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Molecular palaeontology: understanding catalytic mechanisms in the RNA world by excavating clues from a ribozyme three-dimensional structure

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Ribozymes and the RNA world

The discovery in the last decade that RNA can act as an enzyme refuted one of the main tenets of the so-called central dogma of molecular biology, i.e. that all enzymes must be proteins. The finding that RNA possesses catalytic activity, and in some cases can act as a true enzyme, has inspired research efforts dedicated not only to understanding the mechanisms of RNA-catalysed cleavage and ligation reactions [1,2], but also to developing ribozyme-based therapies targeted against RNA viruses and oncogene mRNA [3].

The discovery that RNA can be an enzyme also provides an escape from a paradox posed by the central dogma: if nucleic acids contain the heritable genetic information required for protein synthesis, and if proteins are required for nucleic acid synthesis and replication, which evolved first? One possible answer is that RNA in principle can function both as a heritable information-encoding molecule and as an enzyme. Life therefore may have evolved from molecules, or assemblies of molecules, in a prebiotic 'RNA world' populated by self-replicating RNAs [4]. Indeed, the development of experimental techniques which allow *in vitro* selection and evolution of RNA sequences possessing desired catalytic traits by screening randomized RNA libraries offers the exciting prospect of discovering RNAs that can replicate themselves efficiently [5]. Although such a discovery would not prove the existence of a prebiotic RNA world, it would clearly demonstrate that such a hypothesis of evolution is reasonable.

Much of our understanding of the details of physicochemical mechanisms and function of protein enzymes is based upon our knowledge of the three-dimensional structures of these macromolecules obtained by high-resolution techniques such as X-ray crystallography and NMR. In much the same way that three-dimensional structures of protein enzymes have been of paramount importance for understanding classical enzymes, so too would the three-dimensional structures of RNA enzymes influence our understanding of ribozyme catalysis, both in the current biological world and in a prebiotic RNA

world. However, since RNA enzymes were discovered relatively recently, comparatively little is known about the relationship between structure, function and catalytic mechanism.

A ribozyme three-dimensional structure

Recently, however, the three-dimensional structure of a relatively simple ribozyme, the hammerhead RNA, has been elucidated [6,7]. The hammerhead RNA is a small catalytic RNA motif found in a number of RNA virus genomes and virus-like RNAs [8]. Two research groups using different approaches determined the crystal structure of this ribozyme. The first structure was that of a hammerhead ribozyme in which the catalytic, or 'enzyme', strand was composed of RNA, and the RNA substrate was replaced with a 'substrate analogue' strand composed of DNA [6]. The DNA strand was employed as a competitive inhibitor to prevent catalytic cleavage. The second hammerhead ribozyme structure was composed entirely of RNA with a single 2′-methoxy modification at the active site to prevent cleavage [7]. Despite superficial differences, the largely conserved catalytic core region of both ribozyme structures is quite similar.

The hammerhead RNA secondary-structural motif consists of three base-paired stems flanking a central core of 15 conserved nucleotides [8] (Figure 1*a*). The conserved central bases are essential for ribozyme activity. Most of these conserved bases cannot form conventional Watson–Crick base-pairs, but instead form more complex structures which mediate RNA folding and catalysis. The hammerhead RNA, like other naturally occurring ribozymes, is a metalloenzyme and requires a bivalent metal ion, such as Mg^{2+}, to mediate catalytic cleavage. The bivalent metal ion is thought to be hydrated, and becomes active when it binds to the RNA and becomes ionized, i.e. the active form is an RNA-bound metal hydroxide which acts by abstracting a proton from the 2′-hydroxy group at the cleavage site. The rate of bivalent-metal-ion-assisted catalytic cleavage generally increases with decreasing pK_a of the metal hydroxide, strongly suggesting that the active species is indeed a metal hydroxide [9,10].

Global conformation of the hammerhead ribozyme

The overall fold of the all-RNA hammerhead ribozyme is roughly γ-shaped, with Stem III and its tetraloop forming the apex from which Stem I and Stem II bifurcate. The hammerhead RNA is depicted in Figure 1(*b*). The conformation of the all-RNA hammerhead ribozyme is very similar to that found for the RNA–DNA hybrid ribozyme [7]. Stem II and Stem III are essentially coaxial, with Stem I and the catalytic pocket branching away from this axis. Stem II together with two GA base-pairs and an AU base-pair form an 'augmented' Stem II helix; Stem II forms a standard A-form RNA double helix, and these three additional base-pairs extend or augment the helix of Stem II. The augmented Stem II helix stacks directly upon the helix of Stem III, forming one long pseudo-continuous distorted A-form helix. Stacking of the augmented Stem II helix upon the Stem III helix forces C^{17}, which joins Stem III to Stem I, outwards to stack upon the end of Stem I itself, and concomitantly into close proximity to the conserved $C^3U^4G^5A^6$ turn.

The three augmenting helical base-pairs of Stem II consist of two absolutely conserved reversed-Hoogsteen GA base-pairs (G^{12}–A^9 and A^{13}–G^8) followed by a singly hydrogen-bonded AU base-pair (A^{14}–U^7). Although A^{14} is absolutely conserved, the U may be replaced with C, G or A and the ribozyme will still maintain some catalytic activity. This AU pair of the augmented Stem II helix in turn stacks upon the absolutely conserved AU base-pair of Stem III ($A^{15.1}$–$U^{16.1}$). Together with a second semi-conserved Watson–Crick base-pair ($G^{15.2}$–$C^{16.2}$) in Stem III, these base-pairs form an imperfect helical duplex region of continuously stacked bases joining Stem II to Stem III. The role of the conserved bases of the augmented Stem II helix as it stacks upon the conserved bases of Stem III is to force the cleavable nucleotide into the catalytic pocket of the hammerhead ribozyme.

The conserved base-pairs in Stem III ($A^{15.1}$–$U^{16.1}$ and $C^{15.2}$–$G^{16.2}$) lie adjacent to the active-site C^{17} and are stacked upon the A^{14}–U^7 non-Watson–Crick base-pair of the abutting augmented Stem II helix. U^7 stacks upon $U^{16.1}$ of Stem III, but the divergent phosphate backbone paths at the three-strand junction are the consequence of different helical conformations in the augmented Stem II helix and the abutting Stem III. The unusual structure of the $A^{15.1}$–$U^{16.1}$ pair in Stem III locally broadens the minor groove relative to a standard A-form RNA helix, whereas the unusual A^{14}–U^7 pair narrows it. Together, these two rather different non-canonical AU

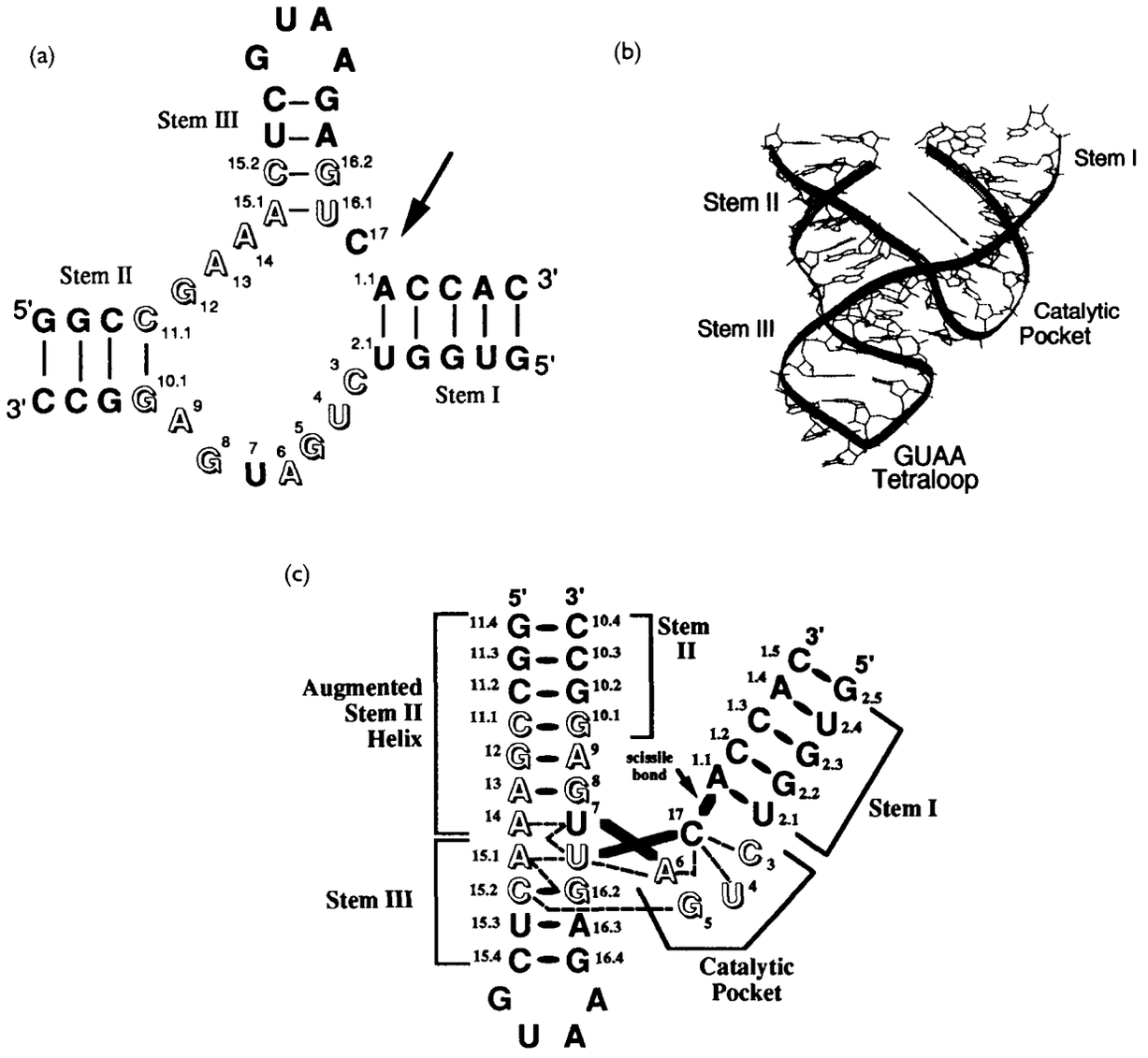
base-pairs therefore act to accommodate the three-stranded junction where the phosphodiester backbone chains leaving Stem III and the augmented Stem II helix in turn continue into the active-site cytosine and the catalytic pocket respectively, and then both finally come together to form Stem I. These aspects of the structure are represented schematically in Figure 1(*c*).

This four-nucleotide uridine turn, or catalytic pocket, is identical in sequence and conformation with the so-called uridine turn found in the anticodon loop of $tRNA^{Phe}$. An important

Figure 1

Structure of hammerhead RNA

(*a*) The sequence and predicted secondary structure of the hammerhead RNA used to determine the three-dimensional structure reported here. The letters outlined are absolutely or highly conserved in all hammerhead RNAs. Those that do not form conventional base-pairs are involved in mediating the three-dimensional structure of the hammerhead RNA necessary for catalysing the self-cleavage reaction. The arrow denotes the self-cleavage site. (*b*) The three-dimensional structure of the hammerhead RNA sequence shown in (*a*). The ribbons trace through the phosphate backbones of the two strands and highlight the global fold of the hammerhead RNA. (*c*) A schematic diagram which shows the structural architecture of the hammerhead RNA, corresponding to (*a*) and (*b*). Stems I, II and III as well as the catalytic pocket formed by the uridine turn and C^{17} are labelled, as is the augmented Stem II helix, as described in the text. Note that the augmented Stem II helix stacks directly upon Stem III, forming a pseudo-continuous long helix. Watson–Crick and reversed-Hoogsteen bases are denoted with black ovals. Single hydrogen bonds between non-Watson–Crick bases are shown as dashed lines, as are single hydrogen bonds between bases and backbone riboses and the two aromatic stabilization interactions between C^{17} and the uridine turn of the catalytic pocket. The scissile bond between C^{17} and $A^{1.1}$ is marked with an arrow.



structural feature of the catalytic pocket is the presence of π–aromatic interactions which stabilize the enzyme–substrate interaction. In addition to the hydrogen bond which stabilizes the active-site cytosine, two π–aromatic interactions in the catalytic pocket between the active-site base and the conserved uridine turn add additional stabilization to C^{17} [7]. One of these aromatic interactions is the perpendicular stacking interaction between the furanose oxygen of C^{17} and the platform formed by the base of A^6. This interaction is reminiscent of that described in Z-DNA between the guanine base in the *syn* position which stacks on the furanose oxygen of the adjacent cytosine. The other is a stacking interaction between the base of C^{17} and the exocyclic oxygen at position 2 of U^4 in the uridine turn, which makes a 3 Å aromatic–nπ stabilizing interaction. These interactions are significant not only because they can in principle stabilize any base at the active site, but also because they may allow sufficient flexibility to stabilize the active-site base throughout the catalytic reaction pathway, a point invoked in formulating a proposed cleavage mechanism.

A structure-based proposal for the ribozyme catalytic mechanism

Difference Fourier maps reveal a $Mg(H_2O)_6^{2+}$ complex ion found near the catalytic pocket which can make hydrogen-bonding contacts with the exocyclic amines on C^3 in the catalytic pocket, and on C^{17}, the cleavage-site nucleotide [7]. In addition, the structurally analogous $tRNA^{Phe}$ uridine turn binds bivalent metal ions such as Mg^{2+} and Pb^{2+} [11,12], suggesting that the catalytic pocket in the hammerhead ribozyme is also capable of binding the catalytically active bivalent metal ion. The cytosine corresponding to C^3 in the hammerhead RNA makes similar contacts with hydrated metal ions in the $tRNA^{Phe}$ uridine turn.

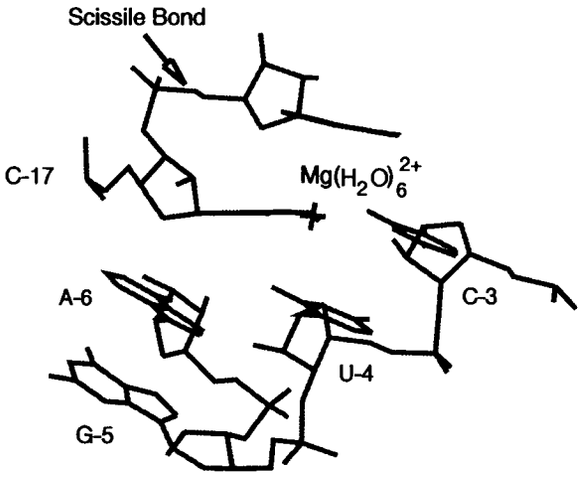
Based upon these observations, a mechanism in which the $Mg(H_2O)_6^{2+}$ complex ion first 'docks' in the catalytic pocket by interacting with C^3 and C^{17} as described above may be proposed. Independent experimental corroboration for this initial interaction has recently emerged; removal of the exocyclic amine from either C^3 or C^{17} causes the dissociation constant for the catalytic $Mg(H_2O)_6^{2+}$ to increase by almost an order of magnitude [13]. Although both hammerhead RNA crystal structures have C at position 17, this C may be replaced with A or U. A^{17} works almost as well as C^{17}, but the activity with U^{17} is somewhat reduced [14]. Note that essentially the same 'docking' interaction could still take place with A, where the $Mg(H_2O)_6^{2+}$ complex ion now interacts with the exocyclic amines on A^{17} and C^3, but the analogous interaction would be somewhat weaker in the case of U^{17} which lacks an exocyclic amine, and thus could explain the observation that A replaces C at position 17 more effectively than does U.

According to this proposed mechanism [7], the metal complex ion is then drawn in towards the cleavage site 2′-hydroxy group until it is within striking distance. (The trajectory and final position of the complex ion are both inferred from the metal positions in the uridine turn of $tRNA^{Phe}$.) As the metal is positioned, one of the six water molecules bound to the metal ion is displaced by the *pro*-R phosphate oxygen at the cleavage site, and direct co-ordination with this phosphate oxygen assists in orienting and perhaps in ionizing one of the remaining water molecules which is now close to the 2′-hydroxy group, i.e. binding the phosphate oxygen may lower the effective pK_a of the hydrated Mg^{2+} ion, thus activating it. Loss of a proton generates a metal hydroxide nucleophile which in turn will act by abstracting the 2′-hydroxy proton from the cleavage-site ribose, initiating nucleophilic attack at the phosphorus and formation of the pentacoordinated 2′,3′-cyclic phosphate transition state or intermediate (Figure 2).

As mentioned, substitution of C^{17} by adenosine or uridine at the active site maintains a

Figure 2

Illustration of the approach of the catalytic hydrated Mg^{2+} ion in the ribozyme catalytic pocket



functional ribozyme, although U^{17} functions less well than A^{17} and C^{17} [14]. This fact is accounted for in both stages of the proposed mechanism. In the first step, the interaction with the exocyclic amine could still take place with A, but the analogous interaction would be somewhat weaker in the case of U^{17} which lacks an exocyclic amine. In the second step, the base itself of the cleavage-site nucleotide stacks upon A^6 in the catalytic pocket. Such a stabilization interaction may also take place with adenosine or uridine substituting for cytidine at the cleavage site.

Enzyme catalysis in the RNA world

Whether the hammerhead RNA and other modern ribozymes are molecular fossils, bypassed by the evolution of protein enzymes, or are highly evolved and adapted biological catalysts, preserved by evolution because of their superiority as catalysts in the context of nucleic acid biochemistry, an understanding of the relationship between structure, function and catalytic mechanism in RNA enzymes will enhance our understanding of how catalysts may have functioned in a prebiotic RNA world. In particular, the hammerhead ribozyme catalyses a reaction which, though simplistic compared with RNA self-replication, is of fundamental importance, i.e. RNA oligonucleotide cleavage and ligation via metal-mediated phosphodiester isomerization. Thus the three-dimensional structure and cleavage mechanism of the hammerhead RNA gives us the first evanescent glimpse (at molecular resolution) into the distant past of a possible prebiotic RNA world.

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Hairpin ribozyme: current status and future prospects

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Introduction

In this paper, I present a brief overview of a fascinating small RNA enzyme, the hairpin ribozyme. I will then comment on the prospects for future progress in the areas of basic ribozyme biochemistry and targeted RNA inactivation. Since its discovery in 1989 [1–3], the hairpin ribozyme has been the subject of increasingly intense experimental analysis. Contributions from a number of research groups have led to a sound understanding of basic structure–function relationships and prospects for use of engineered ribozymes as highly specific endoribonucleases to achieve targeted RNA cleavage for basic science and, potentially, therapeutic applications.

Abbreviation used: sTRSV RNA, satellite tobacco ringspot virus RNA.

Reaction

The hairpin ribozyme acts as a reversible endoribonuclease (Figure 1). It uses single-stranded RNA as its substrate, cleaves via a transesterification reaction pathway using the 2′-hydroxy