activation of a nucleophilic water was required. These conclusions are supported by the efficient turnover of R_P- and S_P-phosphorothioate substrate analogs

Figure 1



A summary of proposed mechanisms for metal-promoted ribozyme catalysis. **(a,b)** Two proposed mechanisms for hammerhead ribozyme catalysis; both use an inner-sphere Mg^{2+} which is involved in the cleavage reaction. **(c)** A proposed mechanism for cleavage of the hairpin ribozyme. The multivalent metal ion is involved as an outer-sphere entity and is not directly involved in the chemistry of the cleavage reaction.

by both Mg^{2+}-promoted and $Co(NH_3)_6^{3+}$-promoted ribozyme reactions and demonstrate that the hairpin ribozyme falls into a mechanistic category distinct from the hammerhead and *Tetrahymena* ribozymes, by utilizing hydrated divalent metal ions as the catalytically active cofactor. By considering the influence of monovalent cations on ribozyme activity, we further demonstrate that the metal cofactor has both structural and catalytic roles.

Results and discussion

Table 1 summarizes the activity data for the hairpin ribozyme and substrate (Figure 2) obtained with a variety of ionic conditions. The activity of the cobalt-promoted reaction is around 40% of that of the Mg^{2+}-promoted reaction. In the presence of $Co(NH_3)_6^{3+}$ and the absence of monovalent salt, addition of Mg^{2+} does not restore the relative activity to 100%, although upon addition of 100 mM Na^+ the activity in the cobalt-promoted reactions is equivalent to or exceeds that of the Mg^{2+}-promoted reactions. In fact, Na^+ appears to inhibit the Mg^{2+}-promoted reaction (with 500 mM Na^+, activity is reduced to 1%). Spermidine assists both the Mg^{2+}- and cobalt-promoted reactions, and at high concentrations (25 mM) of spermidine alone there is a residual activity (1%). In the presence of Na^+, spermidine appears to have a negligible influence, and it is most likely that Na^+ competes for the spermidine-binding sites. There is also a very low level of activity at high Na^+ levels (1% at 500 mM Na^+).

To determine whether the activities observed with $Co(NH_3)_6^{3+}$ could be ascribed to the presence of contaminating divalent ions, we examined the influence of increasing EDTA concentrations on activity. The results are summarized in Figure 3. Clearly the activity of the cobalt-promoted reactions is not reduced by EDTA, so $Co(NH_3)_6^{3+}$, which is not bound by EDTA, is truly promoting the reaction. In contrast, when sufficient EDTA is added to complex all of the Mg^{2+}, the activity is eliminated. These results clearly indicate that $Co(NH_3)_6^{3+}$ is indeed the metal cofactor that promotes ribozyme activity in this reaction. $Co(NH_3)_6^{3+}$ is a substitutionally inert transition metal complex ($k_{ex} < 10^{10} s^{-1}$ even at acidic pH) [15], so activation arises neither from inner-sphere binding (Lewis acid catalysis), nor by activation of a bound water molecule. Moreover, the relative activities of the cobalt-promoted and magnesium-promoted reactions argues that the catalytic mechanism for each of these metal cofactors is similar.

To investigate further the influence of monovalent ions on ribozyme activity, the effect of both the cation and th counteranion were examined (Table 2). Each of the background electrolytes used (NaCl, KCl, NaOAc) further activated the cobalt-promoted reaction. Each of these salts

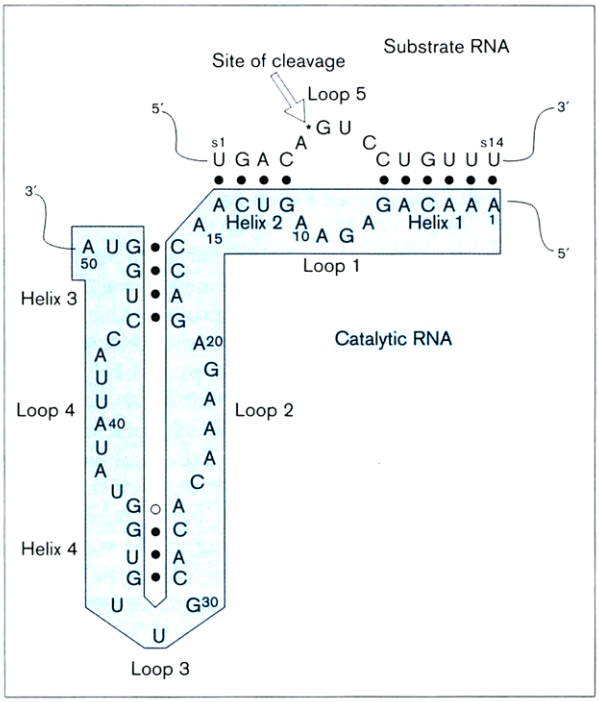
Table 1

A survey of metallocofactor requirements for catalysis by hairpin ribozyme.

Buffer composition (mM)					
Tris	NaCl	$MgCl_2$	Spermidine	$Co(NH_3)_6Cl_3$	Activity*
40	–	12	2	–	1.0
40	–	12	–	–	0.63
40	–	–	2	–	NC
40	–	–	–	–	NC
40	–	–	2	12	0.38
40	–	–	–	12	0.29
40	100	–	2	12	1.0
40	100	–	–	12	1.1
40	100	12	2	–	0.4
40	100	12	–	–	0.35
40	100	–	–	–	NC
40	100	–	2	–	NC
40	500	–	–	–	0.01
40	–	–	25	–	0.01
40	–	12	–	12	0.39
40	–	12	2	12	0.5

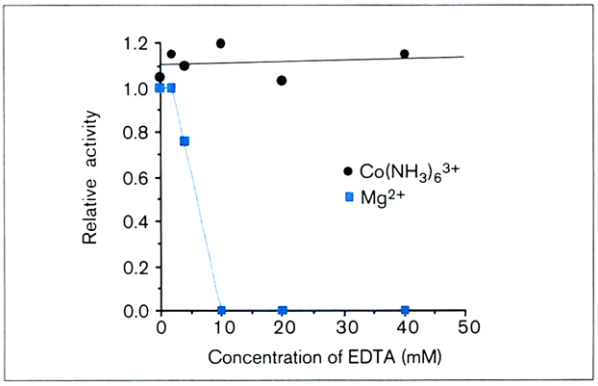
*Activity is defined as the initial velocity of the reaction, relative to that determined under standard conditions (12 mM $MgCl_2$, 2 mM spermidine, 40 mM Tris pH 7.5, 37°C) [33]. Reactions were monitored over either 10 min or 15 min with ribozyme concentrations of 10 nM or 2 nM, respectively, and a substrate concentration of 100 nM. NC, no cleavage. Data were reproducibly obtained from at least two sets of independent experiments.

Figure 2



The hairpin ribozyme. The ribozyme (blue) and substrate of the native sTRSV (satellite RNA of tobacco ringspot virus) sequence are shown.

Figure 3



The influence of EDTA on the reaction velocity when activity is mediated by $Co(NH_3)_6^{3+}$ relative to Mg^{2+}(aq). An activity of 1.0 is obtained under standard reaction conditions (12 mM $MgCl_2$, 2 mM spermidine, 40 mM Tris pH 7.5, 37°C) [33]. Reactions were monitored over 10 min, using 12 mM of either $Co(NH_3)_6^{3+}$ or Mg^{2+}(aq) cofactor. Data were reproducibly obtained from at least two sets of independent experiments.

inhibited the Mg^{2+}-promoted reaction, however, to approximately equal extents. The nature of the monovalent cation or its counteranion appear to be relatively insignificant. Previous studies of the magnesium binding of nucleic acids have demonstrated the importance of hydrogen bonds from metal-bound water molecules in stabilizing structural elements of RNA molecules [16–20]. Although $Co(NH_3)_6^{3+}$ can provide electrostatic stabilization in RNA folding, it is a poor hydrogen-bond donor [16], so further stimulation of ribozyme activity by monovalent cations probably reflects the occupation of key sites in the ribozyme where hydrated cations are required for catalysis. The inhibitory effect of monovalent salts on the Mg^{2+}-required reaction is probably due to competition of monovalent cations for Mg^{2+}-binding sites [17,21].

These results point to a catalytic pathway excluding direct coordination of either a phosphate oxygen or ribose hydroxyl by magnesium. Previous studies with the hammerhead and *Tetrahymena* ribozymes have demonstrated that substitution of phosphate oxygen by sulfur is accompanied by a dramatic decrease in Mg^{2+}-promoted activity [22–24]. For the hammerhead ribozyme, direct interaction of Mg^{2+} with a nonbridging phosphate oxygen is supported by results obtained from phosphorothioate derivatives and a manganese-rescue experiment [25]. Nuclear magnetic resonance (NMR) data also suggest that the magnesium cofactor does not have a significant structural role [26]. Direct binding of Mg^{2+} by both bridging and nonbridging phosphate oxygen is also suggested for the *Tetrahymena* ribozyme, again on the basis of results from phosphorothioate substrate derivatives [27,28], although binding of Mg^{2+} by the 2′-OH group has also been suggested [29]. Previously, it has been shown that the hairpin ribozyme cleaves a phosphorothioate substrate [30]. Although this result suggests that there is no direct binding to the pro-R_p

Table 2

The effect of monovalent salts on Mg^{2+}- and $Co(NH_3)_6^{3+}$-promoted reactions of hairpin ribozyme.

Buffer composition (mM)						
Tris	NaCl	KCl	NaOAc	$MgCl_2$	$Co(NH_3)_6Cl_3$	Activity*
40	50	–	–	–	12	1.2
40	50	–	–	–	12	1.2
40	100	–	–	–	12	1.1
40	100	–	–	12	–	0.35
40	150	–	–	–	12	1.1
40	200	–	–	–	12	1.15
40	500	–	–	–	12	1.05
40	–	100	–	12	–	0.38
40	–	100	–	–	12	0.93
40	–	–	100	12	–	0.32
40	–	–	100	–	12	0.95

* Activity is defined in the legend to Table 1. Reactions were monitored over 15 min with ribozyme concentrations of 2 nM and a substrate concentration of 100 nM. Data were reproducibly obtained from at least two sets of independent experiments.

Table 3

The relative activity of phosphorothioate derivatives towards Mg^{2+}- and $Co(NH_3)_6^{3+}$-promoted reactions.

	Metal cofactor	
Substrate	Mg^{2+}	$Co(NH_3)_6^{3+}$
Native	1	1
S-analog	0.75 (1)	(0.72)

Unit activity in the first column is defined as the initial velocity of the reaction, relative to that determined under standard conditions of 12 mM $MgCl_2$, 2 mM spermidine, 40 mM Tris pH 7.5, 37°C [33] and for the second column, the initial velocity is that obtained in 12 mM $Co(NH_3)_6Cl_3$, 100 mM NaCl, 2 mM spermidine, and 40 mM Tris pH 7.5 37°C. The reactions were monitored over 0–240 min, with a substrate concentration of 100 nM and a ribozyme concentration of 10 nM. The values in parentheses are a paired comparison.

phosphate oxygen, it does not exclude binding to the R_S phosphate oxygen or to other bridging or hydroxyl oxygen centers. To further analyze the effect of cations on catalysis, we prepared the substrate with nonbridging sulfur at the scissile phosphate, by RNA chemical synthesis [14]. This synthesis is expected to give an approximately equal mix of R_P and S_P isomers. If Mg^{2+} ions were directly involved in catalysis by an inner-sphere mechanism, inhibition of cleavage would be expected for one or the other isomer and therefore a relative activity of ~50% would be expected. The extent of cleavage, and cleavage rates, under standard cleavage conditions, were similar for the native and thio-substituted substrates in Mg^{2+} cleavage buffer, except for a small decrease in activity due to the sulfur itself being involved in the reaction (Table 3). As a control, the thio-containing substrate was cleaved in the presence of $Co(NH_3)_6^{3+}$ and compared to cleavage in the presence of Mg^{2+}. No significant differences in cleavage rate or extent of cleavage were seen. These results support our earlier conclusion that Mg^{2+} is unlikely to be directly involved in catalysis via an inner-sphere mechanism. The measured thio effect of $(k_{cat}/K_m)^O/(k_{cat}/K_m)^S \sim 1$ indicates that any step in the ribozyme reaction could be rate-limiting. A detailed discussion of the magnitude of the thio effect and its interpretation has been reported previously by Cech and coworkers [27].

There is considerable evidence for outer-sphere complexation of Mg^{2+}(aq) to oligonucleotides in solution and in the solid state [16–20]. The fact that activation of the ribozyme by $Co(NH_3)_6^{3+}$ is kinetically comparable to that by Mg^{2+}(aq) supports an outer-sphere mechanism for metal activation of hairpin ribozyme; direct metal coordination to the phosphate is not necessary for substrate activation. As monovalent ions are known to have a function in correct folding of the ribozyme, the lack of significant activation by either Na^+ or spermidine alone (≤1%) suggests that the divalent cofactor has both a structural and catalytic role [21]. This could possibly indicate that there is outer-sphere stabilization of the developing negative charge on the phosphate diester during hydrolysis, rather than Lewis acid activation or promotion of nucleophilic attack by HO^- by way of a template mechanism. The similarity in K_m values for both Mg^{2+}- and $Co(NH_3)_6^{3+}$-promoted reactions suggests that binding of the ribozyme and substrate is not significantly influenced by the nature of the metal cofactor, again supporting an effect on k_{cat} rather than K_m in defining the activation barrier ΔG^*.

These results provide clear support for a model of RNA catalysis that does not require direct inner-sphere coordination by the divalent cofactor. A mechanism in which catalysis is mediated by functional groups on the RNA ribozyme itself is possible, if such functional groups are likely to have pK_a values that are tuned to favor this kind of reaction pathway. In this case the divalent metal cofactor serves principally to define the architecture of the catalytic pocket and — or alternatively — may serve to stabilize the transition state of the hydrolysis reaction by an outer-sphere mechanism through hydrogen bonding to the water molecules of solvation. Such a mechanism is likely to be especially prevalent in the case of magnesium-promoted nuclease reactions [12–14,31].

Significance

Ribozymes, which are RNA catalysts, typically require magnesium ions for activity, but it has been difficult to determine whether Mg^{2+} is required in a structural or catalytic role. Here, we show that the inert metal complex $Co(NH_3)_6^{3+}$ also promotes hairpin ribozyme activity, indicating that inner-sphere pathways are not used by hairpin ribozymes for catalysis. It is likely that the metal cofactor has a structural role and contributes to the stability of transient species in the reaction. The hairpin ribozyme has a different catalytic mechanism from that of the *Tetrahymena* and hammerhead ribozymes. The hairpin ribozyme therefore has a unique catalytic mechanism that could have consequences for the design of novel ribozymes.

Materials and methods

Preparation of RNA

Native hairpin ribozyme substrate 5′ UGACA*GUCCUGUUU 3′ was prepared both with and without nonbridging sulfur at the scissile phosphate (*). RNA was chemically synthesized by an ABI 392 DNA/RNA instrument using RNA phosphoramidites (ABI user Bulletin 69). Sulfur was inserted at the scissile phosphate by the TETD method (ABI user Bulletin 58). This yields an approximate equal mixture of Rp and Sp thio-isomers. Chemically synthesized material was 5′ labeled with ^{32}P by the T4 polynucleotide kinase method and purified by polyacrylamide gel electrophoresis. Time courses of cleavage of these substrates was carried out in both magnesium-containing standard cleavage buffer and in buffer with the magnesium replaced by cobalt(III) hexaammine at 37°C.

Ribozyme assays

All assays used the native sequence of the hairpin ribozyme and its substrate [32]. The ribozyme consisted of the required 50 nucleotides plus an additional GGG sequence at the 5′ terminus (R53) and the

substrate consisted of the required 14 nucleotides plus an additional GCG sequence at the 5′ terminus. RNA was prepared by the T7 transcription method from synthetic DNA templates with double stranded promoter [33] using ^{32}P-labeled α-CTP and purified by polyacrylamide gel electrophoresis [3]. RNA cleavage reactions were carried out under various buffer conditions including those in which magnesium was replaced by hexaammine cobalt(III) chloride (Aldrich) at 37°C. Reaction velocities were determined by measuring relative amounts of product and reactant as a function of time for each condition [34]. Activity was initial velocity of a reaction relative to the velocity under standard buffer conditions of 12 mM $MgCl_2$, 2 mM spermidine and 40 mM Tris pH 7.5. Data from independent experiments were reproducible within 15% of the mean. All sample preparation and reactions with recrystallized $Co(NH_3)_6^{3+}$ were conducted under reduced lighting, and solutions were retained in amber bottles or vials, to avoid decomposition of the photolabile cobalt complex. Specific conditions are given in the legends to each Table.

Acknowledgements

We thank Jane Grasby and coworkers for a preprint of related work. This work was supported by the National Institutes of Health (A.H.), the donors of the Petroleum Research Fund (administered by the American Chemical Society, J.A.C.), and the NSF (CHE-9706904, J.A.C.). J.A.C. is a Camille Dreyfus Teacher-Scholar (1994–1999), and a National Science Foundation Young Investigator (1992–1997; CHE-9258168).

References

1. Yang, J., Usman, N., Chartland, P. & Cedergren, R. (1992). Minimum ribonucleotide requirement for catalysis by the RNA hammerhead domain. *Biochemistry* **31**, 5005-5009.
2. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* **35**, 849-875.
3. Hampel, A. & Tritz, R. (1989). RNA catalytic properties of the minimum (–)sTRSV sequence. *Biochemistry* **28**, 4929-4933.
4. McSwiggen, J.A. & Cech, T.R. (1989). Stereochemistry of RNA cleavage by the *Tetrahymena* ribozyme and evidence that the chemical step is not rate-limiting. *Science* **244**, 679-683.
5. Zaug, A.J. & Cech, T.R. (1986). The intervening sequence RNA of *Tetrahymena* is an enzyme. *Science* **231**, 470-475.
6. Zaug, A.J., Been, M.D. & Cech, T.R. (1986). The *Tetrahymena* ribozyme acts like an RNA restriction endonuclease. *Nature* **324**, 429-433.
7. Fedor, M.J. & Uhlenbeck, O.C. (1990). Substrate sequence effects on 'hammerhead' RNA catalytic efficiency. *Proc. Natl Acad. Sci. USA* **87**, 1668-1672.
8. Uhlenbeck, O.C. (1987). A small catalytic oligoribonucleotide. *Nature* **328**, 596-600.
9. Pyle, A.M. (1996). The role of metal ions in ribozymes. In *Metal Ions in Biological Systems,* Vol 32 (Sigel, H. & Sigel, A., eds) pp. 479-520, Marcel Dekker, N.Y.
10. Long, D. & Uhlenbeck, O. (1993). Self-cleaving catalytic RNA. *FASEB J.* **30**, 25-30.
11. Steitz, T.A. & Steitz, J.A. (1993). A general two-metal-ion mechanism for catalytic RNA. *Proc. Natl Acad. Sci. USA* **90**, 6498-6502.
12. Jou, R. & Cowan, J.A. (1991). Ribonuclease H activation by inert transition metal complexes. Mechanistic probes for metallocofactors: insights on the metallobiochemisty of divalent magnesium ion. *J. Am. Chem. Soc.* **113**, 6685-6686.
13. Black, C.B., Foster, M. & Cowan, J.A. (1996). Evaluation of the free energy profile for ribonuclease H metal-promoted catalysis of phosphate ester hydrolysis. Use of inert chromium complexes to evaluate hydrogen bonding and electrostatic contributions to transition state stabilization. *J. Biol. Inorg. Chem.* **1**, 500-506.
14. Black, C.B. & Cowan, J.A. (1997). Inert chromium and cobalt complexes as probes of magnesium dependent enzymes. Evaluation of the stoichiometry and mechanistic role of the essential metal cofactor in *Escherichia coli* exonuclease III. *Eur J. Biochem.* **243**, 684-689.
15. Basolo, F. & Pearson, R. (1968). *Mechanisms of Inorganic Reactions* (2nd edn) pp. 164-165, Wiley, N.Y.
16. Black, C.B. & Cowan, J.A. (1994). Quantitative evaluation of electrostatic and hydrogen bonding contributions to metal cofactor binding to nucleic acids. *J. Am. Chem. Soc* **116**, 1174-1178.
17. Reid, Susan S. & Cowan, J.A. (1991). Metallobiochemistry of a ribosomal RNA. A possible role for Na^+ and K^+ in the regulation of Mg^{2+} binding sites on *E. coli* 5S rRNA. Implications for activity. *J. Am. Chem. Soc*, **113**, 673-675.
18. Cowan, J.A., Huang, H.-W. & Hsu, L.-Y. (1991). Sequence selective coordination of Mg^{2+}(aq) to DNA. *J. Inorg. Biochem.* **52**, 121-129.
19. Gessner, R.V., Quigley, G.J., Wang, A.H.-J., van der Marel, G.A., van Boom, J.H. & Rich, A. (1985). Structural basis for stabilization of Z-DNA by cobalt hexaammine and magnesium cations. *Biochemistry* **24**, 237-240.
20. Gessner, R.V., Frederick, C.A., Quigley, G.J., Rich, A. & Wang, A.H.-J. (1989). The molecular structure of the left-handed Z-DNA double helix at 1.0–.ANG. atomic resolution. Geometry, conformation, and ionic interactions of d(CGCGCG). *J. Biol. Chem.* **264**, 7921-7935.
21. Chowrira, B., Berzal-Herranz, A. & Burke, J. (1993). Ionic requirements for RNA binding, cleavage and ligation by the hairpin ribozyme. *Biochemistry* **32**, 1088-1095.
22. Christian, E.L. & Yarus, M. (1993). Metal coordination sites that contribute to structure and catalysis in the group I intron from *Tetrahymena*. *Biochemistry* **32**, 4475-4480.
23. Koizumi, M. & Ohtsuka, E. (1991). Effects of phosphorothioate and 2-amino groups in hammerhead ribozymes on cleavage rates and magnesium binding. *Biochemistry* **30**, 5145-5150.
24. Kuimelis, R.G. & McLaughlin, L.W. (1995). Hammerhead ribozyme-mediated cleavage of a substrate analog containing an internucleotidic bridging 5′-phosphorothioate: implications for the cleavage mechanism and the catalytic role of the metal cofactor. *J. Am. Chem. Soc.* **117**, 11019-11020.
25. Dahm, S.C. & Uhlenbeck, O.C. (1991). Role of divalent metal ions in the hammerhead RNA cleavage reaction. *Biochemistry* **30**, 9464-9469.
26. Heus, H.A. & Pardi, A. (1991). Nuclear magnetic resonance studies of the hammerhead ribozyme domain. Secondary structure formation and magnesium ion dependence. *J. Mol. Biol.* **217**, 113-124.
27. Herschlag, D., Piccirilli, J.A. & Cech, T.R. (1991). Ribozyme-catalyzed and nonenzymic reactions of phosphate diesters: rate effects upon substitution of sulfur for a nonbridging phosphoryl oxygen atom. *Biochemistry* **30**, 4844-4854.
28. Piccirilli, J.A., Vyle, J.S., Caruthers, M.H. & Cech, T.R. (1993). Metal ion catalysis in the *Tetrahymena* ribozyme reaction. *Nature* **361**, 85-88.
29. Sugimoto, N., Tomka, M., Kierzek, R., Bevilacqua P.C. & Turner, D.H. (1989). Effects of substrate structure on the kinetics of circle opening reactions of the self-splicing intervening sequence from *Tetrahymena thermophila*: evidence for substrate and magnesium binding interactions. *Nucleic Acids Res.* **17**, 355-371.
30. Chowrira, B.M. & Burke, J.M. (1991). Binding and cleavage of nucleic acids by the hairpin ribozyme. *Biochemistry* **30**, 8518-8522.
31. Casareno, R.L.B., Li, D. & Cowan, J.A. (1995). Rational redesign of a metal-dependent nuclease. Engineering the active site of magnesium-dependent ribonuclease H to form an active 'metal-independent' enzyme. *J. Am. Chem. Soc.* **117**, 11011-11012.
32. Hampel, A., Tritz, R., Hicks, M. & Cruz, P. (1990). 'Hairpin' catalytic RNA model: evidence for helixes and sequence requirement for substrate RNA. *Nucleic Acids Res.* **18**, 299-304.
33. Milligan, J.F., Groebe, D.R., Witherell, G.W. & Uhlenbeck, O.C. (1987). Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**, 8783-8798.
34. Anderson, P., Monforte, J., Tritz, R., Nesbitt, S., Hearst, J. & Hampel, A. (1994). Mutagenesis of the hairpin ribozyme. *Nucleic Acids Res.* **22**, 1096-1100.