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The Hammerhead Ribozyme Structure Brought in Line

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RNA catalysis seems to be considerably more wide spread than originally thought, with the most prominent example being the ribosome, where RNA catalyses the peptidyl-transferase reaction.^[1] Among the most and longest studied catalytic RNAs are the small nucleolytic ribozymes, such as the hairpin, VS, HDV and hammerhead ribozymes. They all catalyse the site-specific cleavage of their own phosphodiester backbone *in cis* or that of a substrate RNA *in trans* through a transesterification reaction involving the 2'-OH. A novel crystal structure of the hammerhead ribozyme has just been reported,^[2] and this should help to clarify a long-standing debate on the mechanism of catalysis.

First identified in the 1980s as a catalytically active element in the replication cycle of certain viroids and the satellite RNA of plant viruses,^[3-5] the hammerhead ribozyme is the smallest naturally occurring RNA endonuclease. The motif has also been found in transcripts from the satellite DNA of amphibians, schistosomes, cave cricket and, most recently, encoded in the genomes of other eukaryotic organisms.^[6,7] The hammerhead ribozyme consists of a catalytic core of 11 conserved nucleotides that are flanked by three helices (Figure 1A). In the absence of divalent metal ions, the structure is extended, but upon addition of Mg²⁺, the RNA folds in two well-defined steps into a Y-shaped structure (Figure 1B), as deduced by Lilley and co-workers in studies using comparative gel electrophoresis, FRET, NMR and calorime-

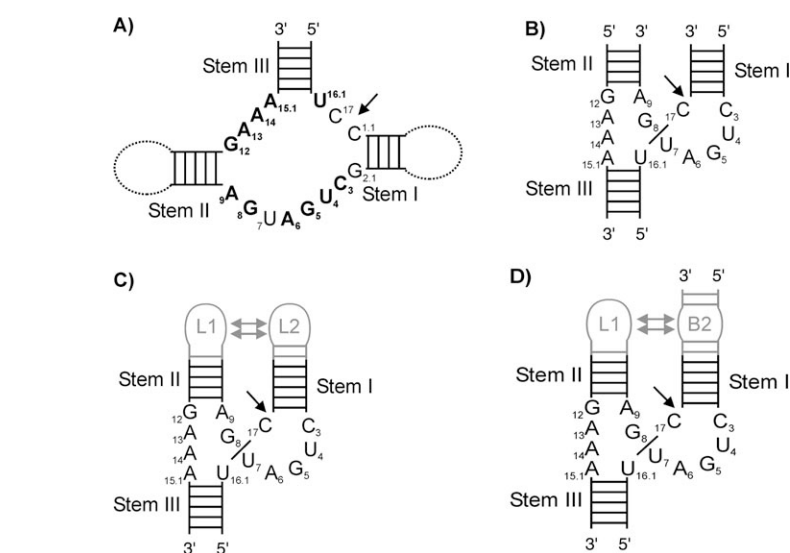


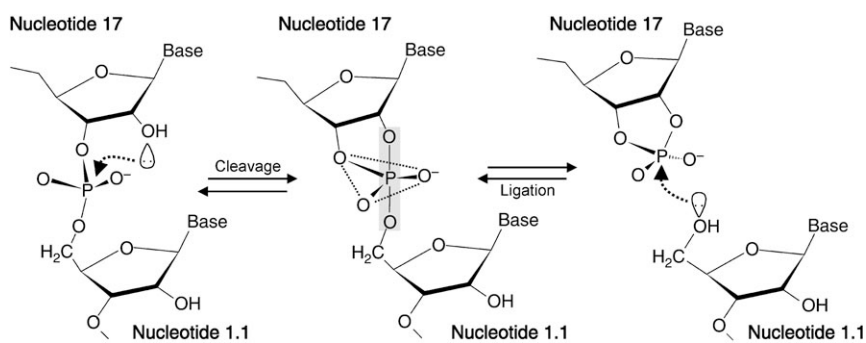
Figure 1. The hammerhead ribozyme. A) Secondary structure with stems I, II and III and the 11 conserved nucleotides (bold). Cleavage takes place between nucleotides 17 and 1.1, as indicated by an arrow. Numbers are given according to the conventional scheme.^[38] In minimal versions of the ribozyme, either stem I or II is closed by loops (dashed lines). B) Y-shaped conformation of the minimal version of the ribozyme upon addition of magnesium. Naturally occurring ribozymes are endowed with tertiary stabilising structures formed between C) loops L1 and L2 or D) loop L1 and bulge B2 in stem II. The ribozyme format shown in (D) was used for crystallisation.

try.^[8] In this active conformation, a reversible transesterification reaction is catalysed by the hammerhead ribozyme (Scheme 1).^[8,9] During cleavage, the 2'-OH of nucleotide C17 is deprotonated and attacks the scissile 3',5' phosphodiester bond. Of the two cleavage products one carries a 2',3'-cyclic phosphate, the other a 5'-hydroxy terminus. In the reverse (ligation) reaction, the 5'-oxygen attacks the cyclic phosphate. For the hammerhead ribozyme, however, the ligation does not proceed as efficiently as seen for the hairpin ribozyme.^[10]

Both reactions proceed through the same, trigonal-bipyramidal pentacoordinated transition state (Scheme 1), thus meeting the principle of microscopic reversibility. This transition state was deduced from the observation that the chirality of the scissile phosphate, when

exchanged for a phosphorothioate, was inverted during the course of the reaction,^[11-13] a hallmark of the S_N2 mechanism. In the transition state, the 2'-OH of C17 has to be in line with the adjacent phosphorus and the 5'-oxygen of nucleotide 1.1 (Scheme 1). This requirement and other data detailed below gave rise to presumably the longest-standing debate in the ribozyme field. The first hammerhead ribozyme crystal structures showed a maximal deviation from the required in-line orientation of the three atoms, at 90°.^[14,15] Hammerhead cleavage, however, could be achieved by soaking all RNA crystals with divalent metal ions.^[16,17] While the first observation argued for a ground-state structure to be present in the crystal, the second would indicate that no major rearrangements were necessary to reach the tran-

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Scheme 1. The S_N2 mechanism of the hammerhead ribozyme reaction. A reversible transesterification is catalysed that proceeds via a trigonal-bipyramidal pentacoordinated transition state, in which the attacking 2'-oxygen of nucleotide 17 is in line with the adjacent phosphorus and the 5'-oxygen of nucleotide 1.1 (boxed in grey).

sition state. The global shape of the ribozyme in the crystals was also in good agreement with an extensive set of in solution data.^[8]

Another set of biochemical data, however, was substantially incompatible with the crystal structure and could only be explained if a large-scale rearrangement of the core region took place during catalysis.^[9,18–21] In particular, rescue experiments with thiophilic metal ions on phosphorothioate-substituted molecules indicated that a single metal ion bridged nonesterified phosphate oxygen atoms of residue A9 and the phosphate of the cleavage site. In the crystal structure, however, these were 20 Å apart.^[9,18]

The basis for the solution of this conundrum was laid in 2003 when the groups of Khvorova and Flores showed independently that the minimal version of the hammerhead ribozyme used until then in structural and biochemical studies was suboptimal.^[22,23] It consists of the core region and three helices only, one of which might be closed by a loop (Figure 1B). In complete ribozymes, however, loops or bulges in stems I and II permit tertiary interactions (Figure 1C and D) that might alter the structure and folding and lead to increased activity of the ribozyme. Furthermore, the presence of these auxiliary elements^[24] allowed for activity at physiological, submillimolar $MgCl_2$ concentrations, while minimal ribozymes were inactive under these conditions. The observation that tertiary contacts were required for full activity^[22,23] proved to be a stimulus to the hammerhead ribozyme field. Magnesium-induced folding of tertiary stabilised

A. thaliana.^[7] Similarly, kinetic data could also be obtained for natural viroid, viral satellite RNA and *Schistosoma mansoni* sequences,^[22–26] which defined the cleavage reaction in detail and established ligation as feature of tertiary stabilised hammerhead ribozymes.

The sequence from *S. mansoni* has now been crystallised, and its structure solved to 2.2 Å by Martick and Scott (Figure 2).^[2] This represents the first structure of a natural hammerhead ribozyme with tertiary-loop interactions in place.^[22,23] Importantly, the folding of the

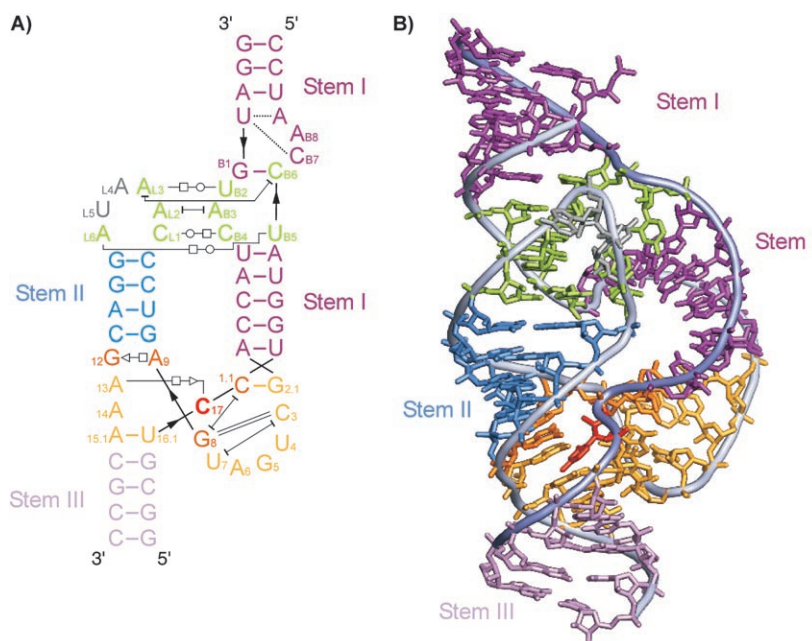


Figure 2. Structure of a tertiary stabilised hammerhead ribozyme determined by Martick and Scott.^[2] A) Sequence, secondary structure and tertiary interactions of the *Schistosoma mansoni* ribozyme. Stems I, II and III are in purple, blue and lilac, respectively. Nucleotides that participate in stabilising tertiary interactions between loop L1 and bulge B2 are shown in green. Residues at the catalytic core are shown in gold with those that are in close proximity to C17 (red) in orange. Thick black lines with arrows denote backbone continuity separated for graphical clarity. Thin black lines denote tertiary interactions; T-termini are shown for stacking interactions, and the Leontis and Westhof nomenclature is used for non-Watson–Crick interactions^[27]—□□ denotes a Watson–Crick/Hoogsteen interaction, and □▷ denotes a Hoogsteen/sugar edge interaction. B) The crystal structure of the *Schistosoma mansoni* ribozyme solved at 2.2 Å, colour coded as in (A). The figure was adapted from ref. [2] and was created with PyMOL.^[39]

molecules was shown to take place in a single step at physiological metal ion concentrations,^[24] whereas minimalist hammerhead ribozymes do so in two steps at elevated Mg^{2+} concentrations.^[8] Also, new examples of this RNA motif were found in a database search,^[6] and, based on kinetic analyses, we obtained a model of tertiary-loop interactions in the novel sequences encoded in

hammerhead in this new structure, particularly in the core region, is substantially different from that seen in previous crystallographic studies.^[8]

The three-way junction folds with a coaxial stack of helices II and III, similar to crystal and in solution structures of minimal hammerhead ribozymes. The expected tertiary interaction between helices I and II is observed in the crystal,

leading to a surprising coaxial stacking of these helices (Figure 2B). This changes the global Y-shape that is seen in minimal ribozymes and is accompanied by an extensive network of tertiary interactions between the loop of stem II and the bulged helix I. As seen in 3D models of other natural ribozyme sequences,^[7,23] the loops also interact by noncanonical base pairing^[27] and stacking of individual, nonadjacent bases (Figure 2A). These interactions lead to a partial unwinding of helix I, which adopts an A-form helix in other crystal structures,^[14,15] a conformation that is incompatible with an in-line mechanism.

Crucially, these tertiary contacts distant from the catalytic core have a marked effect on the structure of the active site. Overall, it is characterised by a more compact arrangement with novel base interactions, as was also suggested recently by Burke and co-workers based on photo cross-linking experiments.^[21] Functionally most relevant, however, is the now perfect in-line geometry of the 2'-oxygen of C17 with the adjacent phosphate and the 5'-oxygen of nucleotide 1.1 (Figure 3A). Nucleotide A9 is now much more closely positioned to the scissile bond, at a distance of only 4 Å compared to the 20 Å observed in the previous studies.^[14,15] The proposed vicinity of these groups to each other had been one of the discrepancies between biochemical and structural data,

as mentioned above, and the arrangement seen now is in accordance with the phosphorothioate rescue data by Herschlag and co-workers.^[18] The bridging metal ion proposed from these experiments was, however, not observed in the new crystal structure, neither were any others. This might be caused by an excess of monovalent metal ions present during crystallisation. Divalent metal ions could actually be expected to be tightly bound to the RNA, particularly since they have been shown by EPR and FRET to be involved in the folding of tertiary stabilised hammerhead ribozymes.^[24,28]

A mechanism by which hammerhead ribozymes accelerate the transesterification reaction, can be proposed from the novel arrangement of specific residues in the catalytic core (Figure 3B). Atom N1 of nucleotide G12 is within hydrogen-bonding distance of the attacking 2'-oxygen of C17; this suggests its role as general base—the 2'-proton would be abstracted by a deprotonated N1 (Figure 3). This is supported by biochemical data on G12 base substitutions that show that the pH dependence of the reaction shifts in accordance with the pK_a of the base analogues.^[29] However, for crystallisation, an RNA with a 2'-OMe modification at C17 was used; this prevented this step and thus cleavage.

Earlier biochemical results had suggested that the G8 nucleobase acts as

the general acid in the hammerhead catalytic reaction.^[29] Surprisingly, however, G8 forms a Watson–Crick base pair with C3 (Figure 2) thereby blocking atom N1 of G8, which cannot stabilise the developing negative charge at the leaving group by proton donation. Exchanging the C3–G8 base pair did not abolish cleavage activity,^[2] this supports the idea that the G8 nucleobase does not act as the general acid in the catalytic reaction. In the crystal, the base of G8 also stacks on nucleotide C1.1, and this results in the 2'-oxygen of the former being oriented so that it could serve as the general acid (Figure 3A). While this hypothesis is supported by loss of activity upon changes of the 2'-hydroxyl group of G8,^[30] it is not clear how exactly the positive charge at the ribose moiety of G8 that is proposed for the transition state of the reaction develops (Figure 3B). A reduction of the unfavourable pK_a of the 2'-hydroxyl group would seem mandatory and this might be achieved by the presence of a divalent metal ion, which, possibly due to the crystallisation conditions, was not seen in this structure.

In summary, the new structure by Mar-tick and Scott^[2] reconciles a number of contradictory results from biochemical analyses and crystallography. The substantial structural rearrangement of the hammerhead ribozyme core region proposed earlier^[18] is indeed seen now, accompanied by an in-line arrangement of

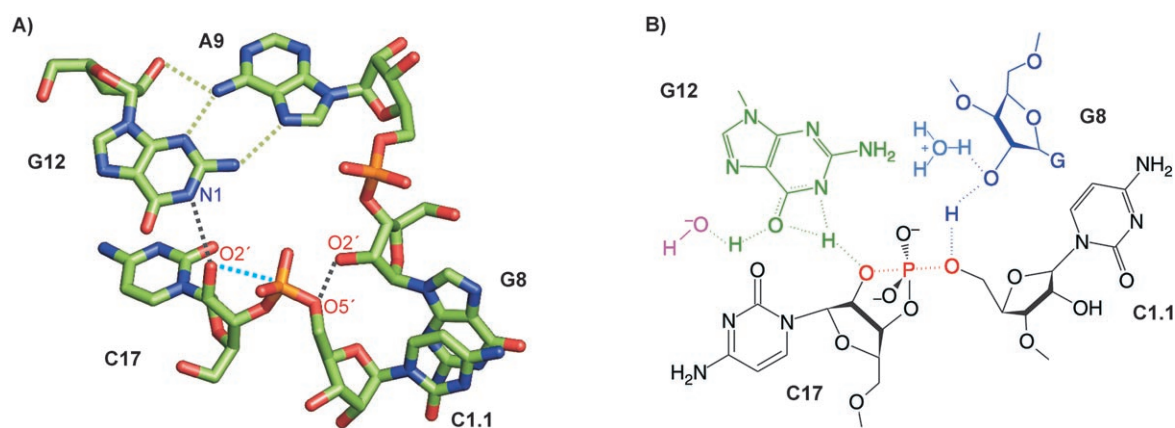


Figure 3. The active-site residues in the hammerhead ribozyme core and implications for their involvement in acid–base catalysis; adapted from ref. [2]. A) Arrangement of nucleotides in the vicinity of the scissile bond between C17 and C1.1 as seen in the crystal structure. Dotted lines denote hydrogen bonds. The 2'-oxygen of C17 is in-line with the adjacent phosphorus and the 5'-oxygen (blue dotted line), as required for the S_N2 mechanism (Scheme 1). Black lines denote hydrogen bonds that might act in acid base catalysis. Figure 3A was created with PyMOL.^[39] B) Transition-state configuration as deduced from (A). In this, the nucleobase of G12 serves as general base and the sugar of G8 as general acid. A hydroxide (purple) and/or a hydronium ion (light blue) might additionally be involved in the reaction.

the attacking nucleophile and the scissile bond, as required for an S_N2 mechanism. The faster cleavage rates of complete ribozymes indicate that the minimal versions only occasionally slip into this active conformation, whereupon they can undergo a reaction, but that they are mainly confined to the inactive form. Thus, the rearrangement presumably had also taken place in crystals of minimal versions,^[16,17] but escaped notice. This structure clearly furthers our understanding of RNA catalysis, and specific nucleobases acting as general acids or bases—which is also seen in other small nucleolytic ribozymes^[31–37]—are emerging as a unifying picture. Divalent metal ions clearly have an important role in folding and stabilising the active conformation of these ribozymes; however, their direct participation, at least in hammerhead catalysis, is not fully understood. Fortunately for ribozymologists, this and other questions about that catalytic RNA, still await elucidation, despite the advances in understanding of its biochemistry. For example, further analyses will be required to define exactly how acid catalysis at G8 occurs. Also, in a more biological context, the cellular function of hammerhead ribozyme containing transcripts encoded in satellite DNA still awaits elucidation.

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- [1] P. Nissen, J. Hansen, N. Ban, P. B. Moore, T. A. Steitz, *Science* **2000**, *289*, 920–930.
- [2] M. Martick, W. G. Scott, *Cell* **2006**, *126*, 309–320.
- [3] G. A. Prody, J. T. Bakos, J. M. Buzayan, I. R. Schneider, G. Bruening, *Science* **1986**, *231*, 1577–1580.
- [4] C. J. Hutchins, P. D. Rathjen, A. C. Forster, R. H. Symons, *Nucleic Acids Res.* **1986**, *14*, 3627–3640.
- [5] A. C. Forster, R. H. Symons, *Cell* **1987**, *49*, 211–220.
- [6] S. Gräf, R. Przybilski, G. Steger, C. Hammann, *Biochem. Soc. Trans.* **2005**, *33*, 477–478.
- [7] R. Przybilski, S. Gräf, A. Lescoute, W. Nellen, E. Westhof, G. Steger, C. Hammann, *Plant Cell* **2005**, *17*, 1877–1885, and references therein.
- [8] C. Hammann, D. M. Lilley, *ChemBioChem* **2002**, *3*, 690–700, and references therein.
- [9] K. F. Blount, O. C. Uhlenbeck, *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 415–440.
- [10] D. M. Lilley, *ChemBioChem* **2001**, *2*, 729–733.
- [11] H. van Tol, J. M. Buzayan, P. A. Feldstein, F. Eckstein, G. Bruening, *Nucleic Acids Res.* **1990**, *18*, 1971–1975.
- [12] G. Slim, M. J. Gait, *Nucleic Acids Res.* **1991**, *19*, 1183–1188.
- [13] M. Koizumi, E. Ohtsuka, *Biochemistry* **1991**, *30*, 5145–5150.
- [14] H. W. Pley, K. M. Flaherty, D. B. McKay, *Nature* **1994**, *372*, 68–74.
- [15] W. G. Scott, J. T. Finch, A. Klug, *Cell* **1995**, *81*, 991–1002.
- [16] W. G. Scott, J. B. Murray, J. R. P. Arnold, B. L. Stoddard, A. Klug, *Science* **1996**, *274*, 2065–2069.
- [17] J. B. Murray, D. P. Terwey, L. Maloney, A. Karpeisky, N. Usman, L. Beigelman, W. G. Scott, *Cell* **1998**, *92*, 665–673.
- [18] S. Wang, K. Karbstein, A. Peracchi, L. Beigelman, D. Herschlag, *Biochemistry* **1999**, *38*, 14363–14378.
- [19] K. Suzumura, M. Warashina, K. Yoshinari, Y. Tanaka, T. Kuwabara, M. Orita, K. Taira, *FEBS Lett.* **2000**, *473*, 106–112.
- [20] J. E. Heckman, D. Lambert, J. M. Burke, *Biochemistry* **2005**, *44*, 4148–4156.
- [21] D. Lambert, J. E. Heckman, J. M. Burke, *Biochemistry* **2006**, *45*, 7140–7147.
- [22] M. De La Pena, S. Gago, R. Flores, *EMBO J.* **2003**, *22*, 5561–5570.
- [23] A. Khvorova, A. Lescoute, E. Westhof, S. D. Jayasena, *Nat. Struct. Biol.* **2003**, *10*, 708–712.
- [24] J. C. Penedo, T. J. Wilson, S. D. Jayasena, A. Khvorova, D. M. Lilley, *RNA* **2004**, *10*, 880–888.
- [25] M. D. Canny, F. M. Jucker, E. Kellogg, A. Khvorova, S. D. Jayasena, A. Pardi, *J. Am. Chem. Soc.* **2004**, *126*, 10848–10849.
- [26] J. A. Nelson, I. Shepotinovskaya, O. C. Uhlenbeck, *Biochemistry* **2005**, *44*, 14577–14585.
- [27] N. B. Leontis, E. Westhof, *RNA* **2001**, *7*, 499–512.
- [28] N. Kisseleva, A. Khvorova, E. Westhof, O. Schiemann, *RNA* **2005**, *11*, 1–6.
- [29] J. Han, J. M. Burke, *Biochemistry* **2005**, *44*, 7864–7870.
- [30] D. B. McKay, *RNA* **1996**, *2*, 395–403.
- [31] A. T. Perrotta, I. Shih, M. D. Been, *Science* **1999**, *286*, 123–126.
- [32] S. Nakano, D. M. Chadalavada, P. C. Bevilacqua, *Science* **2000**, *287*, 1493–1497.
- [33] D. A. Lafontaine, T. J. Wilson, Z. Y. Zhao, D. M. Lilley, *J. Mol. Biol.* **2002**, *323*, 23–34.
- [34] P. C. Bevilacqua, *Biochemistry* **2003**, *42*, 2259–2265.
- [35] F. D. Jones, S. A. Strobel, *Biochemistry* **2003**, *42*, 4265–4276.
- [36] Y. I. Kuzmin, C. P. Da Costa, M. J. Fedor, *J. Mol. Biol.* **2004**, *340*, 233–251.
- [37] T. J. Wilson, J. Ouellet, Z. Y. Zhao, S. Harusawa, L. Araki, T. Kurihara, D. M. Lilley, *RNA* **2006**, *12*, 980–987.
- [38] K. J. Hertel, A. Pardi, O. C. Uhlenbeck, M. Koizumi, E. Ohtsuka, S. Uesugi, R. Cedergren, F. Eckstein, W. L. Gerlach, R. Hodgson, R. H. Symons, *Nucleic Acids Res.* **1992**, *20*, 3252.
- [39] W. L. DeLano, *The PyMOL Molecular Graphics System* **2003**, <http://www.pymol.org>.

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