The hairpin ribozyme: structure, assembly and catalysis

Nils G Walter and John M Burke*

Recent studies of the hairpin ribozyme have revealed a distinct catalytic mechanism for this small RNA motif. Inner-sphere coordinated metal ions are not required, as the inert metal ion complex cobalt hexammine promotes catalysis. Detailed kinetic analyses have defined rates of individual steps in the catalytic cycle. Functional group modification, NMR studies of subdomains and cross-linking experiments, in combination with computer modeling, have led to a proposal for domain interactions in the substrate–ribozyme complex.

Addresses
Department of Microbiology and Molecular Genetics, Markey Center for Molecular Genetics, 306 Stafford Hall, University of Vermont, Burlington, VT 05405, USA
*e-mail: jburke@zoo.uvm.edu

Current Opinion in Chemical Biology 1998, **2**:24–30

http://biomednet.com/elecref/1367593100200024

 ISSN 1367-5931

Abbreviations
sTRSV tobacco ringspot virus satellite RNA

Introduction

Tobacco ringspot virus satellite RNA (sTRSV) depends on the tobacco ringspot virus for its propagation in tobacco plants. The 359 nucleotide satellite RNA acts as a parasite of the single stranded RNA nepovirus by using its enzyme machinery for replication and its coat protein for encapsidation. During rolling-circle replication, the positive-polarity strand, which later becomes encapsulated, yields multimeric forms of its complementary, negative-polarity strand and vice versa. The positive- and negative-polarity strands each harbor a distinct catalytic RNA motif, or ribozyme. Both motifs catalyze site-specific cleavage and ligation reactions of their RNA phosphodiester backbone through a transesterification mechanism, yielding monomeric products of rolling-circle replication with 5′-hydroxyl and 2′,3′-cyclic phosphate termini. The naturally occurring *cis*-acting ribozymes can be truncated and converted to act on substrates *in trans* by removal of their internal substrate and addition of an external one. *Trans*-acting ribozymes have been valuable for studies of structure–activity relationships and have led to the development of ribozymes as potential laboratory tools and therapeutic agents to inhibit gene expression.

Recent studies on the RNA motif derived from the positive strand of sTRSV, the hammerhead ribozyme, have revealed details of its structure–function relationships, through extensive biochemical and structural studies including several solved crystal structures (most recently reviewed in [1]). Until recently, little was known about the catalytic motif of the negative strand of sTRSV, the hairpin ribozyme (previously reviewed in [2,3•,4,5•]). It shows distinctive primary and secondary structure requirements for catalysis (Figure 1a), and *trans*-acting derivatives have been used successfully for intracellular inhibition of pathogenic viruses (reviewed in [6•]).

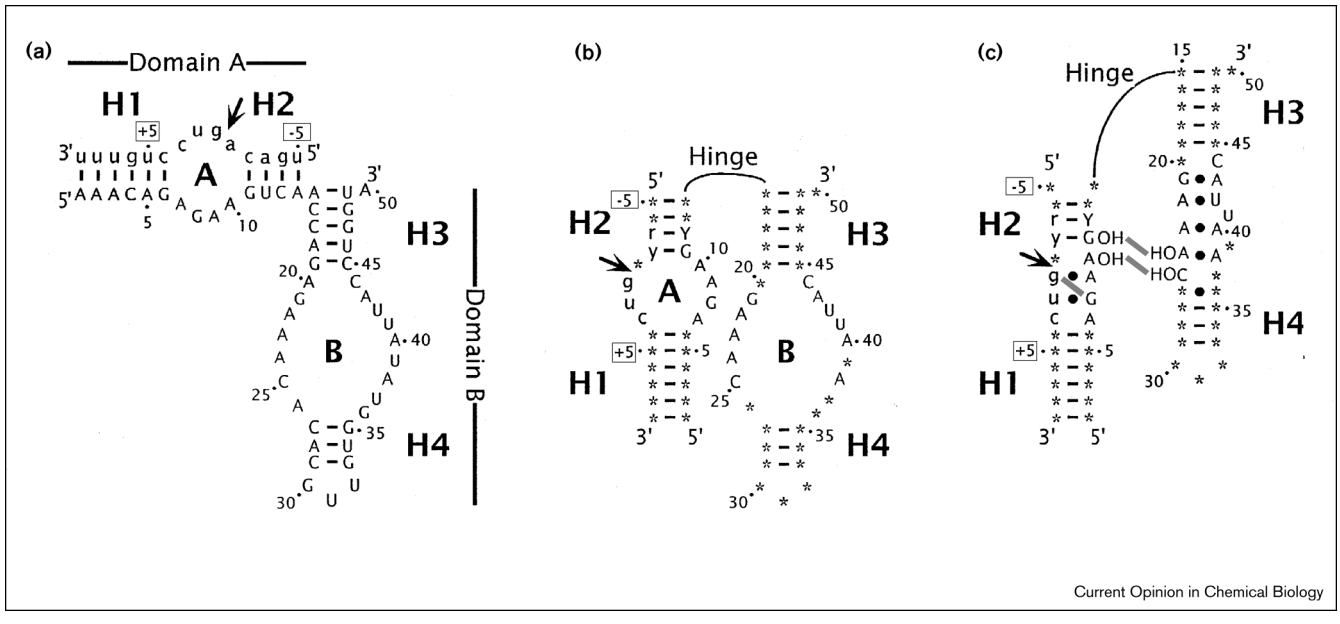
The reactions catalyzed by the hammerhead and hairpin ribozymes are strongly stimulated by metal cations: magnesium ions function to support the activity *in vivo*. Recent results indicate that direct contacts between the metal ions and the RNA chain are not required for catalysis by the hairpin ribozyme, as complex ions that are unable to form inner-sphere complexes (for example, Cobalt[III]hexamine) still support catalytic function. Here, we review our current knowledge on structure and activity of the hairpin ribozyme, including a proposed tertiary structure model, and will then focus on the role of metal ions in the catalytic mechanism of this unique RNA motif.

Structure of the hairpin ribozyme

The secondary structure of the ribozyme–substrate complex (Figure 1a) has been established mainly by analysis of phylogenies derived from natural evolution, *in vitro* selection and mutational analysis [7–9]. It contains two independently folding domains, termed A and B, each with an internal loop flanked by two helices (H1, H2 in domain A, H3 and H4 in domain B). The RNA substrate is bound in domain A through Watson–Crick base pairs in helices 1 and 2, and becomes reversibly cleaved within loop A. Both loops were found to contain highly conserved nucleotides (Figure 1b), whereas the helical regions can largely be changed in sequence, provided that Watson–Crick base pairing is maintained . Insertion of linkers of variable length between the 5′ end of the substrate and the 3′ end of the ribozyme has suggested that the catalytically proficient conformer requires close proximity of loops A and B, using the junction between helices 2 and 3 as a flexible hinge [10,11] (Figure 1b). In the natural sTRSV RNA, as isolated from tobacco plants, this hinge is occupied by a four-way junction, that might regulate interdomain interactions by alternative co-axial stacking of helices [12].

Strand linkage experiments have been used to probe the global arrangement of folding domains in the hairpin ribozyme. With a 5′ extension of the substrate and introduction of a complementary sequence at the 3′ end of the ribozyme, a three-way junction hairpin ribozyme has been designed [13•]. Its activity is highest when a single stranded linker is included between H2 and the added helix, emphasizing the importance of a flexible junction to facilitate the approach of internal loops A and B. Using a flexible hexanucleotide linker, an additional

Figure 1



Hairpin ribozyme structure. **(a)** Secondary structure domains in the wild type (–)sTRSV RNA. The two independently folding domains A and B each consist of two helices (H1, H2 and H3, H4; black lines represent Watson–Crick base pairing) that flank internal loops A and B, respectively. Ribozyme nucleotides are numbered 1 to 50. The external substrate (lower case letters in boxes, nucleotides numbered -5 to +9) is bound in the A domain. Its cleavage site is marked by an arrow. **(b)** Consensus sequence and bent structure. *In vitro* selection [8] and mutational analysis [9] have defined essential nucleotides, mostly in the loop regions. The letter r represents purine nucleotides, y/Y, pyrimidines. Stars indicate variable bases. A sharp bend around the hinge between domains A and B enables the conserved regions to approach each other [10,11]. **(c)** Schematic representation of the current tertiary structure model [29••]. Non-Watson–Crick base pairs are marked by black dots, hydrogen bonds involving the phosphoribose backbone are indicated by gray bars. To enable appropriate alignment of the 2′ hydroxyls of base A_{10} with C_{25} and of G_{11} with A_{24}, for interaction in a proposed ribose 'zipper', the base pair in H2 adjacent to the hinge is frayed, and the sugar–phosphate backbone near the hinge is extended. Note that the bulging interaction between G_{+8} and the ribose of U_{+2} as seen in the ground-state NMR structure of loop A [19••] is changed to a non-Watson–Crick base–base interaction by flipping the bulging u_{+2} back into the minor groove.

domain A with a closing loop on H1 can be connected to the 3′ end of the ribozyme, creating a catalyst that can act on both its A domains to trim its 3′ end *in cis* and cleave an external substrate *in trans* [14]. Inserting one or two nucleotides between A_{14} of domain A and A_{15} of domain B was found to improve catalytic activity [15]. If the phosphodiester bond between A_{14} and A_{15} is removed, the two independently folding domains of the ribozyme become physically separated. At high RNA (100 µM) and Mg^{2+} (≥50 mM) concentrations, the two domains reassemble, using tertiary structure interactions, to give full catalytic activity [15,16]. Alternatively, rejoining the domains by connecting H1 and H4 with either a single-stranded RNA linker [17] or complementary pairing arms [18] restores activity at standard Mg^{2+} concentrations (12 mM).

A recent NMR study has led to a proposal for the ground state structure of domain A [19••]. G_{+1}, an essential base located immediately to the 3′ side of the scissile phosphodiester linkage [20], is proposed to form a canonical base pair with A_2. The geometry of this sheared base pair forces U_{+2} to bulge out into the expanded major groove (Figure 1a, b) and brings G_8 in position to hydrogen bond with the riboses of U_{+2} and G_{+1} (Figure 1c).

For the upper part of loop B, a covalent cross-link between G_{21} and U_{42} can be induced by UV irradiation, indicative of a nine nucleotide structural motif in that region [4,21]. This distinct motif (G_{21}–A_{24} and U_{39}–A_{43}) is also found in loop E of eukaryotic 5S rRNA and in the sarcin/ricin loop of 28S rRNA, the structures of which have been studied by NMR [22,23]. Accordingly, G_{21} and A_{43}, A_{22} and U_{42} as well as A_{23} and A_{40} in the hairpin ribozyme have been proposed to interact in non-Watson–Crick base pairs, while U_{41} is bulged out into the major groove [21] (Figure 1c).

This motif serves as a general recognition element for specific RNA–RNA interactions.

For the lower part of loop B, no high resolution structural information is yet available; however, base pairing between A_{26} and G_{36} adjacent to H4 has been suggested by chemical footprinting experiments utilizing chemical modification reagents [24], as well as extensive mutational analysis [25]. U_{39} has been found to serve as a

molecular spacer, because this base is strongly accessible to chemical modification reagents [24] and replacement with an abasic site or a propyl linker has no effect on cleavage rates [26]. Gait and co-workers [27] used a synthetic two piece ribozyme (with an extended H4 and lacking its closing loop) to study functional group requirements of essential purine residues throughout the ribozyme–substrate complex, as well as sugar and base requirements in loop B [26]. Their results indicate that, unlike in the hammerhead ribozyme, most functional group alterations on hairpin ribozyme bases impede catalytic rates. These effects are difficult to interpret, however, because they could stem from lost hydrogen bonding interactions, both cross-strand or interdomain, or from impaired Mg^{2+} binding in the ground and/or transition state of the reaction pathway. By contrast, only four of all 2′ hydroxyl groups have been found to be essential [26,28], namely those of A_{24} and C_{25} in loop B and A_{10} and G_{11} in domain A (the hydroxyls involved in the ribose zipper). Higher Mg^{2+} concentrations could rescue only the activity of deoxy modifications in positions 24 and 11.

Using site-specific cross-linking of 2′ hydroxyls in domains A and B with disulfide linkages of known spacing, distance constraints for their interaction were obtained [29••]. Combining these constraints with the ground-state structural data for loops A and B and using molecular modeling, Earnshaw *et al.* [29••] generated a tertiary structure model for the hairpin ribozyme. The essential 2′ hydroxyls of positions 24 and 11 (both rescued by high Mg^{2+} concentrations), and those of positions 25 and 10 are proposed to interact with each other to form a ribose zipper [29••] (Figure 1c). Docking of the two domains is accommodated mainly by a sharp turn in the sugar–phosphate backbone between A_{14} and A_{15}, opening the base pair of H2 closest to the hinge, by exploiting the flexibility of the bulging and therefore unconstrained residues U_{+2} and U_{39}, and by flipping U_{+2} into the shallow minor groove of loop A. As a major addition, the authors also propose a sheared base pair between A_{24} and A_{38} (Figure 1c). This model is expected to be a very valuable starting point for experimental tests of ribozyme tertiary structure.

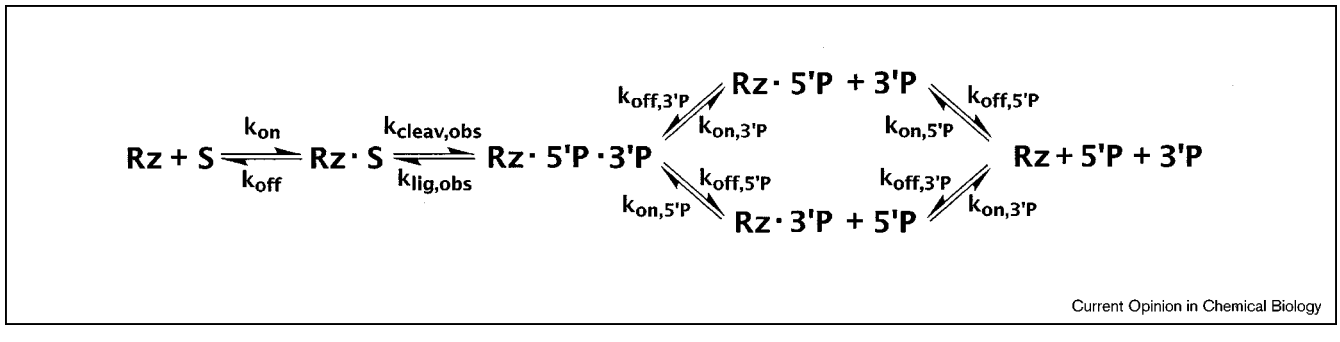
Kinetic studies of catalysis by the hairpin ribozyme

The reaction pathway of intermolecular catalysis by the hairpin ribozyme comprises at least four reversible steps (Figure 2): substrate binding to ribozyme, cleavage in the ribozyme–substrate complex, and release of 5′ and 3′ cleavage products. Rate and equilibrium constants of individual steps have been studied recently by both radioactive [30,31•] and fluorescent methods [32•]. Substrate binding (k_{on}) can be as slow as $6\times10^{6}\,M^{-1}\,min^{-1}$ for the original sTRSV sequence and as fast as $5\times10^{8}\,M^{-1}min^{-1}$ if the substrate sequence is modified to avoid self-complementarity. Substrate dissociation is slow (typically $0.01\,min^{-1}$ for a substrate with six base pairs in H1 and four base pairs in H2) compared to the cleavage rate (see below), so that cleavage of bound substrate is strongly favored over dissociation. There is, however, evidence for biphasic kinetics cleavage; the fast phase is due to correctly folded substrate–ribozyme complex and the slow phase is believed to be due to an inactive conformer with H2 and H3 co-axially stacked. The two conformers are nonexchangeable, so that the inactive complex must dissociate before its substrate can rebind to a catalytically active ribozyme [31•].

Typical cleavage rates are in the range of $0.1–0.3\,min^{-1}$ at 25°C in standard buffer (50 mM Tris-HCl, pH 7.5, 10–12 mM $MgCl_2$) for substrates with different sequences and lengths of intermolecular helices [30,31•]. This rate is somewhat slower than that observed for the hammerhead ribozyme ($1\,min^{-1}$) [33].

Stabilization of H4 and a $U_{39}C$ mutation improves *trans*-cleavage rates. Ligation by the hairpin ribozyme, which is believed to be a simple reversal of the cleavage

Figure 2



Simplified reaction pathway for reversible intermolecular cleavage by the hairpin ribozyme (Rz). Binding and dissociation of substrate (S), 5′ and 3′ products (5′P and 3′P) are characterized by individual k_{on} and k_{off} rates. Note that the observed cleavage ($k_{cleav,\,obs}$) and ligation ($k_{lig,\,obs}$) rates might be composed of several individual reaction steps including conformational changes and transesterification.

reaction pathway, is about 10-fold faster than cleavage (1–3 min^{-1}). This result is in clear contrast to the hammerhead ribozyme, which favors cleavage over ligation by 130-fold due to a significant entropy gain upon cleavage [33]. For the typical short (14–18 nucleotides) substrates used in hairpin ribozyme cleavage, product release is rapid, resulting in similar rates for single- and multiple-turnover reactions [30]. However, when the length of H1 is increased, product release becomes rate-limiting, and unconventional cleavage sites can be observed due to formation of an asymmetric loop A with alternative base pairing in H2 [34].

Fluorescence quenching assays independent of cleavage, employing 3′-fluorescein labeled substrates, were used to demonstrate that domain B provides binding energy for the substrate in addition to that from interactions within domain A [32•]. At least part of this additional binding energy appears to stem from the co-axial stacking of H2 and H3. Whether direct or metal-ion mediated tertiary contacts between loops A and B contribute to this enhanced stability of the ribozyme–substrate complex remains to be determined.

The role of metal ions in hairpin ribozyme structure and catalysis

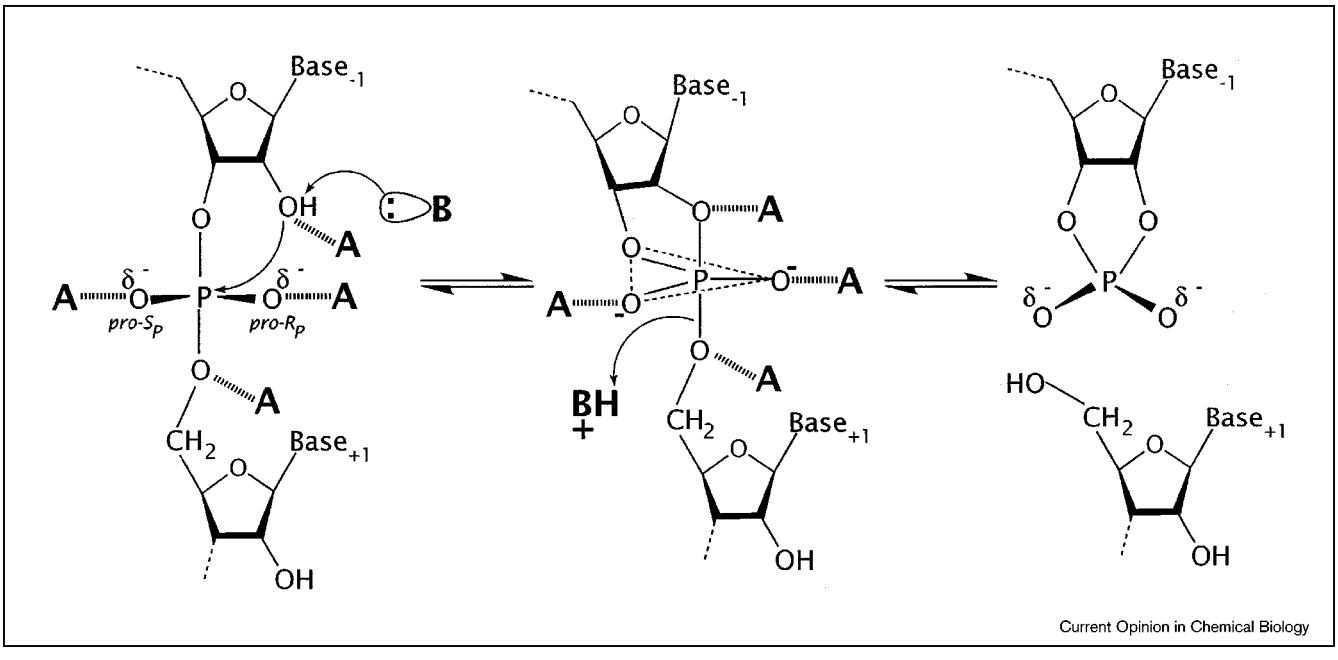
As it is a highly charged polyanion, RNA requires cations to screen negative charge and bind into specific sites, thus enabling folding into compact, biologically active tertiary structures. Divalent alkaline earth metals are particularly well suited to RNA folding as a result of their high charge density and hard electron shell, and serve as the cations used for folding *in vivo*. They can mediate close packing of RNA strands, as is expected for docking of domains A and B of the hairpin ribozyme, either through electrostatic outer-sphere binding of their hydrates or through inner-sphere coordination to the hard oxygen and nitrogen ligands of RNA. In addition, most catalytic RNAs are believed to require divalent metal ions to play a direct role in catalysis, for example by utilizing a bound hydroxide ion as general base catalyst or by inner-sphere coordination to functional groups in the transition state (Lewis acid catalysis; Figure 3) [35]. These roles in structure and catalysis . have proven difficult to separate experimentally. In the case of the hammerhead ribozyme, there is some evidence for both functions of Mg^{2+}; a conformational intermediate of cleavage with several bound metal ions, including one coordinated with the pro-*R* phosphate oxygen of the scissile phosphodiester linkage (Figure 3a), was directly observed by X-ray diffraction of 'freeze-trapped' crystals of catalytically active RNAs [36••].

For the hairpin ribozyme (sTRSV sequence), Mg^{2+}, Ca^{2+} and Sr^{2+} were found to promote catalysis, while Mn^{2+} only activated the ribozyme–substrate complex in the presence of 2 mM of the organic polyamine spermidine, which is known to stabilize RNA structure. Fourteen other monovalent and divalent cations did not yield cleavage products at concentrations of up to 25 mM [37]. Since the addition of up to 2 mM spermidine enhanced the reaction at limiting Mg^{2+} concentrations, two potential functions for cations were inferred: a structural function, satisfied by various cations including spermidine, and a catalytic function, requiring specific divalent metal ions such as Mg^{2+}. However, high concentrations (>5 mM) of spermidine promoted a very slow site-specific substrate cleavage reaction in the presence of EDTA and EGTA [37], indicating a limited suitability of spermidine to fulfil both functions.

Recently several laboratories have reported that the hairpin ribozyme has a unique property: the substitutionally inert transition metal complex cobalt(III)hexammine ($Co[NH_3]_6^{3+}$) can be as active as hydrated Mg^{2+} in promoting the hairpin ribozyme reaction [38•,39••,40•]. Because the ammonia ligands of $Co(NH_3)_6^{3+}$ do not exchange with water or phosphate ligands on the time scale of cleavage reactions, a role in catalysis for metal-bound hydroxide as a general base catalyst can be ruled out (Figure 3). This conclusion is further supported by the shallow pH dependence of cleavage and ligation rates in buffers containing either Mg^{2+}(aq) or $Co(NH_3)_6^{3+}$ [39••]. Second, there appears to be no requirement for inner-sphere coordination of metal ions as Lewis acid catalysts by activating functional groups at the cleavage site (Figure 3). Accordingly, thiophosphate substitutions of the nonbridging oxygens at the cleavage site, in both the *S*P and *R*P diastereomeric configurations, have only a small (≤13-fold) effect on cleavage and ligation rates. This observation was verified in buffers with Mg^{2+}, Mn^{2+} or $Co(NH_3)_6^{3+}$ and demonstrates that the rate-limiting step does not depend on coordination of metal ions to nonbridging oxygens [39••,40•]. Instead, it seems likely that metal ions serve their catalytic function in a unique way, either through electrostatic, outer-sphere stabilization of the developing negative charge on the phosphodiester backbone during transesterification, or through defining the exact architecture of the catalytic pocket, or both.

This proposed catalytic function of metal ions in the hairpin ribozyme resembles their function in folding RNA structure in general; indeed a low level of cleavage activity was observed at high sodium ion concentrations (500 mM) [38•], implying that monovalent cations are sufficient to fulfil this catalytic function to some extent. They (monovalent metal ions) are, however, more efficient in supporting the structural function, as different monovalent cations (Na^+, K^+) significantly enhance activity in the presence of cobalt(III)hexammine [38•]. This stimulation could reflect the requirement for hydrated cations to stabilize the catalytic structure at key sites through hydrogen bonds. By contrast, the Mg^{2+}-catalyzed reaction is inhibited by monovalent cations [37,38•], indicating that hydrated monovalent and divalent cations can compete for these sites.

Figure 3



Reaction chemistry of reversible transesterification by small ribozymes. First, a general base with a lone electron pair (B) deprotonates the 2′ hydroxyl of the upstream nucleotide ($Base_{-1}$), resulting in an oxyanion that attacks the phosphodiester as a nucleophile (arrows). (Note that the stereomeric configuration around the scissile phosphodiester results in two pro-chiralic, distinct phosphate oxygens, pro-S_p and pro-R_p). Second, in the trigonal-bipyramidal transition state, the leaving 5′ oxygen group of the downstream nucleotide ($Base_{+1}$) becomes protonated by a general acid (BH^+, arrow). Third, the upstream 5′ product is released with a 2′,3′ cyclic phosphate group, while the downstream 3′ product carries a 5′ hydroxyl. Possible functions of metal ions in reaction chemistry could either be that of a general base catalyst (for example, in the form of a metal-bound hydroxide, B), deprotonating the attacking the 2′ hydroxyl group, or as Lewis acid catalysts (for example, in the form of directly coordinated metal ions, A), stabilizing developing negative charges in the transition state as indicated. Note that a strategically placed divalent metal ion could fulfil several of these roles at once. In the case of the hairpin ribozyme, however, these potential roles of metal ions are ruled out by recent evidence (see text).

It has been pointed out that several models could account for the pH-independence of the hairpin ribozyme [39••]. First, functional groups of different pK_a values within the RNA could function directly in acid/base catalysis at the active site. Second, the rate-limiting steps for both cleavage and ligation could be one or more slow conformational changes that are independent of pH and divalent metal ions. Third, chemical steps independent of metal-ion coordination or general base catalysis could be rate-limiting, for example, breaking and formation of the 5′ oxygen–phosphorus bond.

However, slow cleavage observed in the presence of only monovalent cations suggests that the fundamental role of metal ions in hairpin ribozyme catalysis may be to organize the active site structure in a way that supports functional groups within the RNA chain in reaction chemistry. When they are available, hydrated divalent metal ions strongly stimulate catalysis (and may participate in active site chemistry). It is clear, however, that some RNA catalysts, such as the hairpin, can entirely dispense with divalent cations and their equivalents. Recruitment of hydrated divalent metal ion cofactors for more direct participation in, and acceleration of, the mechanism would be favorable (at least for some small motifs); however, this is not essential. Interestingly, a DNA enzyme has been generated recently by *in vitro* selection, which is capable of a slow (0.007 min^{-1} at 25°C) self-cleavage reaction at an internal single ribose moiety. This 'DNAzyme' is active with high concentrations of monovalent cations (typically 500 mM Na^+) in the absence of divalent or multivalent metal ions and cleaves with a shallow pH dependence [41•,42•], suggesting that this artificially evolved nucleic acid enzyme may utilize a catalytic strategy that is analogous to that of the hairpin ribozyme.

Conclusions

The hairpin ribozyme consists of two independently folding domains that must interact through tertiary contacts to form a functional complex. Domain interactions are facilitated by metal ions; however, inner-sphere coordination of these metal ions is not required to catalyze site-specific cleavage and ligation reactions. Catalytic rates vary only slightly with pH, ruling out metal-bound hydroxide as a general base catalyst, at least for the rate-limiting step. These observations, together with the

finding that thiophosphate modifications at the cleavage site do not inhibit activity, suggest that the hairpin ribozyme uses a different strategy for RNA cleavage than all other well characterized ribozymes. The function of metal ions in this mechanism might be to organize the active site structure in such a way that functional groups within the RNA itself can catalyze the reaction.

Acknowledgements

This work was supported by grants from the National Institutes of Health and a Feodor-Lynen fellowship by the Alexander-von-Humboldt foundation to NGW. The authors wish to thank Joyce E Heckman, Kenneth J Hampel and Jeff P Bond for helpful comments on the manuscript.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Birikh KR, Heaton PA, Eckstein F: **The structure, function and applications of the hammerhead ribozyme.** *Eur J Biochem* 1997, **245**:1-16.

2. Tuschl T, Thomson JB, Eckstein F: **RNA cleavage by small catalytic RNAs.** *Curr Opin Struct Biol* 1995, **5**:296-302.

3. • Burke JM, Butcher SE, Sargueil B: **Structural analysis and modifications of the hairpin ribozyme.** *Nucleic Acids Mol Biol* 1996, **10**:129-143.
This comprehensive review summarizes our knowledge on hairpin ribozyme structure through 1995.

4. Burke JM: **Hairpin ribozyme: current status and future prospects.** *Biochem Soc Trans* 1996, **24**:608-615.

5. • Earnshaw DJ, Gait MJ: **Progress toward the structure and therapeutic use of the hairpin ribozyme.** *Antisense Nucleic Drug Dev* 1997, **7**:403-411.
This most recent, well-written and concise review covers both structure analysis and potential antiviral applications of the hairpin ribozyme.

6. • Welch PJ, Hampel A, Barber J, Wong-Staal F, Yu M: **Inhibition of HIV replication by the hairpin ribozyme.** *Nucleic Acids Mol Biol* 1996, **10:**315-327.
This concise review focuses on progress and future prospects of hairpin ribozymes applied to the inhibition of HIV replication.

7. DeYoung MB, Siwkowski AM, Lian Y, Hampel A: **Catalytic properties of hairpin ribozymes derived from chicory yellow mottle virus and arabis mosaic virus satellite RNAs.** *Biochemistry* 1995, **34**:15785-15791.

8. Berzal-Herranz A, Joseph S, Chowrira BM, Butcher SE, Burke JM: **Essential nucleotide sequences and secondary structure elements of the hairpin ribozyme.** *EMBO J* 1993, **12**:2567-2574.

9. Anderson P, Monforte J, Tritz R, Nesbitt S, Hearst J, Hampel A: **Mutagenesis of the hairpin ribozyme.** *Nucleic Acids Res* 1994, **22**:1096-1100.

10. Feldstein PA, Bruening G: **Catalytically active geometry in the reversible circularization of 'mini-monomer' RNAs derived from the complementary strand of tobacco ringspot virus satellite RNA.** *Nucleic Acids Res* 1993, **21**:1991-1998.

11. Komatsu Y, Koizumi M, Nakamura H, Ohtsuka E: **Loop-size variation to probe a bent structure of a hairpin ribozyme.** *J Am Chem Soc* 1994, **116**:3692-3696.

12. Duckett R, Murchie AIH, Lilley DMJ: **The global folding of four-way helical junctions in RNA, including that in U1 snRNA.** *Cell* 1995, **83**:1027-1036.

13. • Komatsu Y, Shirai M, Yamashita S, Ohtsuka E: **Construction of hairpin ribozymes with a three-way junction.** *Bioorg Med Chem* 1997, **5**:1063-1069.
Using an original ribozyme design, the authors could improve both substrate binding affinity and catalytic performance of the hairpin ribozyme, emphasizing the importance of a flexible hinge between the separately folding domains.

14. Komatsu Y, Kanzaki I, Shirai M, Ohtsuka E: **A new type of hairpin ribozyme consisting of three domains.** *Biochemistry* 1997, **36**:9935-9940.

15. Shin C, Choi JN, Song SI, Song JT, Ahn JH, Lee JS, Choi YD: **The loop B domain is physically separable from the loop A domain in the hairpin ribozyme.** *Nucleic Acids Res* 1996, **24**:2685-2689.

16. Butcher SE, Heckman JE, Burke JM: **Reconstitution of hairpin ribozyme activity following separation of functional domains.** *J Biol Chem* 1995, **270**:29648-29651.

17. Komatsu Y, Kanzaki I, Koizumi M, Ohtsuka E: **Modification of primary structures of hairpin ribozymes for probing active conformations.** *J Mol Biol* 1995, **252**:296-304.

18. Komatsu Y, Kanzaki I, Ohtsuka E: **Enhanced folding of hairpin ribozymes with replaced domains.** *Biochemistry* 1996, **35**:9815-9820.

19. •• Cai Z, Tinoco I: **Solution structure of loop A from the hairpin ribozyme from tobacco ringspot virus satellite.** *Biochemistry* 1996, **35**:6026-6036.
This first high resolution structural study of a complete folding domain of the hairpin ribozyme gives valuable insights in noncanonical base interactions within loop A and offers an explanation for the importance of functional groups for catalysis.

20. Chowrira BM, Berzal-Herranz A, Burke JM: **Novel guanosine requirement for catalysis by the hairpin ribozyme.** *Nature* 1991, **354**:320-322.

21. Butcher SE, Burke JM: **A photo-cross-linkable tertiary structure motif found in functionally distinct RNA molecules is essential for catalytic function of the hairpin ribozyme.** *Biochemistry* 1994, **33**:992-999.

22. Wimberly B, Varani G, Tinoco I: **The conformation of Loop E of eukaryotic 5S ribosomal RNA.** *Biochemistry* 1993, **32**:1078-1087.

23. Szewczak AA, Moore PB, Chan YL, Wool IG: **The conformation of the sarcin/ricin loop from 28S ribosomal RNA.** *Proc Natl Acad Sci USA* 1993, **90**:9581-9585.

24. Butcher SE, Burke JM: **Structure-mapping of the hairpin ribozyme. Magnesium-dependent folding and evidence for tertiary interactions within the ribozyme-substrate complex.** *J Mol Biol* 1994, **244**:52-63.

25. Siwkowski A, Shippy R, Hampel A: **Analysis of hairpin ribozyme base mutations in loops 2 and 4 and their effects on cis-cleavage *in vitro*.** *Biochemistry* 1997, **36**:3930-3940.

26. Schmidt S, Beigelman L, Karpeisky A, Usman N, Sorensen US, Gait MJ: **Base and sugar requirements for RNA cleavage of essential nucleoside residues in internal loop B of the hairpin ribozyme: implications for secondary structure.** *Nucleic Acids Res* 1996, **24**:573-581.

27. Grasby JA, Mersmann K, Singh M, Gait MJ: **Purine functional groups in essential residues of the hairpin ribozyme required for catalytic cleavage of RNA.** *Biochemistry* 1995, **34**:4068-4076.

28. Chowrira BM, Berzal-Herranz A, Keller CF, Burke JM: **Four ribose 2′-hydroxyl groups essential for catalytic function of the hairpin ribozyme.** *J Biol Chem* 1993, **268**:19458-19462.

29. •• Earnshaw DJ, Masquida B, Müller S, Sigurdsson ST, Eckstein F, Westhof E, Gait MJ: **Inter-domain cross-linking and molecular modelling of the hairpin ribozyme.** *J Mol Biol* 1997, **274**:197-212.
This well written report describes in detail the use of available structural data together with results from a recently developed cross-linking method to model a tentative structure of the active ribozyme–substrate complex at high resolution. This model is valuable as an aid in visualizing structure–function relationships in the hairpin ribozyme.

30. Hegg LA, Fedor MJ: **Kinetics and thermodynamics of intermolecular catalysis by hairpin ribozymes.** *Biochemistry* 1995, **34**:15813-15828.

31. • Esteban JA, Banerjee AR, Burke JM: **Kinetic mechanism of the hairpin ribozyme. Identification and characterization of two nonexchangeable conformations.** *J Biol Chem* 1997, **272**:13629-13639.
A detailed kinetic characterization of catalysis by the hairpin ribozyme that identifies two inherent structural conformers, only one of which is catalytically active.

32. • Walter NG, Burke JM: **Real-time monitoring of hairpin ribozyme kinetics through base-specific quenching of fluorescein-labeled substrates.** *RNA* 1997, **3**:392-404.

The use of fluorescence-based assays for substrate binding and dissociation reveals for the first time energetics of the interaction between domains A and B.

33. Hertel KJ, Herschlag D, Uhlenbeck OC: **A kinetic and thermodynamic framework for the hammerhead ribozyme reaction.** *Biochemistry* 1994, **33**:3374-3385.

34. Moosbauer J, Tabler M: **A helix 1-extended hairpin ribozyme exhibits altered cleavage behavior *in vitro*.** *Antisense Nucleic Acid Drug Dev* 1997, **7**:79-87.

35. Pyle AM: **Ribozymes: a distinct class of metalloenzymes.** *Science* 1993, **261**:709-714.

36. •• Scott WG, Murray JB, Arnold JRP, Stoddard BL, Klug A: **Capturing the structure of a catalytic RNA intermediate: the hammerhead ribozyme.** *Science* 1996, **274**:2065-2069.
An imaginative approach to solve the crystal structure of freeze-trapped intermediates of hammerhead ribozyme cleavage helps to identify the role of conformational changes and bound metal ions in the reaction pathway.

37. Chowrira BM, Berzal-Herranz A, Burke JM: **Ionic requirements for RNA binding, cleavage, and ligation by the hairpin ribozyme.** *Biochemistry* 1993, **32**:1088-1095.

38. • Hampel A, Cowan JA: **A unique mechanism for RNA catalysis: the role of metal cofactors in hairpin ribozyme cleavage.** *Chem Biol* 1997, **4**:513-517.
The first description and discussion of the unusual ability of cobalt(III)hexammine to promote hairpin ribozyme cleavage.

39. •• Nesbitt S, Hegg LA, Fedor MJ: **An unusual mechanism for hairpin ribozyme catalysis: pH and metal ion independence.** *Chem Biol* 1997, **4**:619-630.
A thorough kinetic analysis and detailed discussion of the implications of the unique metal-ion requirement and pH independence of hairpin ribozyme catalysis.

40. • Young KJ, Gill F, Grasby JA: **Metal ions play a passive role in the hairpin ribozyme catalysed reaction.** *Nucleic Acids Res* 1997, **25**:3760-3766.
This report thoroughly discusses the different roles divalent metal ions are expected to have in ribozyme-mediated RNA cleavage and shows that they fulfill only an indirect structural function in hairpin ribozyme catalysis.

41. • Geyer CR, Sen D: **Evidence for the metal-cofactor independence of an RNA phosphodiester-cleaving DNA enzyme.** *Chem Biol* 1997, **4**:579-593.
This paper describes the variation of an existing *in vitro* selection scheme to isolate a novel DNA catalyst for cleavage of an RNA backbone in the absence of divalent or multivalent metal ions.

42. • Faulhammer D, Famulok M: **Characterization and divalent metal-ion dependence of *in vitro* selected deoxyribozymes which cleave DNA/RNA chimeric oligonucleotides.** *J Mol Biol* 1997, **269**:188-202.
A thorough kinetic and structural analysis of several classes of *in vitro* selected DNA enzymes similar to the study in [41•], cleaving an RNA backbone independent of the presence of divalent metal ions.