The hammerhead ribozyme
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Abstract
The hammerhead ribozyme is a small RNA motif consisting of three helices that intersect at a conserved core. When correctly folded, the hammerhead ribozyme stimulates nearly complete cleavage of the phosphodiester chain at a defined internal site to give 2',3'-cyclic and 5'-hydroxy termini. The cleavage rate is approx. 1 min⁻¹ at 25 °C, pH 7.5, and increases proportionally with hydroxide ion concentration between pH 5 and pH 9. As the rate of non-catalysed cleavage of RNA to form the same products is approx. 10⁻⁴ min⁻¹ under similar conditions, an important goal is to understand how the folded structure of this small ribozyme increases the rate of alkaline cleavage 10⁶-fold at this specific site.

The structure–function dilemma
X-ray crystal structures of two different hammerhead sequences appeared in the mid-1990s [1,2]. Despite different crystallization conditions and crystal packing arrangements, the two structures were virtually identical. The three helices form a 'Y', intersecting at an 11-nucleotide conserved core, which folds into two independent domains lying next to each other. Domain II consists of four non-canonical base pairs that connect the coaxial helices 2 and 3, whereas domain I contains the cleavage site and a four-base CUGA loop and connects helix I to the remainder of the molecule. In 1996, McKay reviewed a large number of papers that had measured the rate of cleavage of hammerheads containing a unique modified nucleotide and compared the results with the crystal structure [3]. Two conclusions emerged from this review. First, there were numerous inconsistencies between the crystal structure and the functional data in the sense that many nucleotide functional groups that are essential for cleavage either protrude into the solvent or interact with other functional groups that are not correspondingly essential. Secondly, the biochemical data were not always self-consistent in the sense that the same modification had different effects on the observed rate of cleavage when assayed in a different lab using a different hammerhead.

This second inconsistency could be resolved by considering more carefully the kinetics of the hammerhead cleavage reaction [4]. Hammerhead cleavage reactions are usually initiated by mixing two different oligonucleotides that form the hammerhead through annealing either helices 1 and 2 or helices 1 and 3. The oligomer that contains the cleavage site is called the substrate and the other oligomer is the ribozyme. Thus the reaction pathway involves an initial bimolecular assembly step, the chemical cleavage step and the sequential release of the two oligonucleotide products. If the experimental goal is to relate the crystal structure to the cleavage reaction, it is critical that the observed cleavage rate reflects the rate of chemical cleavage and not the rate of assembly or product release. It is usually straightforward to avoid rate-limiting product release by performing the reaction in excess ribozyme, so that only a single turnover occurs. However, avoiding rate-determining assembly can be complicated by the propensity of many short oligomers to form alternative self-structures that slow or prevent assembly. This 'alternative conformer hell' problem is best avoided by choosing 'well-behaved' hammerheads that do not form self-structures. When this is done, all phases of hammerhead kinetics can be understood as a combination of

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Abbreviations used: RP, ribozyme-product complex; RS, ribozyme-substrate complex.

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the kinetic properties of the appropriate RNA helices forming and dissociating, and the uniform properties of the catalytic core. Indeed, it is possible to accurately predict the kinetic properties of ‘well-behaved’ hammerheads with any set of helical arms [4].

In 2001, Blount examined all the available literature that report cleavage of modified hammerheads and only selected those results where the observed cleavage rate was likely to reflect the chemical cleavage step [5]. Although more consistent results were now observed for each modification, a considerable amount of the data still did not agree well with the crystal structure in the same ways as previously noted by McKay [3]. Of course, the interpretations of such structure–function experiments are complicated by the fact that any modified nucleotide often has several different properties from the parent nucleotide and it is not always possible to determine which property is responsible for the effect. For example, changing G5 in the hammerhead to I reduces cleavage substantially, even though the crystal structure shows the 2'-amino group (and the entire hydrophobic binding face) of G5 protruding into the solvent and, therefore, offers no clear explanation for the effect. Perhaps rather than ablating the hydrogen bond, the reduced stacking potential of I or a change in the local solvent structure could be responsible for the loss of activity. This opens the question of whether it is even possible to usefully interpret the activities of uniquely modified RNAs in terms of a crystal structure. Experiments with a substantial number of RNA–protein complexes [6,7] and several other ribozymes [8–10] indicate that correlations between structure and function are usually quite good. Thus the structure–function dilemma for the hammerhead must be regarded as an unusual situation.

Are bivalent cations the answer?

One possible resolution to the structure–function dilemma is that the unexplained essential functional groups are sites that are specific for the binding of bivalent cations essential for hammerhead cleavage. It is known that bivalent cations dramatically stimulate cleavage and at least six different bivalent ions have been located in the available X-ray crystal structure [11]. Since individual metal ions can only be identified by crystallography if they are present at high occupancy in well localized sites, there is a possibility that more specifically bound ions may be present at lower occupancy. This explanation is especially attractive since there is abundant precedence that nucleic acid transesterifications that are catalysed by protein enzymes utilize specifically bound bivalent ions, acting as either Lewis acids or bases [12]. Indeed, the hammerhead is often referred to as a metalloenzyme and numerous mechanisms involving one or more metal ions have been proposed [13–15].

Since RNA can bind a large number of bivalent cations through non-specific electrostatic interactions, deconvoluting the effects of a limited number of specifically bound cations involved in catalysis is a serious experimental challenge. It is also difficult to distinguish metal ions needed for the specific folding of the hammerhead from those directly involved in the catalytic mechanism. The most valuable approach to date has been to study the effects of thiophilic metal ions on the cleavage of hammerheads containing a single phosphorothioate residue. There are two sites in the hammerhead where introduction of a R–phosphorothioate inhibits cleavage dramatically, but the addition of a low concentration of Cd$^{2+}$ fully rescues cleavage [16,17]. At the scissile phosphate, P1.1, there is no observed effect on increased binding of the Cd$^{2+}$ to phosphorothioate, while at P9 (in domain II), the Cd$^{2+}$ binds slightly tighter to the phosphorothioate linkage, indicating a ground state effect. Quite satisfactorily, crystal structures show the expected direct co-ordination of a bivalent ion at both phosphates. Rescue experiments of a series of modified hammerheads that contain a phosphorothioate at P1.1 indicate that the metal ion bound to P9 is responsible for rescue [18]. Thus, in the transition state, a single bivalent ion bridges the two phosphates, despite the fact that P1.1 and P9 are far apart in the crystal structure. The role of this metal ion is presumably to position the scissile phosphate for cleavage and possibly to neutralize the non-bridging oxygen to allow more efficient attack by the 2' oxygen nucleophile.

It is striking that hammerhead cleavage can also be observed at high concentrations of numerous univalent cations at rates only slightly lower than those in Mg$^{2+}$ [19]. Since NH$_4^+$ is also effective, most cations must be used for electrostatic stabilization of the folded state and inner sphere co-ordination is not needed. However, unlike in Mg$^{2+}$ buffers, phosphorothioates at P1.1 and P9 do not reduce the cleavage rate in high concentrations of univalent ions [20]. This suggests that there is only one catalytically important bivalent ion that binds the hammerhead using
inner sphere co-ordination. This also implies that there are no additional bivalent ions specifically involved in cleavage chemistry, despite numerous proposals suggesting that they act as Lewis acids and/or bases. Finally, these results suggest that essential specific bivalent metal ion binding only partially resolves the structure–function dilemma.

**A transient conformational change**

NMR, gel mobility shift, transient electric birefringence, and fluorescent resonance energy transfer experiments suggest that the solution structure of the hammerhead closely resembles the crystal structure. How, then, can the structure–function dilemma be reconciled? A simple explanation is that a conformational change occurs before the cleavage reaction. Thus the predominant solution conformation, RS (ribozyme–substrate complex), converts a minor active conformation into RS* prior to cleavage to RP (ribozyme–product complex), where $K_{\text{conf}}$ is the equilibrium constant for the two conformers and $k_{\text{chem}}$ is the rate of the chemical reaction:

$$RS \xleftrightarrow{K_{\text{conf}}} RS^* \xrightarrow{k_{\text{chem}}} RP$$

This model would require RS and RS* to interconvert rapidly and the equilibrium would greatly favour RS. Indeed, NMR experiments suggest that the hammerhead core is dynamic in the millisecond time scale, unlike many RNAs of similar size and complexity [21]. Presumably the structure of RS* would be more consistent with unexplained chemical modification data and would allow close approach of P9 and P1.1. Since the observed cleavage rate, $k_{\text{obs}} = K_{\text{conf}} \cdot k_{\text{chem}}$, the model correctly predicts the existence of faster hammerheads where RS* is favoured [22,23].

If the above view of hammerhead catalysis is correct, an important goal is to learn the structure of RS*. In order to permit close approach of P1.1 and P9, Wang et al. [18] suggest that C3, G5 and A6 in domain I dock in some way onto domain II, but have not proposed molecular details. Based on modelling studies, Murray and Scott [24] claim that such a model is not structurally feasible. We have performed two types of experiments that attempt to better define RS*. The first involves adding sterically bulky groups at 2′-hydroxyl positions in the hammerhead where the crystal structure indicates that bulk can be accommodated and where a deoxynucleotide substitution has no effect on catalysis [25]. If the structure of RS* involves close approach of these 2′-hydroxyl groups to a different part of the hammerhead, the introduction of steric bulk would affect the rate of cleavage. The experiments show that, whereas quite large, bulky groups can be introduced throughout helices 1 and 2, there are three sites in domain 1 and three sites in domain 2 where even a small bulky group reduces the cleavage rate. This suggests that the minor groove side of domains 1 and 2 (where the 2′ groups are located) approaches other parts of the molecule in RS*. Since the crystal structure can accommodate steric bulk at these positions, it must differ from RS*.

A second approach is an attempt to use chemical cross-linking methods to stabilize RS* and thereby increase the cleavage rate. Using a variation of an existing cross-linking strategy [26], chemical cross-linkers of different lengths were used to join pairs of 2′-amino groups introduced at defined positions in the hammerhead. Hammerheads containing cross-links between 2′ positions 11.5 and 2.5 or 2.6, which are close in space and remote from the catalytic core, showed normal cleavage rates, but dramatically increased rates of ligation [27,28]. This can be explained by simply proposing that the cross-links reduce molecular motions available to the cleaved hammerhead, thereby allowing the reverse reaction to proceed more efficiently. Interestingly, for the 11.5–2.5 cross-link, ligation is only efficient when the linker is of sufficient length, suggesting that quite specific positioning is required for efficient ligation. Eight additional hammerhead cross-links, primarily between helices 1 and 2, showed varying effects of cleavage and ligation, but none showed faster cleavage. Thus we were unable to find a cross-linked hammerhead that stabilized RS*.

**References**

The Neurospora Varkud satellite ribozyme

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Abstract

Presented is a review of the discovery and characterization of the Neurospora Varkud satellite ribozyme. It outlines the approaches and observations that have led to our current level of understanding of the structure and function of this ribozyme, and highlights its distinctive features compared with other naturally occurring small ribozymes.

The discovery of Varkud satellite (VS) RNA

The discovery of the VS ribozyme was a classic case of serendipity that developed from our previous studies of the mitochondrial (mt) genomes of natural isolates of Neurospora. As is typical of fungi and other simple eukaryotes, the standard laboratory strains of Neurospora contain Group I introns in several of their mt genes. We, and others, had previously found that many natural isolates of Neurospora contained several introns not found in the laboratory strains, and some isolates contained plasmid DNAs that were not derived from the mt chromosome [1,2]. The best characterized of these plasmids is the Mauriceville plasmid, which encodes a reverse transcriptase (RT) that selectively makes cDNA copies of its own mRNA [3].

In addition to containing several novel introns and a plasmid of the Mauriceville type, we noticed that the Varkud-1c isolate also contained two very abundant RNAs of approx. 0.9 kb, as deduced from electrophoretic mobility studies on agarose gels. The RNAs are so abundant that they were easily detectable by ethidium bromide staining of total mt RNA, suggesting that these novel RNAs are present at concentrations similar to those of the mt rRNAs and tRNAs, and much higher than the concentration of any mRNA or excised Group I intron RNA. The Varkud-1c isolate also contains a Group II intron which was also present at concentrations detectable by ethidium bromide staining (because of their covalently closed 'lariat' structure, Group II introns are thought to be less susceptible to degradation than linear RNAs). So, our initial hypothesis was that the novel 0.9 kb RNAs might be previously unrecognized, stable, excised Group II intron RNAs.

To identify the genes on the mt chromosome which encoded these putative introns, we excised the 0.9 kb RNAs from a gel, and made radioactive

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