Metal ions in ribozyme folding and catalysis

Raven Hanna* and Jennifer A Doudna†

Current research is reshaping basic theories regarding the roles of metal ions in ribozyme function. No longer viewed as strict metalloenzymes, some ribozymes can access alternative catalytic mechanisms depending on the identity and availability of metal ions. Similarly, reaction conditions can allow different folding pathways to predominate, with divalent cations sometimes playing opposing roles.

Addresses
Department of Molecular Biophysics and Biochemistry and †Howard Hughes Medical Institute, Yale University, 266 Whitney Ave, New Haven, CT 06520, USA
*e-mail: hanna@csb.yale.edu
†e-mail: doudna@csb.yale.edu

Abbreviation
HDV   hepatitis delta virus

Introduction

Metal ions, particularly divalent cations, are essential for the function of most ribozymes. Structural and biochemical studies have revealed two roles for bound divalent cations: firstly, the majority are required for structural stability, facilitating the formation of an active site; and secondly, a few apparently play a direct role in catalysis. Recent observations, however, reveal a more complex relationship between metal ions and ribozyme function. Several excellent reviews have been written on nucleic acid enzyme structure and catalysis in the past year [1–5]. This review focuses on recent data that challenge traditional paradigms of the relationships between metal ions and RNA.

Metal ions in catalysis
Ribozymes as metalloenzymes

One of the central goals in the study of ribozymes has been to understand how RNA can function as a catalyst despite the relatively restricted variety of functional groups available to perform chemistry. Furthermore, the pK_a values of the free RNA nucleotides are outside the range considered useful for general acid/base catalysis at physiological pH. The requirement of specific divalent metals for activity suggested that RNA structure serves to position metal ions and substrates in the correct context to perform chemistry. In this model, RNA acts as a metalloenzyme in the strictest sense [6]. Steitz and Steitz [7] proposed that the large ribozymes (group I introns, group II introns, and RNase P) might utilize a two-divalent-metal-ion mechanism, similar to that proposed for *Escherichia coli* DNA polymerase and alkaline phosphatase. In this model, one ion (M_B in Figure 1a) activates the nucleophile while the other (M_A) coordinates the leaving group, stabilizing the transition state. This 'two-metal-ion model' became the standard to which subsequent experimental observations were compared, and upon which most current theories are based.
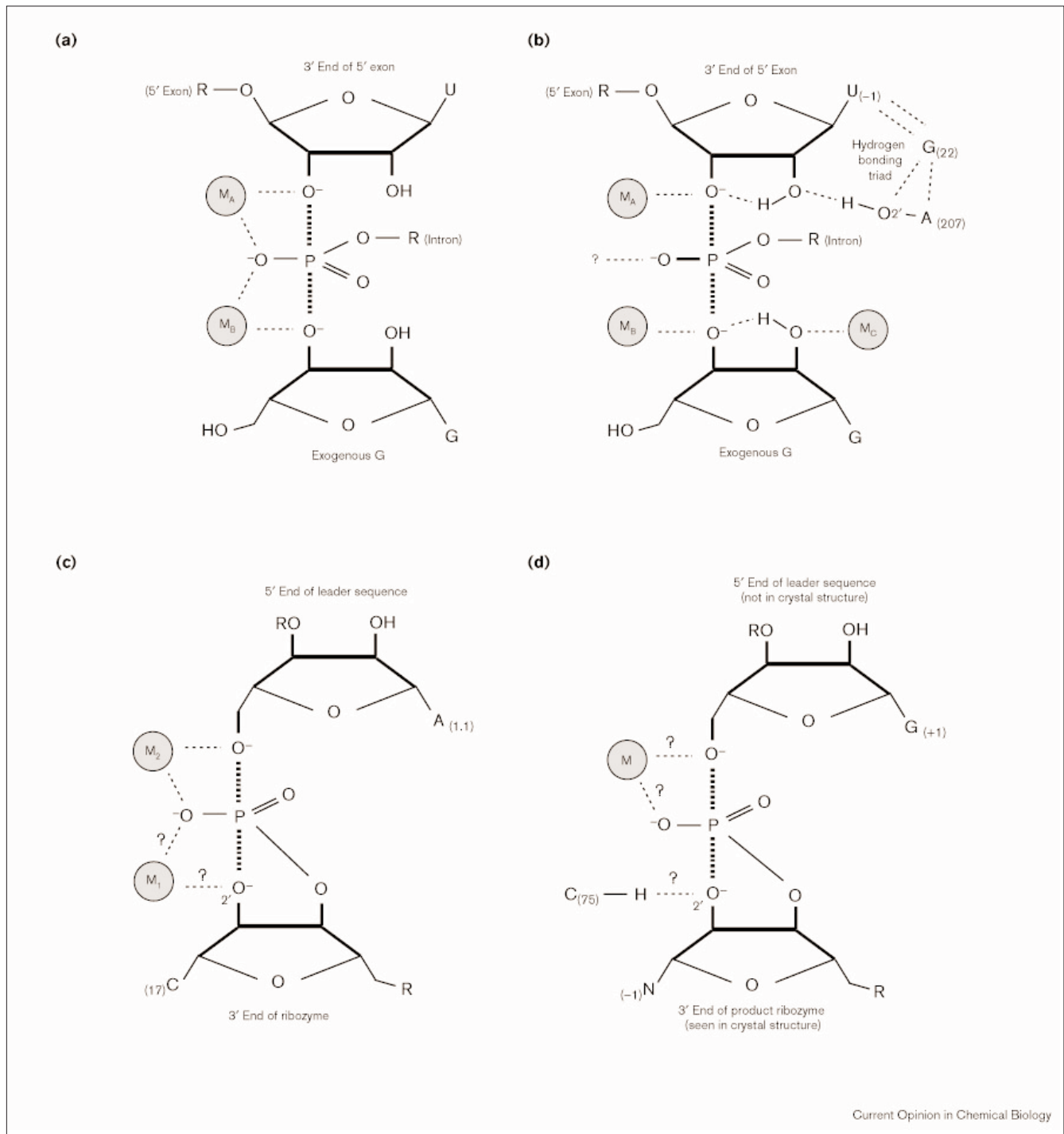
Catalytic metal ions of the group I intron

Of the large ribozymes, most is known about catalysis by the *Tetrahymena thermophila* group I intron, which performs a two-step transesterification reaction using a single active site. It is generally studied in multiple-turnover form, only executing the cleavage portion of the splicing reaction, where the intron catalyzes nucleophilic attack of the 3′ hydroxyl of a bound guanosine on a specific phosphodiester bond in a bound oligonucleotide substrate. Its tightly folded catalytic core positions divalent cations around the labile bond to stabilize the transition state. Assembling details about the chemical environment of the active site is critical for understanding the catalytic mechanism. Unfortunately, the effects of catalytic metal ions are difficult to discriminate from the effects of a multitude of noncatalytic specific and nonspecific ion-binding sites in the RNA. To distinguish catalytic from noncatalytic metal ions, Herschlag and co-workers [8•] developed an innovative general fingerprinting technique to resolve individual metals within this 'sea of bound metal ions.' The latest evidence supports a minimum of three metals participating in catalysis [8•,9,10], illustrated in Figure 1b. As in the two-metal-ion model, one metal (M_B) acts as a base that activates the 3′-hydroxyl nucleophile, and another (M_A) acts as an acid coordinated to the leaving group. The third metal (M_C) might help activate the nucleophile by interacting with the 2′ hydroxyl of the guanosine cofactor.

Because of the reversibility of the reaction, a fourth metal ion might be expected to help stabilize the 3′ leaving group via interactions with the adjacent 2′ hydroxyl. Using nucleotide analog interference mapping (NAIM) [11], Strobel and Ortoleva-Donnelly [12•] found evidence that, instead of a fourth divalent metal ion, a conserved nucleotide triad performs this role through a hydrogen-bond network. This is the first evidence of a hydrogen-bonding network directly stabilizing the transition state in a ribozyme, yet it is known that protein enzymes, such as *E. coli* DNA polymerase and fructose-1,6-biphosphatase, can use hydrogen-bonding networks of amino acids in a similar way to catalyze analogous reactions [10]. The original two-metal-ion model also predicts a metal-ion interaction with the non-bridging phosphate oxygen in the transition state. Piccirilli and co-workers [13•] recently obtained evidence for such a contact, although it is still unclear if the same metal ion (as modeled, M_A and/or M_B in Figure 1a) or additional metals are involved in this interaction.

The group I intron seems to be a true metalloenzyme, specifically binding ions that play roles required for acid–base catalysis. A clear caveat of creating mechanistic

Figure 1

Transition state models of the group I intron, hammerhead, and HDV ribozymes. **(a)** The two-metal-ion model as predicted by Steitz and Steitz [7]. In the first step of the reaction, the 3′ hydroxyl of the exogenous G performs an in-line attack through the phosphate. This nucleophile is activated by one divalent metal ion (M_B), while another metal ion (M_A) acts as an acid to stabilize the leaving group. **(b)** The current model for group I intron chemistry [8•,9–11,12•,13•]. Metal ions in the M_A and M_B positions maintain the functions previously proposed, while a third metal ion(M_c) and a hydrogen-bonding network add to the stability of the transition state. It is unknown which metal ion contacts the non-bridging phosphate oxygen. **(c)** The hammerhead ribozyme transition state, where the 2′ hydroxyl attacks the phosphate bound to its neighboring 3′ hydroxyl. Metal 2 was seen in the crystal structure [19], whereas the presence of metal 1 has been shown through biochemical experiments [21•]. **(d)** One of several current genomic HDV ribozyme transition-state models is shown. A nucleobase (C75) is acting as the general base to activate the 2′ hydroxyl nucleophile. It is possible that a metal ion might function as a general acid to stabilize the 5′ hydroxyl leaving group [15•,16••].

models from the current data is the necessary assumption that the positions of the metals in the ground state imply their participation in the transition state. Ultimately, the role of metals will need to be resolved by more specific means, such as determination of the structure of the intron bound to a transition state analog.

Catalytic metal ions of the hammerhead ribozyme

Given the evidence that the group I intron is a metalloenzyme, it was a surprise that most of the small, viral ribozymes (e.g. hammerhead, hairpin, and VS) can perform catalysis in the absence of divalent metals, when the monovalent cations Li^+ or NH_4^+ are used at molar concentrations [14••]. This suggests that cations are critical for stabilizing the active forms of these ribozymes, but that all of the elements necessary for catalysis are contained within the RNA. These ribozymes are therefore not obligate metalloenzymes, yet they appear to require divalent metal ions under the conditions in which they are typically studied.

Current mechanistic models for several ribozymes focus on the functional groups of the nucleobases as being directly responsible for the chemistry. Catalytic mechanisms in which the local environment allows exocyclic amine functional groups to act as general bases have previously been proposed for two RNAs: the hepatitis delta virus (HDV) ribozyme [15•,16••], and a leadzyme selected *in vitro* [17]. Experiments using NMR spectroscopy have demonstrated that the pK_a of adenosine residues can be perturbed to nearly physiological pH when influenced by the local environment [17]. Riepe *et al.* [18•] provide further evidence that local effects can influence the pK_as of the nucleotides and can stimulate the reaction using an inventive scheme that simulates a hydrophobic local environment.

Studies of the hammerhead ribozyme demonstrate how difficult it can be to discern mechanistic details. Despite a wealth of biochemical and structural data, the mechanism of the hammerhead ribozyme remains unclear. Unlike the group I intron, this ribozyme catalyzes a unimolecular reaction that utilizes a 2′ hydroxyl as the attacking nucleophile, resulting in a 2′,3′-cyclic phosphate (Figure 1c). The crystallographic studies of trapped intermediates of an active hammerhead ribozyme by Scott and co-workers [19,20] showed a bound magnesium ion (M_2 in Figure 1c) making an inner-sphere coordination to the 5′-oxygen leaving group, but there was no evidence for a metal ion contacting the 2′-hydroxyl nucleophile. However, biochemical data from carefully conducted experiments involving phosphorothioate substitution and metal ion specificity switches suggest that there are two distinct divalent metal ions performing these tasks [21•,22••,23]. These seemingly contradictory data have yet to be resolved into a unified model, but could imply that the reaction mechanism is mutable depending on the experimental conditions. It is possible that there are several pathways through a potential energy surface for this reaction, and the preference for a particular path varies according to the reaction conditions.

The existence of multiple mechanisms may be made possible through the simplicity of the hammerhead's unimolecular, one-step reaction and the conformational flexibility of the active site. These features are also likely to characterize the other small ribozymes that have been shown to be non-obligate metalloenzymes.

Catalytic metal ions of the HDV ribozyme

The HDV ribozyme is the only small RNA enzyme that is inactive in the presence of monovalent cations alone [14••], indicating a need for at least one divalent ion to participate during catalysis. Biochemical evidence exists for such a metal-ion coordination in the active-site region of both the genomic and antigenomic ribozyme forms [24,25•]. Thus, it was a surprise that the crystal structure of the genomic HDV ribozyme product showed no well-ordered metal ions bound in the active site [15•]. Instead, the structure revealed the imino nitrogen (N3) position of a cytosine in the active site (C75) to be close to the nucleophile. It is possible that this nucleotide could function in a general acid/base reaction mechanism as either the general acid or base. Subsequent experiments support the hypothesis that, in the antigenomic HDV ribozyme, the cytosine participates in a general base mechanism (see Figure 1d) [16••]. Definitive evidence for the elusive, critical catalytic metal is currently being sought. A metal ion stabilizing the leaving group might only be bound in the precursor form of the ribozyme or may stay attached to the cleaved leader sequence. Therefore, it will be important to obtain structural details for the ribozyme with its substrate in place. It is interesting that the HDV ribozyme, unlike the hammerhead ribozyme, does not appear able to access alternative reaction pathways, which may be partially caused by the comparatively limited flexibility of its tightly packed, pseudoknotted tertiary structure [15•]. If this is true, the reaction mechanism of the HDV ribozyme may prove the easier of the two to define.

Metal ions in ribozyme folding: the group I intron

The complex relationship between metal ions and RNA is also evident from an examination of ribozyme folding. Cations are essential for folding ribozymes into their native states. The beneficial effect of divalent cations on stabilizing structure has led to a general rule: to drive folding, add more magnesium; the more, the better. Although there is no question that cations are necessary to counteract the high negative charge density of RNA globular folds, recent biochemical and structural studies have begun to demonstrate the adverse effect metal ions can have on folding. The *Tetrahymena* group I intron, a favorite model for RNA-folding studies because of its well defined stable domains and abundance of biochemical and structural data, has been the subject for many of the recent developments.

Finding that the optimal magnesium-ion concentration for group I intron folding (2 mM) is just barely above the minimal amount necessary for structure formation, Williamson

and co-workers [26••] postulated that opposing influences determine this surprisingly low value: too little magnesium will not allow occupation of structural metal sites, but too much magnesium inhibits the folding process. The Woodson group [27] reached a similar conclusion, finding that increasing the magnesium-ion concentration decreases the rate of folding by stabilizing a partially folded intermediate. So why can there be too much of a good thing? Treiber *et al.* [28••] approached this question through a selection experiment to create fast-folding mutants. They found that all of the selected molecules contained mutations in a structural subdomain known as the A-rich bulge (Figure 2), which requires several specifically bound divalent cations to fold [29]. This region of the group I intron folds the fastest upon addition of divalent metal ions [30] and it was proposed that the binding of metal ions to this region nucleated the folding process of the entire intron [31]. It now seems likely that if the early folding of the A-rich bulge region into its final, native structure is made too stable through a high concentration of magnesium, it can act as a kinetic trap that energetically blocks further folding [28••]. Alternatively, deletions of this region slow folding by close to fivefold, which suggests that multiple

folding pathways may exist [32•]. Because factors such as temperature, sequence, and ion concentration will alter the predominance of certain pathways, it may be useful to think about RNA folding in the statistical terms of energy landscapes [32•,33•], which have proven successful for modeling and understanding protein folding [34].

Conclusions

Metal ions are essential for the folding and catalysis of ribozymes, but their precise roles are now being questioned. Ribozymes are not all obligate metalloenzymes, as previously thought; specifically coordinated nucleobases may be able to act as general bases and acids to stabilize the transition state. It is also now apparent that, in some ribozymes, different mechanisms may predominate depending on the availability and identity of metal ligands. Similarly, multiple folding pathways can be utilized by ribozymes depending on the environmental conditions. There is a delicate balance between insufficient ions to fold and an overabundance that can hinder folding by stabilizing folding intermediates. These observations challenge what were once considered essential and unrivaled roles of divalent metals for RNA and

Figure 2
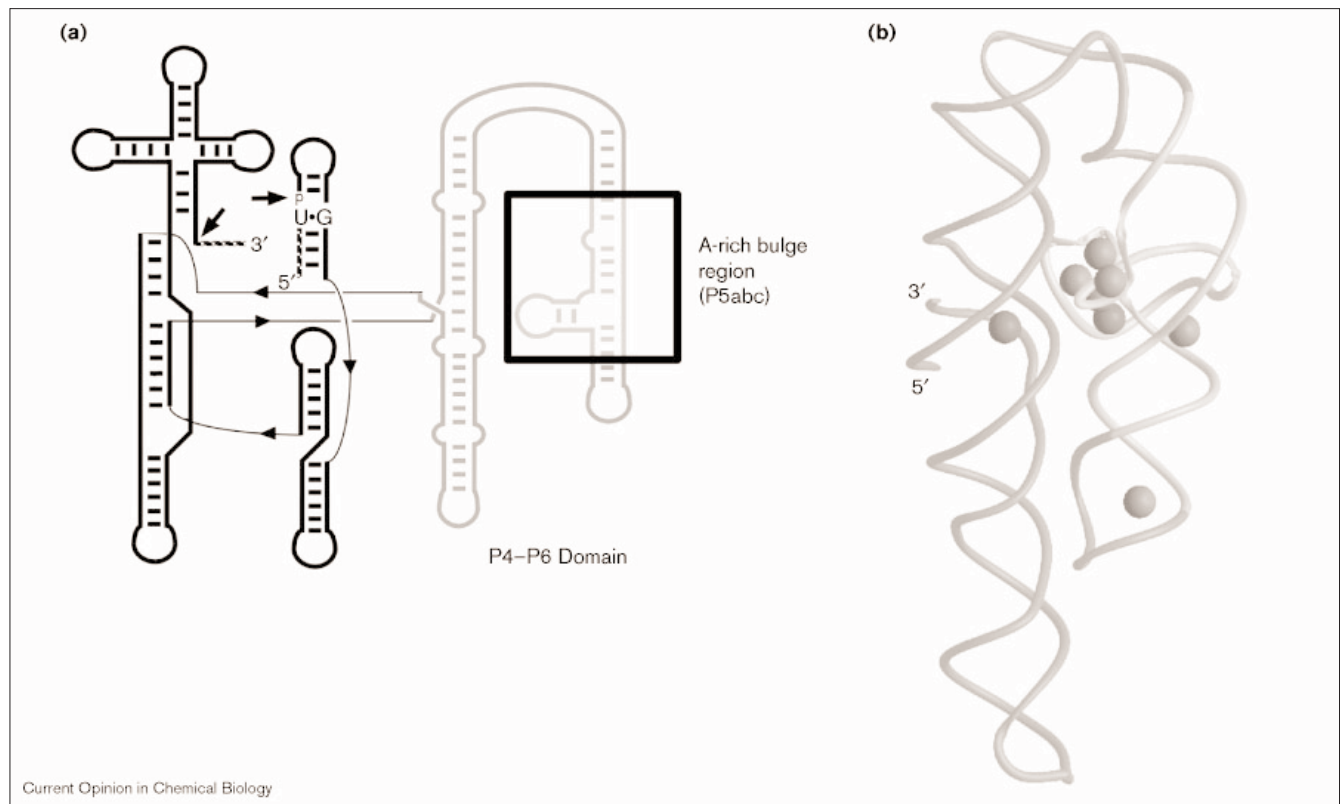
The A-rich bulge motif of the *Tetrahymena* group I intron. **(a)** The secondary structure of the self-splicing group I ribozyme. Arrows denote the 5′ and 3′ cleavage sites. In the first step of the reaction, a guanosine cofactor attacks the 5′ splice site at a specific U·G base pair, which is shown. The A-rich bulge (boxed in the figure) is a subdomain of the P4–P6 structural domain and folds first upon the addition of magnesium. **(b)** Diagram of the crystal structure of the P4–P6 domain showing the well-ordered metal ions (shown as spheres) that are observed [32•]. This region's affinity for divalent cations and stable folded structure might nucleate the folding process in low concentrations of magnesium, but act as a kinetic trap in high concentrations [29].

might foreshadow findings that, *in vivo*, these roles may be usurped by proteins.

Acknowledgements

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. Scott WG: **RNA catalysis.** *Curr Opin Struct Biol* 1998, **8**:720-726.

2. Carola C, Eckstein F: **Nucleic acid enzymes.** *Curr Opin Chem Biol* 1999, **3**:274-283.

3. Lilley DM: **Structure, folding and catalysis of the small nucleolytic ribozymes.** *Curr Opin Struct Biol* 1999, **9**:330-338.

4. Kuimelis RG, McLaughlin LW: **Mechanisms of ribozyme-mediated RNA cleavage.** *Chem Rev* 1998, **98**:1027-1044.

5. Treiber DK, Williamson JR: **Exposing the kinetic traps in RNA folding.** *Curr Opin Struct Biol* 1999, **9**:339-345.

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

7. Steitz TA, Steitz JA: **A general two-metal-ion mechanism for catalytic RNA.** *Proc Natl Acad Sci USA* 1993, **90**:6498-6502.

8. Shan S, Yoshida A, Sun S, Piccirilli JA, Herschlag D: **Three metal**
   • **ions at the active site of the** *Tetrahymena* **group I ribozyme.** *Proc Natl Acad Sci USA* 1999, **96**:12299-12304.
   The authors have developed a quantitative fingerprinting approach to distinguish individual identities of critical metal ions in the group I intron.

9. Weinstein LB, Jones BCNM, Cosstick R, Cech T: **A second catalytic metal ion in group I ribozyme.** *Nature* 1997, **388**:805-808.

10. Piccirilli JA, Vyle JS, Caruthers MH, Cech TR: **Metal ion catalysis in the** *Tetrahymena* **ribozyme reaction.** *Nature* 1993, **361**:85-88.

11. Strobel SA: **A chemogenetic approach to RNA function/structure analysis.** *Curr Opin Struct Biol* 1999, **9**:346-352.

12. Strobel SA, Ortoleva-Donnelly L: **A hydrogen-bonding triad**
    • **stabilizes the chemical transition state of a group I ribozyme.** *Chem Biol* 1999, **6**:153-165.
    Through use of the powerful nucleotide analog interference mapping technique, the transition state of the *Tetrahymena* group I intron was found to be stabilized by a hydrogen-bonding base triad.

13. Yoshida A, Sun S, Piccirilli JA: **A new metal ion interaction in the**
    • *Tetrahymena* **ribozyme reaction revealed by double sulfur substitution.** *Nat Struct Biol* 1999, **6**:318-321.
    Using phosphorothioate substitution, the authors found that a metal-ion interaction with a non-bridging oxygen position at the active site of the group I intron contributes to catalysis.

14. Murray JB, Seyhan AA, Walter NG, Burke JM, Scott WG: **The**
    •• **hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone.** *Chem Biol* 1998, **5**:587-595.
    This study showed that three small ribozymes do not require divalent ions to function in the presence of high concentrations of monovalent cations. The exact role for monovalent ions in catalysis is undetermined.

15. Ferre-D'Amare AR, Zhou K, Doudna JA: **Crystal structure of a**
    • **hepatitis delta virus ribozyme.** *Nature* 1998, **395**:567-574.
    The crystal structure of the product form of the genomic HDV ribozyme shows the active site cleft. It is proposed that Cys75 is involved in the catalytic scheme.

16. Perrotta AT, Shih I, Been MD: **Imidazole rescue of a cytosine**
    •• **mutation in a self-cleaving ribozyme.** *Science* 1999, **286**:123-126.
    Imidazole was found to rescue activity of a mutant antigenomic HDV ribozyme, which supports the theory that the missing nucleobase (Cys76) might be acting as a general base in the reaction mechanism. Mechanistic models are presented.

17. Legault P, Pardi A: **Unusual dynamics and pK_a shift at the active site of a lead-dependent ribozyme.** *J Am Chem Soc* 1997, **119**:6621-6628.

18. Riepe A, Beier H, Gross HJ: **Enhancement of RNA self-cleavage by**
    • **micellar catalysis.** *FEBS Lett* 1999, **457**:193-199.
    The authors used the hydrophobic interior or micelles to simulate the possible effects of a intramolecular environment and found that the reaction rate of a self-cleaving motif was increased compared with the rate in aqueous solution.

19. 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.

20. Murray JB, Terwey DP, Maloney L, Karpeisky A, Usman N, Beigelman L, Scott WG: **The structural basis of hammerhead ribozyme self-cleavage.** *Cell* 1998, **92**:665-673.

21. Lott WB, Pontius BW, von Hippel PH: **A two-metal ion mechanism**
    • **operates in the hammerhead ribozyme-mediated cleavage of an RNA substrate.** *Proc Natl Acad Sci USA* 1998, **95**:542-547.
    Evidence for two separate magnesium ions directly involved in the hammerhead reaction is obtained by titration of La^{3+} into a constant background of Mg^{2+}. Implications of site preference in conjunction with possible ion roles are discussed.

22. Scott EC, Uhlenbeck OC: **A re-investigation of the thio effect at the**
    •• **hammerhead cleavage site.** *Nucleic Acids Res* 1999, **27**:479-484.
    Following recent doubts about the interpretation of phosphorothioate substitution experiments, this paper pioneers a careful way of designing and analyzing thioate substitution experiments.

23. Wang S, Karbstein K, Peracchi A, Beigelman L, Herschlag D: **Identification of the hammerhead ribozyme metal ion binding site responsible for rescue of the deleterious effect of a cleavage site phosphorothioate.** *Biochemistry* 1999, **38**:14363-14378.

24. Lafontaine DA, Ananvoranich S, Perreault JP: **Presence of a coordinated metal ion in a trans-acting antigenomic delta ribozyme.** *Nucleic Acids Res* 1999, **27**:3236-3243.

25. Shih IH, Been MD: **Ribozyme cleavage of a 2,5-phosphodiester**
    • **linkage: mechanism and a restricted divalent metal-ion requirement.** *RNA* 1999, **5**:1140-1148.
    The hammerhead can cleave a 2′-5′ linkage as well as a 3′-5′ linkage when Mg^{2+} or Mn^{2+} are used, but not when Ca^{2+} is used. This metal preference suggests that there is an important metal coordination to the active site. Implications of this catalytic promiscuity are also discussed.

26. Rook MS, Treiber DK, Williamson JR: **An optimal Mg(2+)**
    •• **concentration for kinetic folding of the** *Tetrahymena* **ribozyme.** *Proc Natl Acad Sci USA* 1999, **96**:12471-12476.
    This study leads to a discussion examining the possibility that cations can be harmful as well as helpful to the folding process.

27. Pan J, Thirumalai D, Woodson SA: **Magnesium-dependent folding of self-splicing RNA: exploring the link between cooperativity, thermodynamics, and kinetics**. *Proc Natl Acad Sci USA* 1999, **96**:6149-6154.

28. Treiber DK, Rook MS, Zarrinkar PP, Williamson JR: **Kinetic**
    •• **intermediates trapped by native interactions in RNA folding.** *Science* 1998, **279**:1943-1946.
    Kinetic traps are typically thought of as non-native structures. This study finds that early stable formation of native structure can lead to a folding barrier.

29. Cate JH, Gooding AR, Podell E, Zhou K, Golden BL, Kundrot CE, Cech TR, Doudna JA: **Crystal structure of a group I ribozyme domain: principles of RNA packing.** *Science* 1996, **273**:1678-1685.

30. Sclavi B, Sullivan M, Chance MR, Brenowitz M, Woodson SA: **RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting.** *Science* 1998, **279**:1940-1943.

31. Cate JH, Hanna RL, Doudna JA: **A magnesium ion core at the heart of a ribozyme domain.** *Nat Struct Biol* 1997, **4**:553-558.

32. Russell R, Herschlag D: **New pathways in folding of the**
    • *Tetrahymena* **group I RNA enzyme.** *J Mol Biol* 1999, **291**:1155-1167.
    The authors discuss alternate folding pathways of the group I intron in terms of folding landscapes and discuss the implications of this model.

33. Rook MS, Treiber DK, Williamson JR: **Fast folding mutants of the**
    • *Tetrahymena* **group I ribozyme reveal a rugged folding energy landscape.** *J Mol Biol* 1998, **281**:609-620.
    It was found in this study that fast-folding variants of the group I intron included mutations in the P5abc region that destabilize the native structure of this metal-binding subdomain of the ribozyme.

34. Lazaridis T, Karplus M: **'New view' of protein folding reconciled with the old through multiple unfolding simulations.** *Science* 1997, **278**:1928-1931.