Structure–function relationships of hammerhead ribozymes: from understanding to applications

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The hammerhead ribozyme is the smallest member of the naturally occurring family of RNA molecules that are capable of catalysing the site-specific cleavage of RNA. Functional-group modifications have led to an identification of groups that are important for catalysis, and have helped in the understanding of the role of Mg^{2+}, which is required for catalysis. Recent studies on the three-dimensional structure of the hammerhead ribozyme, including X-ray analysis, have contributed significantly towards an understanding of its mode of action. In addition to contributing to our understanding of RNA catalysis, these studies have also stimulated investigations into the possibility of using ribozymes in gene therapy to cleave specific mRNAs.

The hammerhead ribozyme is an RNA motif capable of the magnesium-dependent site-specific cleavage of RNA, and is utilized by a number of small pathogenic plant-viroid and satellite RNAs for processing during rolling-cycle replication[1]. The hammerhead consists of three helices (Fig. 1), connected by single-stranded regions. Cleavage by the hammerhead ribozyme requires a specific set of three nucleotides at the cleavage site; GUC is the triplet that is most efficiently cleaved (Fig. 1). Mutagenesis experiments have revealed that most of the base pairs in the helices can be altered without reducing the activity of the ribozyme. Conversely, substitution of residues, with the exception of U7, in the single-stranded regions of the catalytic core, dramatically reduces the activity.

The relatively small size of the hammerhead ribozyme, coupled with the rapid advances in automated oligoribonucleotide synthesis over the past decade, has stimulated studies on hammerhead structure–function relationships. Furthermore, the availability of phosphoramidites of modified nucleosides has enabled functional-group modifications in the hammerhead ribozyme that have led to an identification of the functional groups that are important for catalysis. This article focuses on the functional-group modifications in the sugar–phosphate backbone that have shed light on the mechanism of cleavage and led to the development of nuclease-resistant ribozymes. The applications of hammerhead ribozymes, and a summary of recent developments in the quest for a tertiary structure are also presented.

The mechanism and the role of Mg^{2+}

The hammerhead-promoted cleavage of RNA occurs via a nucleophilic attack on the scissile phosphate by the adjacent 2′-hydroxyl group, generating a 5′-hydroxyl group and a 2′,3′-cyclic phosphate (Fig. 2). Valuable information on the stereochemistry of cleavage and the role of Mg^{2+} in catalysis has been obtained by the introduction of phosphorothioates (phosphodiesters in which a non-bridging oxygen has been replaced by sulphur) at the cleavage site. A phosphorothioate with an R_p configuration* dramatically reduces the rate of Mg^{2+}-dependent cleavage; this can be partially restored by changing the metal ion to Mn^{2+} (Refs 2–4). However, a substrate containing a phosphorothioate with the S_p configuration* is cleaved in the presence of Mg^{2+} (Refs 4,5). These results are consistent with a catalytically essential Mg^{2+} coordinating to the pro-R_p oxygen of the internucleotidic linkage, because Mg^{2+} has a much lower affinity for sulphur than oxygen, whereas Mn^{2+} has a similar affinity for both. The phosphorothioate studies also revealed that

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*R_p and S_p refer to the configuration of the phosphorus atom according to the Cahn–Ingold–Prelog system.

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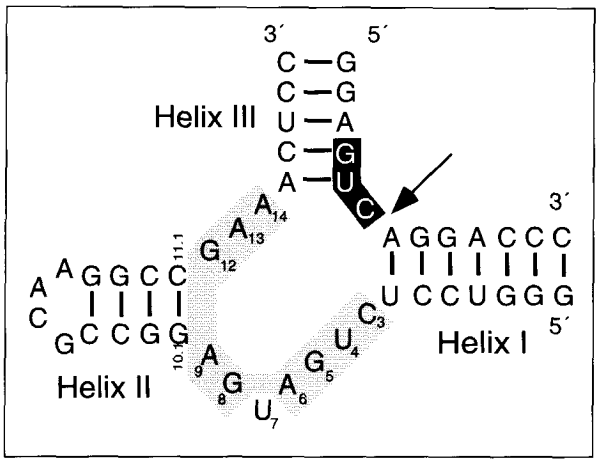

Figure 1
Secondary structure of a hammerhead ribozyme. The conserved residues that are essential for activity are shaded in grey. The GUC cleavage triplet is indicated in black, and the arrow indicates the site of cleavage.

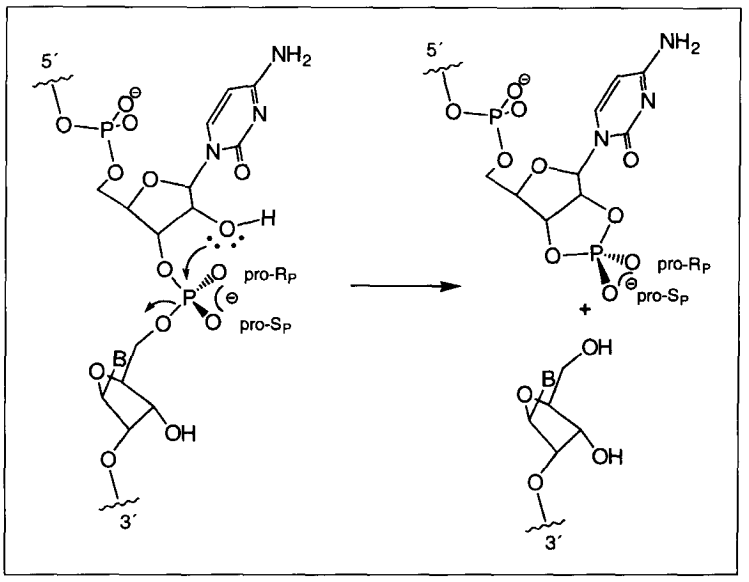

Figure 2
Mechanism and stereochemistry of hammerhead cleavage. R_p and S_p refer to the configuration of the phosphorus atom according to the Cahn–Ingold–Prelog system.

the reaction proceeds with inversion of configuration at phosphorus[2,4]. It has been suggested that magnesium hydroxide (a hydrated Mg^{2+}) not only coordinates to the phosphate, but also acts as a base to promote cleavage[5]; this model was supported by the pH dependence of the reaction[6]. However, based on a comparison of the rates of the hammerhead cleavage reaction in H_2O and D_2O, it has subsequently been postulated that proton transfer does not take place in the rate-determining step[7]. It was also suggested that one Mg^{2+} is directly coordinated to the 2′-oxygen, promoting attack on the phosphorus, while a second Mg^{2+} facilitates the departure of the 5′-oxygen[7].

Apart from playing a direct role in the cleavage reaction, metal ions are are also important for promoting the folding of the hammerhead into its active conformation. For example, the Mg^{2+} concentration required for efficient cleavage can be reduced by the addition of spermine, which is a polyvalent organic cation under physiological conditions[3]. This suggests that a good proportion of the Mg^{2+} required for cleavage is involved in stabilizing tertiary interactions, presumably by reducing the electrostatic repulsion of the negatively charged sugar–phosphate backbones. In addition, Zn^{2+} and Cd^{2+} can only promote cleavage in the presence of spermine, indicating that they can participate in the cleavage reaction, but not in folding of the ribozyme into an active structure.

Importance of the helix-II loop for activity

Helix II is the only helix in the hammerhead ribozyme that is not directly involved in substrate binding, and several studies have been directed towards determining its importance for catalysis. In a systematic study in which the length and base composition of helix II was varied, it was established that the minimal length of the helix was two base pairs with a requirement for a C11.1–G10.1 base pair for maximal activity[8] (Fig. 1). A further reduction in the length of helix II resulted in decreased activity, which originated from the cleavage step rather than from interference with substrate binding. Several groups have replaced the tetranucleotide loop of helix II with polyethylene glycol spacers[9–11], and with abasic nucleotides[12]; this was accomplished without a loss of catalytic activity when at least two base pairs of helix II were retained. These results indicate that the first two base pairs of the helix-II loop are essential for activity, and probably promote the folding of the catalytic core, and/or to stabilize the tertiary structure.

Importance of 2′-hydroxyl groups for activity

It seems obvious that 2′-hydroxyl groups play a key role in ribozyme function, since it is already known that RNA is richer in both structural and functional variation than DNA. The role of the 2′-hydroxyl group has been ascribed to two factors: hydrogen bonding, including possible coordination to Mg^{2+}; and affecting the conformation of the sugar residues. It is possible to distinguish between these effects by a judicious choice of modified residues. For example, 2′-fluoro-modified residues adopt the same conformation as the unmodified ribose, but the fluorine can only act as a hydrogen-bonding acceptor[13]. The activity of ribozymes with this particular modification, as well as with 2′-deoxynucleotide, 2′-alkoxynucleotide, and 2′-aminonucleotide modifications, led to the conclusion that only the 2′-hydroxyl groups of residues G5 and G8 are critical for activity[14,15].

Tertiary structure of the hammerhead ribozyme

The eagerly awaited tertiary structure of the hammerhead ribozyme, based on single-crystal X-ray crystallography, was recently reported[16]. The ribozyme has a wishbone shape, with helices II and III almost colinear and helix I and II close together (Fig. 3). The residues C3–A6 form a U-turn in the X-ray structure;

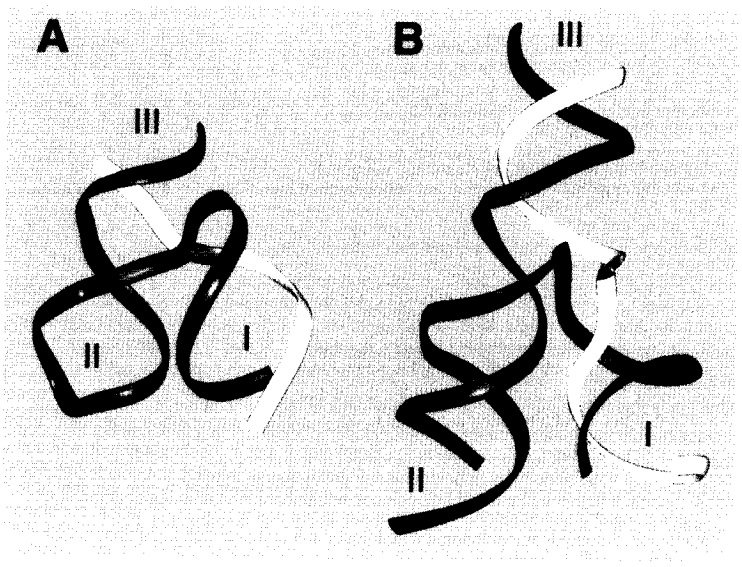

Figure 3
A ribbon representation of the tertiary structure of the hammerhead ribozyme as determined by (**A**) X-ray crystallography, and (**B**) fluorescence resonance energy transfer (FRET). The ribozyme is in blue, substrate in yellow, single-stranded regions of the ribozyme are in red, and the cytidine at the cleavage site in pink.

this motif is also found in the anticodon loop and uridine loop of $tRNA^{Phe}$. The results of most functional-group modifications were consistent with the X-ray structure[16,17]. However, the 2′-hydroxyl required at the cleavage site was not suitably positioned for an in-line attack on the phosphorus, and no metal ion was found close to the cleavage site[16]. These findings raise the frequently asked question: is the structure displayed in the crystal a ground-state structure that bears limited resemblance to the transition-state structure?

Fluorescence resonance energy transfer (FRET) measurements in solution that were carried out in parallel, revealed the relative orientation of the three helices, and allowed the central core to be modelled[18]. A comparison of the FRET model and the X-ray structure revealed striking similarities with regard to the placement of the helices in three-dimensional space (Fig. 3). This global shape has also been supported by electrophoretic mobility studies[19], and by measuring the transient electric bi-refringence of the hammerhead ribozyme[20]. However, the relative orientation of helices I and II in the X-ray structure and the FRET model are different. Qualitatively, when the minor grooves of helices I and II face each other in the X-ray structure, at the same point of reference in the FRET model, the major grooves face each other. It is clear that more experiments will have to be performed in order to assess the catalytic competence of the two models.

Applications of hammerhead ribozymes

The potential application of ribozymes to gene therapy, where a messenger RNA encoding a harmful protein would be intercepted and cleaved, is a fruitful area of research[21]. A major limitation of this approach is the instability of RNA in serum, due to the presence of ribonucleases. The cleavage of RNA by ribonucleases requires a 2′-hydroxyl group at the cleavage sites, so functional-group modifications at the 2′-site have been shown to render RNAs resistant to ribonuclease cleavage. For example, substituting the pyrimidines of the hammerhead ribozyme with their 2′-fluoro derivatives (with the exception of positions U4 and U7 that were substituted with 2′-aminouridines), led to the stabilization of the ribozyme against degradation in serum by a factor of at least 10^3 without any loss of catalytic activity[13,22]. Modifications to the 2′-O-methyl-groups, 2′-O-allyl-groups[23] and 2′-deoxy-groups[24] have also been successfully used to make the hammerhead ribozyme nuclease resistant. Additional stability can be generated by the introduction of phosphorothioates[22] or 3′–3′ linkages at the 3′-end of ribozymes[25]; this confers stability to 3′-exonucleases.

Numerous examples exist in which the hammerhead ribozyme has been successfully used to interfere with gene expression. Most of these studies utilize the endogenous expression of ribozymes from their respective coding sequence carried on a plasmid[21]. Conversely, synthetic ribozymes must be delivered exogenously, so ribozymes need to pass through the cell membrane to exert their activity. This approach has been hampered by the inefficient cellular uptake of oligonucleotides, resulting in few examples of successful applications. However, carriers such as liposomes can improve this process, and might also give a degree of protection against nuclease degradation. For example, the 2′-fluoro-modified ribozymes have been successfully applied by liposome-aided delivery for the inhibition of expression of the multiple drug-resistance gene in a tumour cell line, and of the interleukin 6 (IL-6) gene in human fibroblasts[26,27].

There has been some concern that the catalytic efficiency of ribozyme-mediated cleavage of long RNAs might be too low for practical purposes[22]. Fortunately, some cellular proteins are capable of increasing this efficiency by facilitating the formation of the ribozyme–substrate complex, as well as increasing the rate of product release[28,29].

Future prospects

It has been demonstrated in this brief review how much we have learned about the hammerhead ribozyme in a relatively short period. Continued studies in this exciting area of research will undoubtedly increase our understanding of RNA catalysis and further the development of new RNA catalysts. For example, once the factors that govern the specificity of hammerhead cleavage have been determined, it may be possible to design new catalysts that efficiently cleave triplet sequences other than GUC. Furthermore, modifications of the hammerhead ribozyme that result in a more efficient cellular uptake of oligonucleotides will hopefully result in successful applications of hammerhead ribozymes for therapeutic purposes.

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