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When to Believe What You See

Minireview

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The recent X-ray crystal structure of a hammerhead ribozyme derived from *Schistosoma mansoni* containing the rate-enhancing peripheral domain has a catalytic core that is very different from the catalytic core present in the structure of the "minimal" hammerhead, which lacks a peripheral domain (Martick and Scott, 2006). The new structure reconciles many of the disagreements between the minimal hammerhead structure and the biochemical data on the cleavage properties of chemically modified hammerheads. The new structure also emphasizes the dynamic nature of small RNA domains and provides a cautionary tale for everyone who tries to use structure to understand function.

The *Schistosome* hammerhead is a 63 nucleotide fragment of RNA taken from the sequence of satellite DNA of digenetic trematodes, which can self-cleave at a unique site. Although the biological role of the *Schistosome* hammerhead remains to be established, hammerhead motifs found in viroids and virusoids are believed to be needed to cleave the genome during their rolling circle replication mechanism (Ferbeyre et al., 1998). The RNA secondary structure of the *Schistosome* hammerhead consists of the three helices and catalytic core that define the minimal hammerhead as well as a hairpin loop at the end of stem II and a bulged loop in an extended stem I (Figure 1). These two peripheral elements were believed to interact and, when present, stimulate the hammerhead cleavage rate by at least 50-fold (Canny et al., 2004). It is now clear that all hammerhead motifs derived from natural RNAs contain interacting loops or bulges at the ends of stem I and II that substantially enhance the cleavage rate (De la Peña et al., 2003; Khvorova et al., 2003). Because the sequences of these peripheral tertiary interactions are not phylogenetically conserved and hammerheads lacking them exhibit rapid and complete cleavage, their presence was unappreciated for many years (Uhlenbeck, 2003).

The structure of the catalytic core of the *Schistosome* hammerhead differs substantially from the structures reported for the minimal hammerhead (Martick and Scott, 2006). In the Y-shaped minimal hammerhead (Figure 1), the core consists of two separate domains: a domain 1 "U turn" structure at the end of helix I and a domain 2 that connects helices II and III by forming four noncanonical base pairs (Wedekind and McKay, 1998). In the more elongated *Schistosome* hammerhead, the formation of the loop II-bulge I tertiary interaction results in an overwound helix II and an underwound helix I. This is accompanied by a substantial rearrangement of the core, including three residues in domain 2 and all of the residues in domain 1, resulting in many new base-base and base-backbone interactions. Some of the rearrangements are dramatic. For example, the noncanonical pair between A13 and G8 in domain 2 is disrupted and a new Watson-Crick base pair between G8 and C3 is formed (Figure 1). As a result, the core of the *Schistosome* hammerhead can no longer be considered as having two discrete domains but instead is a single complex network of interactions. In addition, unlike in the minimal hammerhead (Wedekind and McKay, 1998), the phosphodiester bond at the cleavage site is poised for in line cleavage and buried in the core adjacent to several functional groups that could participate in catalysis. In other words, unlike the original minimal hammerhead structure, the new structure resembles a functional RNA catalyst.

Given the important role of the peripheral loop-bulge interactions in rearranging the hammerhead core, how does a hammerhead that lacks these interactions cleave at all? Remarkably, the minimal hammerhead is quite effective at RNA cleavage, enhancing the cleavage rate of the phosphodiester bond by about one million-fold above the uncatalyzed rate. Extending the structure of the hammerhead to include the naturally occurring tertiary interactions only enhances the cleavage rate by an additional 50- to 500-fold. Insight into this conundrum appears in a prescient paper by Peracchi et al. (1998) written well before the importance of the peripheral domains was perceived. As summarized in Figure 2, they proposed that the core of the minimal hammerhead is a mixture of conformations in solution that can interchange rapidly. Most of these conformations are not catalytically active, and the active conformation only forms transiently prior to cleavage. Because the conformational fluctuations are taking place at a time scale much faster than the cleavage rate, they would not be distinguished by experiments that follow the overall cleavage rate. For example, if one assumes a cleavage rate constant of 100 min^{-1} for a hypothetical fully active hammerhead, the typical 1 min^{-1} cleavage rate constant observed for a minimal hammerhead reflects the fact that it only adopts the active conformation 1% of the time.

This dynamic view of the solution structure of the minimal hammerhead is largely consistent with the NMR and other solution data. Many of the resonances associated with hammerhead core residues are hard to detect, indicating a mixture of conformations in rapid exchange (Simorre et al., 1997). However, it is interesting that the NMR data also suggest that the G8-A13 and A9-G12 pairs in domain 2 are formed, indicating that they are present in the majority of the multiple conformations (Heus and Pardi, 1991). Because we now know that G8 is base paired with C3 in the more active *Schistosome* hammerhead, it appears likely that the minimal hammerhead in solution is predominantly in the G8-A13 form and is inactive. Because the G8-A13 conformation is the predominant solution conformation of the minimal

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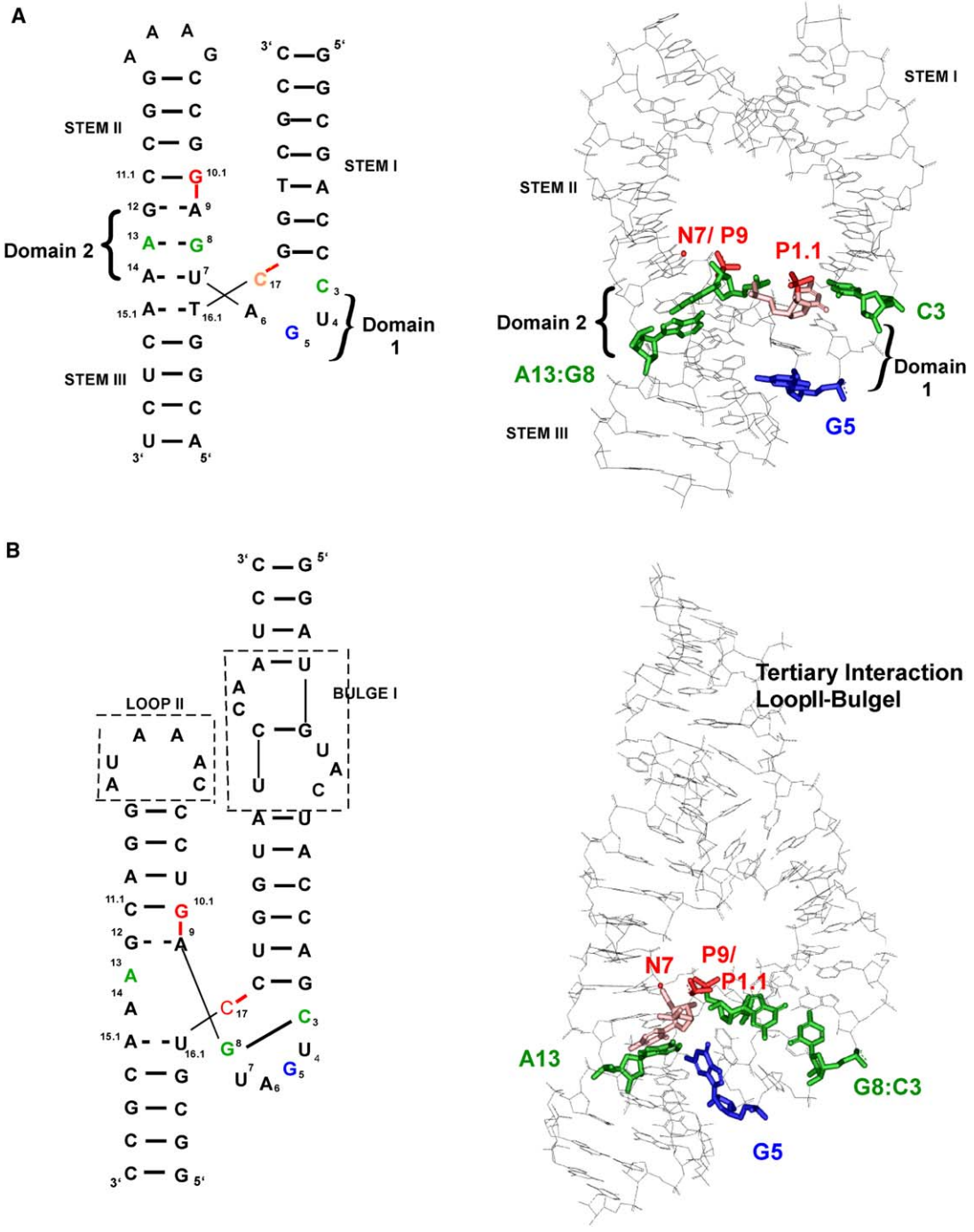

Figure 1. Secondary Structures (Left) and Crystal Structures (Right) of Minimal and *Schistosome* Hammerheads

Critical residues discussed include G8, A13, and C3 (green), G5 (blue) and the putative metal binding pocket (red) comprised of the N7 of G10.1, and phosphates P9 and P1.1. The base 3′ of the site of the cleavage site C17 is shown in pink.

hammerhead, it is not surprising that it crystallizes in that form. Indeed, several different minimal hammerhead sequences crystallize into different space groups but arrange in similar structures that all contain the inactive G8-A13 pair (Wedekind and McKay, 1998). The idea that the minimal hammerhead X-ray structure represents an inactive conformation seems at odds with the observation that cleavage appears to occur in the crystal lattice (Murray et al., 1998). However, as discussed by Martick and Scott (2006), it is possible that the crystal lattice could deform transiently to allow the active G8-C3 conformation to form.

Is the X-ray structure of the *Schistosome* hammerhead close to the active conformation? Or is it, too, trapped in an inactive structure, perhaps as a result of the presence of the 2′ O-methyl group at the cleavage site? Currently, relatively few structure-function experiments have been performed directly on the *Schistosome* hammerhead.

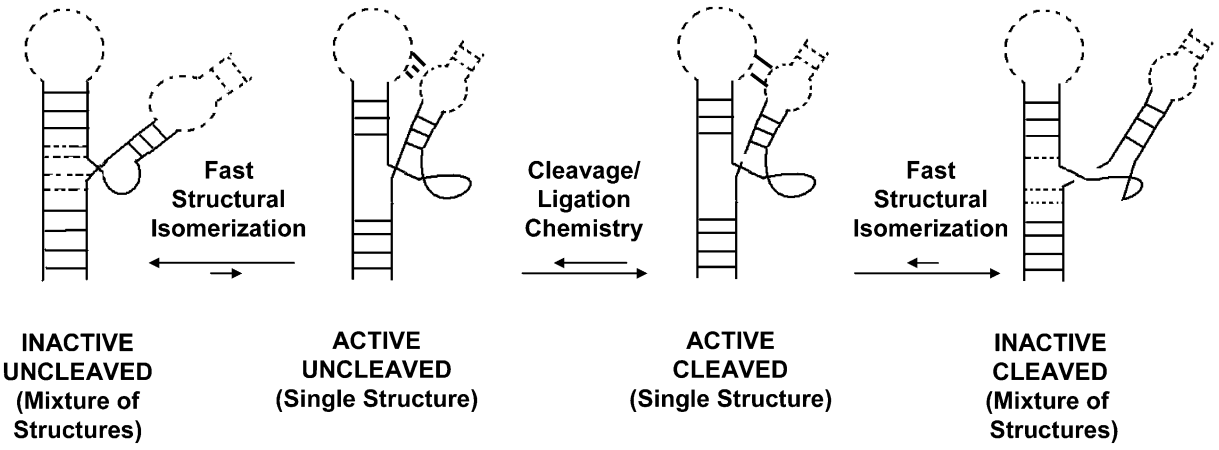

Figure 2. A Schematic of the Possible Dynamic Isomerizations Existing in the Hammerhead Cleavage Reaction Pathway

The minimal hammerhead region (solid line) on which the loop II and bulge I tertiary structures of the *Schistosome* hammerhead (dotted line) are grafted. For the minimal hammerhead, the two isomerization equilibrium constants favor the inactive structures, whereas in the *Schistosome* hammerhad, and presumably other extended format hammerheads, they favor the active structures. However, the values of the equilibrium constants remain unknown.

However, if one assumes that minimal hammerheads and the *Schistosome* hammerhead have the same active conformation and use the same mechanism to promote catalysis, the large body of data assessing the cleavage properties of chemically modified minimal hammerheads can be compared with the *Schistosome* structure. In a recent review, the cleavage rates of 53 different minimal hammerheads containing conservative atomic or functional group changes were correlated with the X-ray structure of the minimal hammerhead (Blount and Uhlenbeck, 2005). In only 26 of the cases (49%) could the effect (or lack of effect) on the cleavage rate be sensibly rationalized in terms of the structure. In many cases, the disagreement was striking. For example, modifying the 2′-hydroxyl or the base functional groups of G5 had a large effect on the cleavage rate although the nucleotide protruded into the solvent. This led to the conclusion that the biochemical data agreed poorly with the structure and supported the idea that a conformational isomerization must occur.

We have reexamined these data to see how well they fit the *Schistosome* structure. Because the *Schistosome* structure contains a 2′O-methyl at the cleavage site, it cannot fully achieve the transition state of the reaction reported on by the cleavage data. Thus, if it is assumed that the positions of residues in the *Schistosome* hammerhead X-ray structure may shift by 1 Å or less to reach the transition state, 43 of the 53 (81%) biochemical experiments agree with the *Schistosome* structure. For example, both the 2′-hydroxyl and the base functional groups of G5 form part of the network of hydrogen bonds that stabilize the rearranged core. At least four more (89%) of the biochemical experiments can be reconciled if slightly greater conformational shifts are allowed. Several of the remaining disagreements are probably the result of the modified nucleotides disrupting RNA folding. We therefore conclude that the agreement between the biochemical data and the *Schistosome* structure is excellent.

Another biochemical experiment that now can be understood is the mysterious 10-fold increase in the cleavage rate observed for minimal hammerheads that replace U7 with a pyridine-4-one (Burgin et al., 1996). Because the functional groups of U7 face the solvent in the active *Schistosome* structure, the pyridine-4-one modification would not be expected to affect cleavage. However, the pyridine-4-one modification would destabilize the U7-A14 pair seen in the inactive minimal hammerhead structure, thereby favoring the active conformation and increasing the cleavage rate. Similar explanations may account for two other sequence variants that are known to stimulate cleavage of minimal hammerheads.

If the *Schistosome* structure is a reliable approximation of the active hammerhead conformation and is reasonably close to the transition state, it can be used to design experiments directed at deducing the cleavage mechanism. Because the hydrogen bonding face of G12 and the 2′-hydroxyl group of G8 are both positioned near the scissile phosphate, Martick and Scott (2006) propose that both of these elements may participate in the proton transfer steps that accompany RNA chain cleavage. In support of such a mechanism, they cite experiments performed with minimal hammerheads that show that the 2′ OH of G8 is essential for cleavage and that when derivatives of G8 or G12 with altered pK_as are introduced, the pH dependence of cleavage is altered (Han and Burke, 2005). However, interpretations of such modification experiments are complicated by the fact that it is impossible to tell whether an individual modification disrupts catalysis by preventing proton transfer or by disrupting the folded structure of the active hammerhead. This is clearly the case for minimal hammerheads where the active structure only forms transiently, because any modification that disrupts the active conformation will lead to an even lower population of active molecules and result in a decreased cleavage rate. Indeed, there are numerous modifications throughout the catalytic core of minimal hammerheads that display equally large effects on catalysis, but these effects are likely due to disrupting RNA folding rather than preventing necessary proton transfers (Peracchi

et al., 1998). Although it is clearly important to assay *Schistosome* hammerheads containing chemical modifications at selected sites, it is very possible that such modifications will also affect the dynamic equilibrium between folded and unfolded states. Thus, independent assays will be needed to evaluate whether a given chemical modification exerts an effect on the conformational isomerization or on the chemical mechanism. Recent fluorescence resonance energy transfer experiments on the *Schistosome* hammerhead (Penedo et al., 2004) suggest that this method will be ideal, perhaps using single molecules similar to those experiments done with the hairpin ribozyme (Rueda et al., 2004).

One of the most striking structural rearrangements in the *Schistosome* hammerhead is the approach of the phosphate at position P9 to within 4 Å of the cleavage site phosphate P1.1. The juxtaposition of these two phosphates had been accurately predicted by Wang et al. (1999) using phosphorothioate "rescue" experiments to define divalent metal ion binding sites in a minimal hammerhead, an approach that was established with the group I intron . Because P9 and P1.1 are 20 Å apart in the minimal hammerhead structure, this result provided critical support for the dynamic isomerization model discussed above. Although soaking experiments were not able to detect a metal ion at this site in the *Schistosome* hammerhead crystal structure (Martick and Scott 2006), it is likely that a magnesium ion does occupy this site under physiological conditions. Phosphorothiotes at either P9 or P1.1 in the *Schistosome* hammerhead inhibit cleavage, and the addition of low concentrations of a thiophilic ion such as Cd^{2+} restores cleavage, in agreement with a divalent ion site (Osborne et al., 2005). In addition, recent EPR experiments have shown that the *Schistosome* hammerhead binds a single Mn^{2+} ion much more tightly than the minimal hammerhead (Kisseleva et al., 2005). This is consistent with the formation of the metal binding pocket only when the hammerhead isomerizes into the active conformation. Although minimal hammerheads can cleave in high concentrations of monovalent ions, the cleavage rate is considerably slower, primarily because monovalent ions bind less tightly (O'Rear et al., 2001). Although the cleavage rate of the *Schistosome* hammerhead in monovalent ions has not yet been carefully measured (Osborne et al., 2005), several other extended hammerheads cleave no better than minimal hammerheads in monovalent ions (Nelson et al., 2005), suggesting that divalent ions are primarily needed to maintain the active fold. A likely role for the magnesium ion bound between P9 and P1.1 is both to help stabilize the tertiary fold and to position the cleavage site phosphate for catalysis. In addition, it probably neutralizes some of the negative charge in the transition state and thus has a role analogous to that of lys41 in the pancreatic ribonuclease mechanism.

This story provides an important lesson about the relationship between RNA structure and function. Minimal hammerheads adopt a structure in solution that is inactive, and they only can cleave when they transiently adopt a very different structure approximated by the *Schistosome* hammerhead crystal structure. This requirement for molecular rearrangement reminds us that crystal structures are only "snapshots" of dynamic processes and that small RNA motifs can rearrange on very fast time scales. Thus, when presented with an RNA structure, one should never assume that it unequivocally represents the functionally relevant structure. Instead, it should be considered a valuable starting point for additional experiments directed at discerning function.

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