

Hammerhead ribozyme structure: U-turn for RNA structural biology

Two crystal structures of the hammerhead ribozyme provide the first atomic-resolution views of an RNA active site, and suggest that the catalytic center may reside in a U-turn motif which was first seen in tRNA^{Phe}.

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RNA catalysts — ribozymes — have intrigued evolutionary biologists and enzymologists since their initial discovery over a decade ago [1,2]. How can a relatively simple polymer, constructed from only four nucleotide building blocks, provide the structural stability and active-site functional groups that are required of an enzyme? Is it possible that the RNA components of such fundamental cellular machinery as ribosomes and spliceosomes are also catalytic? Could ribozymes be designed to cleave specific cellular sequences, thereby making them analogous to restriction enzymes? Although numerous laboratories have used biochemical experiments to explore these questions, the structural basis for RNA catalysis has remained largely unknown. Even though atomic-resolution structures of both DNA and proteins have accrued rapidly in the past two decades, the RNA structures that have been determined have been limited to those of tRNA, simple duplexes, hairpins and pseudoknots. Thus, the recently determined crystal structures of two hammerhead ribozymes, at 2.6 Å [3] and 3.1 Å [4] resolution, respectively, provide the first detailed views of an RNA active site and its structural underpinnings. This work marks the advent of an exciting new phase of ribozyme research in which the continuation of studies in structural biology should offer further major advances in our understanding of RNA architecture and may even facilitate ribozyme engineering for therapeutic purposes.

The hammerhead motif is a self-cleaving RNA structure that is found in small plant-pathogenic RNAs, and which is probably involved in the processing of multimeric genomes during rolling-circle replication [5,6]. Unlike ribozymes such as self-splicing introns and the catalytic RNA subunit of ribonuclease P, the hammerhead motif is small, consisting of three helical regions flanking a core of phylogenetically conserved nucleotides [7–9]. Auto-catalytic 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 changed into a multiple-turnover enzyme by separating the strand containing the cleavage site from the rest of the core [8,9]. In this way, hammerhead ribozymes have been designed to cleave specific target and cellular sequences *in vivo* (see [10] for review). These designs have also proved useful for

crystallization by allowing substitution of the substrate strand with an all-DNA strand ([3]; see Fig. 1a) or with an RNA strand modified at the cleavage site by a 2'-O-methyl group ([4]; construct shown in Fig. 1b). These changes do not interfere with substrate binding, but do prevent cleavage during the crystallization process. The crystal structures of the hammerhead ribozyme answer some important questions about the role of its conserved nucleotides in creating a finely tuned catalytic center containing coordinated magnesium ions. They also open the way for further investigations of hammerhead ribozyme catalysis by making specific predictions about the reaction mechanism.

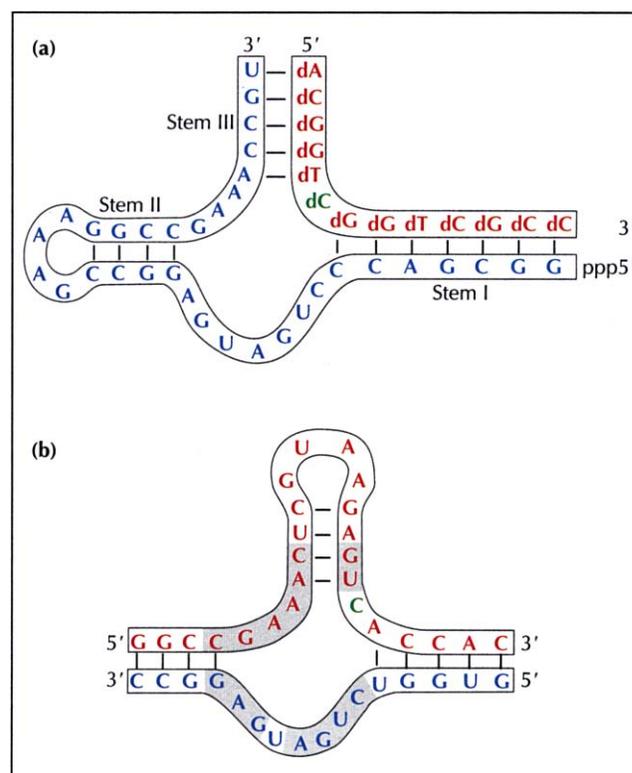


Fig. 1. Secondary structures of the hammerhead motif. In each case the nucleotide at the cleavage site is shown in green, the substrate strand in red and the remainder of the ribozyme in blue. (a) DNA-RNA hybrid construct used in the structure determination by Pley *et al.* [3]. (b) All-RNA construct used in the structure determination by Scott *et al.* [4]. Regions containing invariant residues are shaded gray.

RNA conformation

In three dimensions, the hammerhead is shaped like a wishbone, or γ , with Stems I and II forming the arms and Stem III and the core at the base (Fig. 2a) [3,4]. This conformation is seen in both structural determinations despite their different 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 phylogenetically conserved nucleotide bases. Two G·A base pairs and an A·U base pair are sandwiched between Stems II and III, forming one long pseudo-continuous helix from which Stem I and the catalytic site emanate (Fig. 2b). The highly conserved sequence CUGA between Stems I and II then forms a tight turn. Furthermore, the uridine turn (U-turn) seen previously in the anticodon loop of the yeast phenylalanine tRNA (tRNA^{Phe}) X-ray crystal structure [11,12] is identical in sequence and structure to this CUGA turn [3]. The cytosine that is found at the cleavage site between Stems I and III is positioned near the CUGA cleft by interactions with the cytosine and adenine of the CUGA motif. This proximity, along with the ability of the tRNA^{Phe} U-turn to bind metals such as Mg²⁺ and Pb²⁺, has led Scott *et al.* [4] to propose that the CUGA turn of the hammerhead ribozyme (called domain I by Pley *et al.* [3], and the U-turn hereafter) constitutes the catalytic pocket.

The observed structures are consistent with results obtained by other techniques which were used to investigate the shape of the hammerhead ribozyme in solution. Tuschl *et al.* [13], for example, used fluorescence resonance energy transfer data to build a three-dimensional (3D) model of the hammerhead ribozyme in which Stems I and II are parallel and point away from Stem III. Bassi *et al.* [14] proposed a similar model based on gel electrophoresis and uranyl photocleavage experiments. Amiri and Hagerman [15], using gel electrophoresis and transient electric birefringence, determined that the three stems are roughly coplanar and do not rearrange significantly after cleavage. Further analysis of the solution structure of the hammerhead motif, including the use of NMR spectroscopy, will be necessary to elucidate details of the hammerhead ribozyme's cleavage mechanism.

Importance of being ribose

RNA often forms complex 3D structures, whereas DNA exists primarily as a double helix. An appealing explanation for this difference in structural propensities is that the 2'-hydroxyl groups on RNA may provide a unique means of tethering large RNA structures by virtue of their hydrogen-bonding capacity and accessibility. Indeed, the hammerhead ribozyme structures reveal several 2'-hydroxyl contacts between conserved bases in Stem III and the guanine and adenine of the CUGA turn. This network of interactions explains, in part, why an all-DNA substrate, with a single ribonucleotide at the cleavage site, is 100-fold less reactive than an analogous all-RNA substrate. Pley *et al.* [16] also observed a series of 2'-hydroxyl

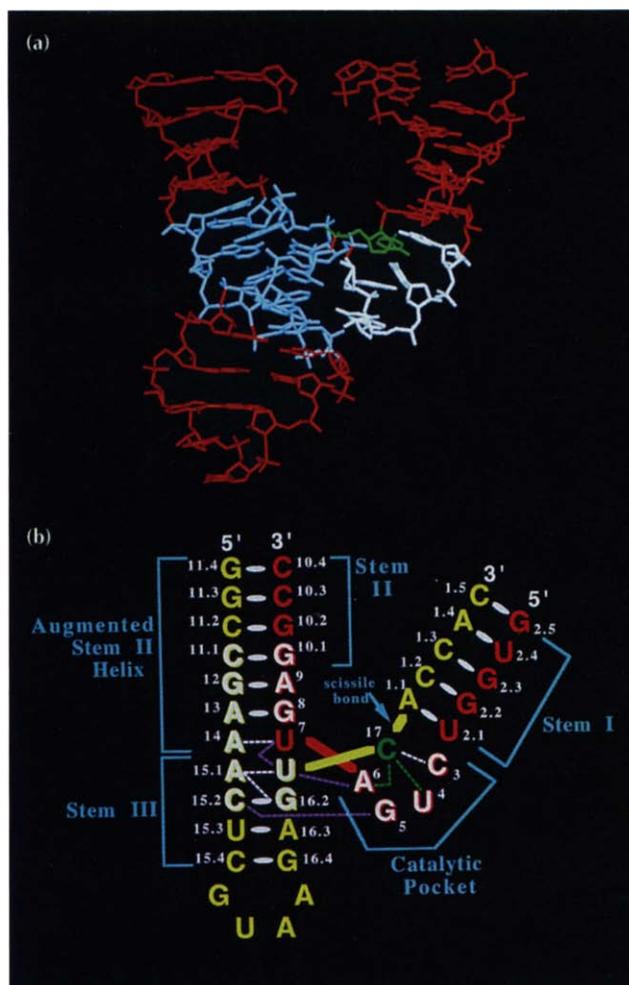


Fig. 2. Wishbone RNA. (a) Representation of the structure as drawn by Scott *et al.* [4], with the cleavage-site nucleotide (green) in the cleft created by the CUGA turn. The CUGA uridine turn is shown in white; the three-base pair augmenting helix joining Stem II to Stem III is shown in cyan; and the other helical residues are shown in red. (b) Diagram of the RNA backbone connectivity. The enzyme strand is shown in red and the substrate strand in yellow with the active-site cytosine in green. (Reproduced from [4], with permission.)

contacts between the GAAA loop of Stem II and the minor groove of Stem II of another molecule in the crystal lattice. These interactions correlate with the specificity of GNRA loops (where N is any nucleotide and R is a purine nucleotide) for sites on RNA duplexes, which was proposed by Michel and Westhof [17]. GNRA and other stable 'tetraloop' sequences occur frequently in ribozymes and rRNAs, suggesting that this mode of loop-helix docking may be a common tertiary structural motif in large, structured RNAs.

Divalent metal binding sites

The hammerhead and other ribozymes require divalent metals such as magnesium or manganese for activity, and biochemical experiments suggest these ions have both structural and catalytic roles (for review, see [18]). The hammerhead ribozyme crystals analyzed by Pley *et al.* [3] grew in high concentrations of monovalent salt, that is, in

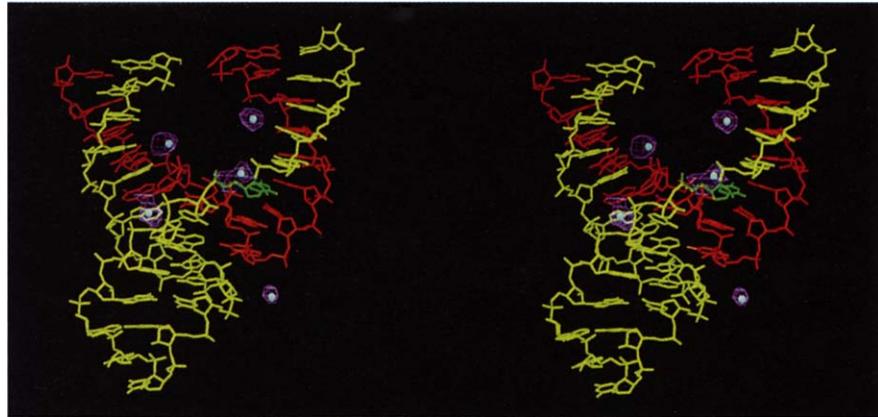


Fig. 3. Stereoview of the five proposed metal-binding sites (magenta) in the Scott *et al.* [4] structure. The enzyme and substrate-analog strands are shown in red and yellow, respectively, with the active-site base shown in green. One of these binding sites was also observed in the Pley *et al.* [3] structure.

conditions which prevent most divalent metals from binding. To circumvent this problem, native crystals were soaked in solutions of manganese or cadmium and anomalous scattering data were collected. This analysis revealed a metal-coordination site formed by the two adjacent G·A base pairs located between Stems II and III. The metal ion fits between the N7 of one of the guanines and this base's neighboring phosphate, consistent with results from previous thiophosphate interference and nucleotide substitution experiments. Interestingly, the same sequence of two consecutive G·A mismatches has been found near the active sites of a Pb^{2+} -dependent self-cleaving ribozyme and of ribozymes that catalyze RNA ligation [19,20]. Thus, this motif may turn out to be a common way of positioning divalent metals within an RNA structure.

Scott *et al.* [4], working with data from crystals grown in low-salt concentrations, interpreted five difference Fourier peaks as being possible hydrated magnesium ions, on the basis of coordination geometry and distances to nearest-neighbour functional groups. One of these corresponds to the site described above. A second site occurs in the proposed catalytic pocket, with possible coordination by the exocyclic amines of the cytosine of the U-turn and the cytosine at the cleavage site.

A catalytic mechanism?

While the crystal structures of the hammerhead ribozyme provide a detailed view of its catalytic center, questions about the reaction mechanism still remain. One challenge is to explain the 'in-line' attack by the 2'-hydroxyl at the cleavage site that has been implicated by biochemical experiments (see earlier discussion). In the Pley *et al.* [3] crystal structure, the nucleotide at the cleavage site is not positioned correctly for an in-line mechanism, possibly because of the absence of the attacking 2'-hydroxyl in the DNA substrate analog. The authors proposed that, during catalysis, a conformational change may occur which repositions the nucleotide at the cleavage site [3]. The magnesium ion that is coordinated near the proposed catalytic pocket in the Scott *et al.* [4] structure is just beyond striking distance of the scissile phosphodiester bond. Again, this could reflect the ground-state conformation of the native hammerhead ribozyme or it could result from having a 2'-O-methyl rather than a 2'-hydroxyl

group at the cleavage site in the substrate strand. On the basis of comparisons of the metal site in their structure with those of the Mg-bound and Pb-bound U-turns in tRNA^{Phe}, Scott *et al.* [4] suggest that, in the native conformation, the magnesium ion can move 3.5 Å closer to the 2'-hydroxyl that is adjacent to the scissile bond, a move facilitated by rotation of the glycosidic bond of the cleavage-site cytosine. These hypotheses will be explored by further structural and functional analyses of the hammerhead ribozyme as the precision of the current structures prevents detailed analyses of hydrogen bonding and other stereochemical effects.

The determination of the crystal structures of two hammerhead ribozymes greatly advances current understanding of RNA architecture and will facilitate future experimental design. One exciting application of this structural information involves the design of hammerhead ribozymes for therapeutic purposes. Research on this front has focused on engineering hammerhead ribozymes that are physiologically stable and have the ability to cleave target RNAs *in vivo*. The hammerhead ribozyme structure now provides a framework for testing nuclease-resistant derivatives of RNA and for optimizing this ribozyme for multiple-turnover reactions.

The solution of these structures marks the beginning of a new era in RNA research. Improved RNA synthesis and crystallization methods, in addition to progress in NMR solution-structure determinations of RNA, ensure that the next few years will be an exciting time for RNA structural biology.

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Jennifer A Doudna, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06522, USA.