Ribozymes: **The hammerhead swings into action**

Jennifer A. Doudna

A new crystal structure of a modified hammerhead ribozyme reveals an intermediate conformation that may explain discrepancies between previous structures and the required orientation of the labile bond in the ribozyme's active site.

Address: Department of Molecular Biophysics and Biochemistry and Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06520-8114, USA.
E-mail: doudna@csb.yale.edu

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RNA catalysts, or ribozymes, have intrigued enzymologists and evolutionary biologists since their discovery in the early 1980s. A major question is how ribozymes achieve catalysis, given the limited repertoire of functional groups available on RNA for active-site formation and chemical rate enhancement. The hammerhead ribozyme, a self-cleaving RNA structure, has been the focus of intense study because of its small size — approximately 50 nucleotides — and the seemingly simple RNA cleavage reaction it performs. In spite of extensive kinetic and structural characterization, however, the hammerhead catalytic mechanism has proved tricky to nail down.

Occurring in small plant pathogenic RNAs, the hammerhead motif is a self-cleaving structure that is thought to process multimeric transcripts into monomers during rolling-circle replication of viroid and virusoid genomes [1]. Unlike large ribozymes, such as self-splicing introns and the catalytic subunit of ribonuclease P, the hammerhead consists of just three helices — stems I–III — flanking a core of conserved nucleotides (Figure 1a). Autocatalytic cleavage occurs via nucleophilic attack by the 2′-hydroxyl of a specific core nucleotide on its adjacent phosphodiester bond, producing 2′,3′-cyclic phosphate and 5′-hydroxyl termini. Normally a single-turnover catalyst, the hammerhead is readily converted into a multiple-turnover enzyme by separating the strand containing the cleavage site from the rest of the core. In this way, hammerhead ribozymes have been designed to cleave specific viral and cellular sequences *in vivo*.

Site-directed mutagenesis and chemical probing of the hammerhead RNA revealed that nucleotides in the core region at the junction of the three helices are essential for activity. Furthermore, divalent metal ions such as Mg^{2+} were found to be necessary for ribozyme-catalyzed cleavage. These experiments implied that a specific structure of the RNA in the core is responsible for positioning divalent metals in the active site. Crystal structures of the hammerhead ribozyme, reported in 1994 and 1995, revealed a wishbone-shaped RNA, in which stems I and II form the arms, with stem III and the conserved core at the base (Figure 1b) [2,3]. This conformation was seen in two independent structure determinations, despite differences in RNA backbone connectivities, substrate-strand identity, crystallization conditions and crystal-packing arrangements.

Although the three stems are all A-form helices, the structure of the central core is created, in part, by non-canonical pairings of the conserved nucleotide bases. The catalytically essential sequence CUGA, between stems I and II, forms a tight turn identical to the 'U-turn' seen in $tRNA^{Phe}$. The cytosine that is found at the cleavage site between stems I and III is positioned near

Figure 1

Structure of the hammerhead ribozyme. (a) The secondary structure of the hammerhead shows stems I, II and III, the CUGA sequence of the catalytic pocket, and the scissile bond (red). (b) As revealed by X-ray crystallography, the hammerhead is shaped like a wishbone, with stems I and II emanating from the catalytic pocket and stem III at the base.

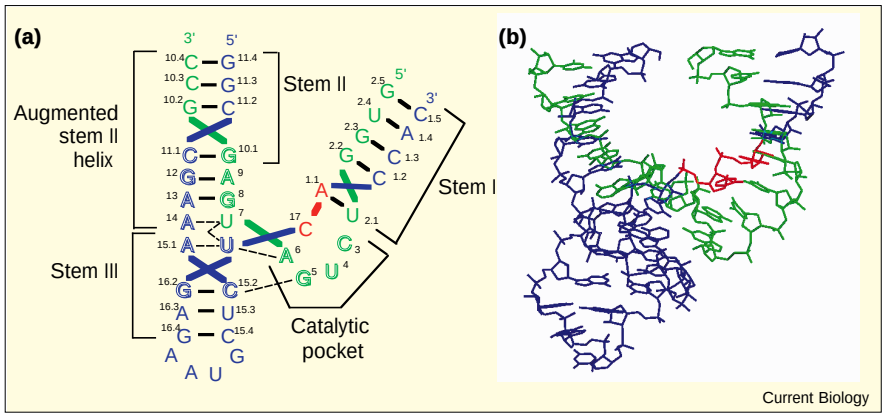
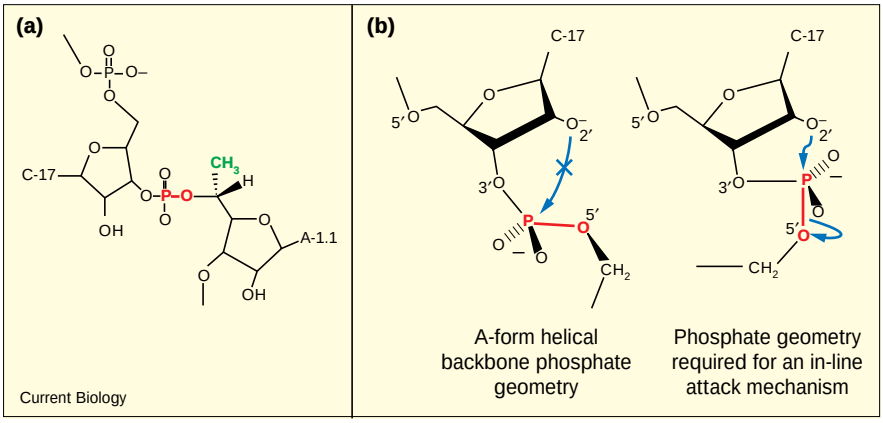


Figure 2



The cleavage-site phosphate conformational switch. **(a)** The modification adjacent to the cleavage site that slows down the cleavage reaction. The methyl group (green), added adjacent to the 5′-oxygen leaving group of the reaction, creates a kinetic bottleneck at the stage of scissile-bond cleavage. **(b)** The cleavage-site conformational switch. As shown on the left, the cleavage-site phosphate in the ground state of the hammerhead ribozyme is 90° away from the geometry required for an in-line attack mechanism. Any significant movement away from the canonical helical conformation would thus position it more favorably for in-line attack, as indicated at right.

the CUGA cleft by interactions with the C and A of the CUGA motif. This proximity, along with the ability of the $tRNA^{Phe}$ U-turn to bind to Mg^{2+} and to be cleaved by Pb^{2+}, led Scott *et al.* [3] to suggest that the CUGA turn of the hammerhead ribozyme, called domain I by Pley *et al.* [2] and the U-turn hereafter, constitutes the ribozyme's catalytic pocket.

The crystal structures of the hammerhead ribozyme provided a detailed view of the catalytic center, but they also raised a puzzling question about the mechanism of cleavage. In both of these original structures, the A-form helical conformation seen for the cleavage-site phosphate is maximally incompatible with the known in-line attack mechanism of the hammerhead reaction. Thus, a conformational change seemed to be necessary to reposition the labile bond during catalysis. To avoid possible complications from the use of 2′-deoxy and 2′-methoxy substrates, as in the original structure determinations [2,3], an all-RNA hammerhead was crystallized and its structure determined in the absence and presence of Mg^{2+} [4]. Without Mg^{2+}, the structure was identical to that determined originally; in the presence of Mg^{2+}, and at low pH to slow down the cleavage rate, an intermediate structure was 'freeze-trapped' in the crystals, in which a 3 Å movement of the scissile phosphate had occurred. While this result suggested that cleavage indeed involves a conformational change around the cleavage site, the details remained tantalizingly obscure.

In an effort to trap a conformational intermediate that would be closer to the transition state, Murray *et al.* [5] synthesized a hammerhead ribozyme with a methyl group on the 5′ carbon atom adjacent to the phosphorous at the cleavage site (Figure 2a). This modification slows down the catalytic rate, presumably by altering the steric or electronic properties of the leaving group. Using 50 mM Co^{2+} as the divalent metal ion in the cleavage reaction, the modified hammerhead ribozyme cleaved 300-fold more slowly than the unmodified ribozyme in the crystals.

This slowing down of the reaction allowed Murray *et al.* [5] to perform a time-course experiment in the crystals. X-ray diffraction data were measured from crystals of the modified hammerhead RNA after soaking in 50 mM $CoCl_2$ for 30 minutes or 2.5 hours. Electron-density maps calculated from these data were compared to those obtained using data from unsoaked crystals and crystals of the original unmodified RNA. While no differences are observed between the unmodified and modified RNA structures after 0 or 30 minutes exposure to Co^{2+}, after 2.5 hours in Co^{2+} the cytosine base and ribose 5′ to the cleavage site have rotated 60° away from the original ground state geometry. This dramatic base swing positions the adjacent phosphorous atom such that it is closer to the conformation required for an in-line attack mechanism (Figure 2b). Murray *et al.* [5] interpret this structure as a 'late' intermediate in the ribozyme reaction pathway.

Murray *et al.* [5] took pains to confirm the conformational changes observed in the crystal by omitting the cytosine base (C-17) immediately 5′ of the cleavage site and the adjacent scissile phosphate from the structure refinement, and asking where electron density reappeared for these residues after simulated annealing [5]. Starting with the ground state structure, and using the late-intermediate data set in this procedure, electron density corresponding to the late-intermediate structure consistently reappeared. It is possible that the trapped structure is on a different pathway than that leading to the transition state for the cleavage reaction. The observation that cleavage occurs faster in the crystal than in solution, however, is reassuring in this regard. As the authors point out, the trapped intermediate approaches the conformation that would be required for the in-line nucleophilic attack mechanism, but it is not a true transition-state intermediate. The 3 Å

resolution limit of the structure prevents unambiguous resolution of the non-bridging phosphate oxygen positions.

This latest chapter in hammerhead ribozyme crystallography suggests that large conformational changes away from the ground-state structure are not required for hammerhead catalysis. Instead, local rearrangements around the scissile bond probably are sufficient to move the cleavage site into the transition-state geometry. Some unanswered questions about the hammerhead mechanism remain. For example, the role of the conserved G5 nucleotide in the core is unclear, though recent experiments suggest that it binds to a Mg^{2+} ion that either helps form the transition state or prevents formation of the transition state [6]. Further experiments are required to distinguish these possibilities. One wonders how this mechanism will compare to those of other ribozymes involved in rolling circle replication, in which different substrate specificities, kinetic parameters and structures are involved.

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