What can the New Hammerhead Ribozyme Structures Teach us About Design?

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Contents

Abstract The hammerhead ribozyme is a small, self-cleaving genomic ribozyme whose substrate-targeting properties are quite flexible. It catalyzes a phosphodiester backbone cleavage reaction that can be exploited for antisense-type applications in which it is desirable to cleave the target RNA. To better understand the requirements for rational hammerhead ribozyme design, the natural history, secondary and tertiary structures, and reaction mechanism are reviewed in detail. Specifically, significant advances in our understanding of how the hammerhead ribozyme works

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have taken place since 2003, rendering previous assumptions about therapeutic hammerhead ribozyme design largely obsolete. The requirement for a tertiary contact between Stems I and II to be present in order to achieve a highly active ribozyme in vivo is described, and design requirements that enable straightforward incorporation of the tertiary contact are explicitly described. This analysis is only possible with crystal structures of two classes of full-length natural hammerhead ribozymes that became available in 2006 and 2008.

Keywords Ribozyme · RNA · Ribozyme gene regulation · Ribozyme mechanism · Ribozyme structure · Ribozyme design

1 Introduction to the Hammerhead Ribozyme

Prior to the 1980s, all enzymes were thought to be proteins. RNA was thought to play a mostly subservient role in cellular biochemistry. tRNAs were merely adapter molecules that were employed by the ribosome translational apparatus to read the genomic message, immortalized in DNA, from an intermediary mRNA transcript, and translate it into the protein sequence corresponding to the DNA sequence. The ribosome itself was recognized to be an RNA–protein complex, but conventional wisdom suggested that the ribosomal rRNA was merely scaffolding that enabled the required collection of ribosomal proteins to assemble. RNA viruses were considered a rare exception to the Central Dogma of Molecular Biology, and genetic regulation via RNA interference mechanisms would remain unimagined for decades. Every step of each of a myriad of biochemical reactions that comprised the complex entangled web of metabolic pathways was catalyzed by an enzyme, as were DNA replication and transcription. These enzymes were always proteins.

The discovery that RNA, like proteins, also can have catalytic activity was therefore a complete surprise. The Group I intron was shown to have self-splicing activity in the absence of protein cofactors (Kruger et al. 1982), and the RNA subunit of the RNA–protein complex enzyme RNase P was shown to be the catalytic subunit of this precursor-tRNA processing enzyme (Guerrier-Takada et al. 1983). Both of these ribozymes were comprised of RNA sequences that were several hundred nucleotides in length and were believed (correctly) to have rather complex secondary and tertiary structures and catalytic mechanisms. The third ribozyme to be discovered was rather more simple and compact; it was the hammerhead self-cleaving motif found in the genome of the satellite RNA of tobacco ringspot virus (Prody et al. 1986). Subsequently, several other self-splicing and self-cleaving RNAs have been discovered (Fedor 2009), and the most profound discovery, both in terms of cellular biochemistry and evolutionary biology, was the realization that the peptidyl transferase activity within the ribosome is a ribozyme (Noller et al. 1992; Steitz and Moore 2003).

1.1 The Genomic Ribozymes

The hammerhead self-cleaving RNA was the first of several self-cleaving RNAs, or ribozymes, to be found in the context of RNA genomes (Cochrane and Strobel 2008). The hammerhead motif, first discovered in the satellite RNA of tobacco ringspot virus (Prody et al. 1986), has been found in a number of other plant satellite virus RNAs, viroid RNAs, and related genomic elements. In every case, the hammerhead RNA is involved in the rolling circle replicative mechanism of RNA genome replication (Fig. 1). In addition, several other self-cleaving ribozymes with different sequences have subsequently been discovered, and all catalyze the same chemical reaction in the same sort of biological context. This self-cleavage reaction is not a hydrolysis reaction but rather a phosphodiester isomerization reaction, wherein nucleophilic attack of the 2′-OH upon the adjacent phosphate results in backbone cleavage, leaving 2′,3′-cyclic phosphate and 5′-OH termini (Fig. 2).

1.2 What is a Hammerhead Ribozyme

The hammerhead RNA sequence within satellite RNA genomes occurs at the interface of two monomeric segments of a linear concatamer following rolling-circle replication (Fig. 1). Although it is, in that context, a single self-cleaving strand of RNA that is capable of catalyzing only a single, albeit highly specific, cleavage reaction, the hammerhead RNA can be artificially engineered to create a true multiple-turnover ribozyme simply by separating the molecule into discrete enzyme and substrate strands. The latter constructs are typically studied in vitro and also correspond to hammerhead ribozyme sequences that have been used for targeting other RNAs. Minimal hammerhead ribozymes have typical K_m values of $\sim$10 μm, and turnover rates of about 1 substrate molecule/minute, whereas full-length hammerhead ribozymes have a similar K_m but may be 1,000-fold faster.

1.3 Minimal and Full-Length Hammerhead Ribozymes

Soon after the discovery of the hammerhead self-cleaving motif, the minimal sequence required for self-cleavage activity was identified (Uhlenbeck 1987; Haseloff and Gerlach 1989). The minimal hammerhead sequence consists of a central core region of 13 mostly invariant nucleotides flanked by three A-form Watson–Crick base-paired helical sequences whose detailed sequence is comparatively less important (Fig. 3a). The highly conserved central region for the most part is incapable of forming canonical Watson–Crick base-pairs and was identified as likely giving rise to a tertiary structure that enabled the RNA to possess catalytic activity.

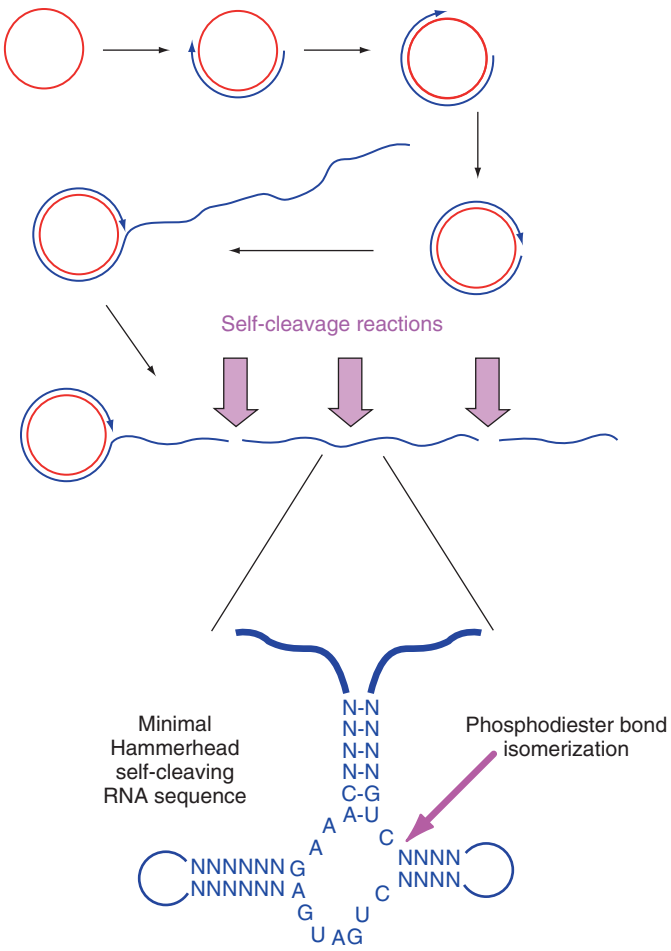

Fig. 1 Rolling circle replication. A single-stranded, covalently-closed circular RNA genome is replicated by the host cell's RNA polymerase. The polymerase copies the template (*red*) processively, creating a long linear complementary concatomeric copy (*blue*) that must then cleave itself into linear monomeric fragments that can then recircularize to form single-stranded templates for the second half of the replicative process. The cleavage sites are autolytic in the absence of protein, and correspond to the minimal hammerhead sequence shown. The cleavage reaction is a readily reversible phosphodiester isomerization reaction, which permits ligation into monomeric circles to take place subsequent to the self-cleavage processing reaction

Minimal hammerhead ribozymes received an immense amount of attention in terms of biochemical and biophysical characterization. Every one of the functional groups on each of the conserved nucleotides has been modified to dissect its particular contribution to catalysis, often with conflicting results (McKay 1996; Wedekind and McKay 1998, Blount and Uhlenbeck 2005), and several crystal structures (Pley et al. 1994; Scott et al. 1995, 1996; Murray et al. 1998a, b, 2000;

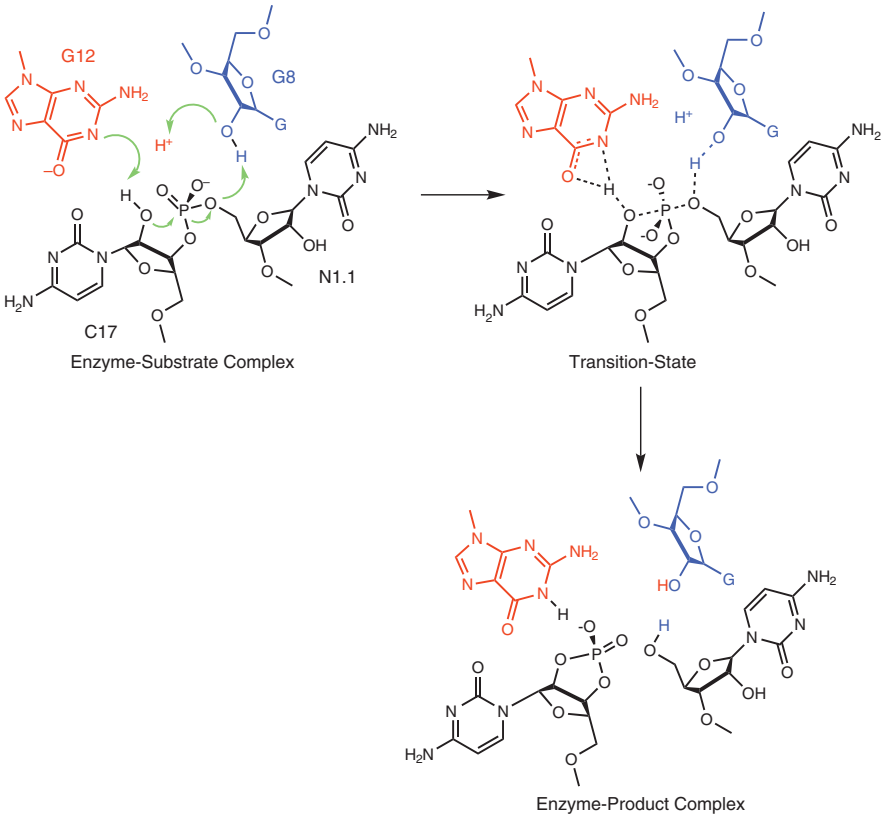

Fig. 2 The chemical mechanism of hammerhead ribozyme self-cleavage. The 2′-H of C17 is abstracted by a base (a transiently deprotonated G12), and the nucleophilic 2′-O of C17 initiates attack upon the adjacent phosphate of nucleotide 1.1. A proton is supplied to the 5′-O leaving group, presumably supplied from the ribose of G8, and the cleavage reaction is completed, generating 2′,3′-cyclic phosphate and 5′-OH termini, as shown. The transition-state is required to be in an in-line conformation, as shown

Martick and Scott 2006; Chi et al. 2008), the first of any ribozyme, have been determined. The crystal structures were only capable of reconciling a subset of the biochemical experiments designed to probe the catalytic mechanism, and considerable discord plagued the hammerhead ribozyme biochemical community (Blount and Uhlenbeck 2005).

All ribozymes, including the hammerhead ribozyme, were originally believed to be metalloenzymes (Pyle 1993), requiring an obligate Mg^{2+} for catalysis (Dahm and Uhlenbeck 1991; Dahm et al. 1993; Peracchi et al. 1997). It has subsequently been revealed, however, that the hammerhead, in addition to other small self-cleaving ribozymes, does not strictly require divalent cations for catalysis. Instead, if a sufficiently high concentration of even nonmetallic monovalent salt is present, permitting the RNA to fold correctly, it will remain catalytically active, even in a

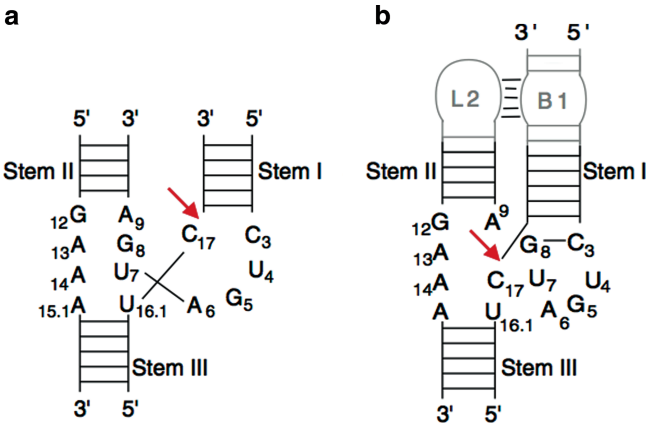

Fig. 3 A schematic secondary structure of (**a**) the minimal and (**b**) the full-length hammerhead ribozyme. The conserved residues in the catalytic core are shown explicitly in each case, and the cleavage site is indicated with a *red arrow*. The tertiary contact in (**b**) is indicated in the grey portion of the schematic diagram. This figure was kindly supplied by Christian Hammann

high concentration of EDTA (Murray et al. 1998a, b). Hence it appeared that the RNA itself, rather than functioning as a passive scaffold to bind metal ions, instead must be an active participant in its own chemical catalysis (Scott 1999). This renewed focus upon the RNA structure itself. However, the crystal structures of the minimal hammerhead could not be reconciled with this conclusion; none of the invariant residues were positioned in a way that made their role in catalysis at all obvious, and the substrate itself was not bound to the enzyme in a way that would permit the known required in-line attack geometry to be stabilized, as one would expect from an enzyme (McKay 1996; Blount and Uhlenbeck 2005).

In 2003, two papers appeared (De la Peña et al. 2003; Khvorova et al. 2003) that had essentially the same conclusion: the minimal hammerhead construct lacked a tertiary contact between helices I and II that had a rather profound effect upon hammerhead ribozyme catalysis, despite being distant from the cleavage site (Fig. 3b). This contact appeared to have little if any sequence conservation between the large number of natural hammerhead ribozyme sequences that had been identified to date and had thus escaped notice. However, when the tertiary contact sequences were included, these natural, full-length hammerheads were observed to be up to 1,000-fold more active than their minimal counterparts (Khvorova et al. 2003; Canny et al. 2004). Clearly, the tertiary contact imparted some change at the active site that stimulated catalytic activity.

1.4 Expanding Biological Context

Although the hammerhead ribozyme was originally discovered in RNA virus-like genomes, it has since been discovered to occur in a few other contexts (Ferbeyre

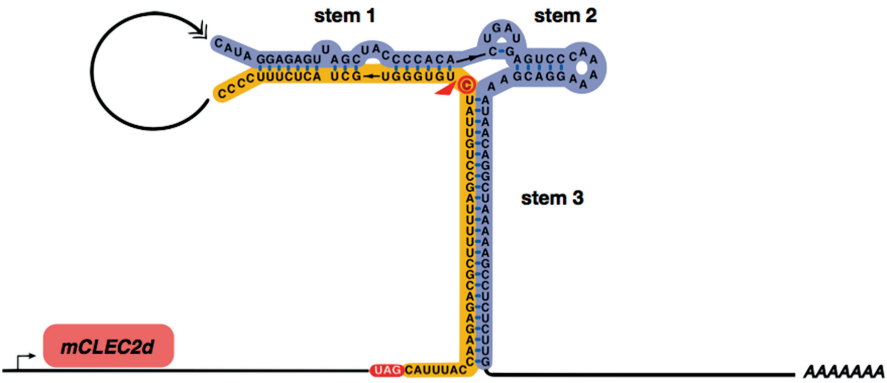

Fig. 4 The hammerhead ribozyme embedded within the 3′-UTR of the clec2d mRNA transcript, immediately downstream from the stop codon. The "enzyme" part of the strand is highlighted in *blue*, and the "substrate" is highlighted in *orange*, with the cleavage site indicated

et al. 2000). Highly repetitive DNA sequences in a *Schistosome* trematode parasite (Ferbeyre et al. 1998) and in a newt genome (Forster et al. 1988; Luzi et al. 1997), when transcribed, give rise to RNA satellites that contain hammerhead ribozyme sequences. More recently, active hammerhead ribozyme sequences have been discovered in the 3′-untranslated regions of mature mRNAs in a variety of mammals (Martick et al. 2008a, b), and these are thought to control export and translation via a riboswitch mechanism that is currently under investigation (Fig. 4). Additional reports of hammerhead ribozyme sequences in bacteria and eukaryotes are now emerging. Hammerhead ribozymes may thus turn out to be far more ubiquitous than originally thought and may play a significant role in RNA-based regulation of gene expression.

2 Hammerhead Ribozyme Structures

The first structures of an RNA appeared in 1974 with the publication of the yeast $tRNA^{Phe}$ crystal structures (Robertus et al. 1974; Kim et al. 1974). These structures revealed that RNA possesses the propensity to fold into comparatively compact globular protein-like three-dimensional structures, and it also illustrated the importance of tertiary contacts in stabilizing complex RNA backbone folds (Klug et al. 1974). Another 20 years passed before another complex RNA structure emerged. In 1994, the first structure of a ribozyme was published by McKay and coworkers (Pley et al. 1994); it consisted of a minimal hammerhead enzyme strand hybridized with a DNA substrate analog. A second structure of a minimal hammerhead, this time with an all-RNA substrate (Scott et al. 1995), appeared shortly thereafter, corroborating the initial structural work.

2.1 Three-Dimensional Structure of Minimal Hammerhead Ribozymes

Both minimal hammerhead ribozyme structures revealed a three-stranded junction in which the cleavage-site nucleotide, C17, is surrounded by invariant residues that formed a structure analogous to the uridine turn in tRNA (Pley et al. 1994). The remaining invariant residues augmented Stem II, permitting it to stack upon Stem III coaxially, at the junction interface (Fig. 5).

The crystal structures of the minimal hammerhead ribozyme frustratingly created many more questions than compelling explanations for RNA catalysis. The 12 years subsequent to the publication of these structures saw only increasing discord; the crystal structure analyses seemed hopelessly irreconcilable with a growing corpus of biochemical evidence (Blount and Uhlenbeck 2005). Meanwhile, crystal structures for many of the other ribozymes, including the Group I intron, the hairpin, HDV, and RNase P, appeared.

Despite observations of hammerhead ribozyme catalysis in a crystal in which the lattice packing contacts by necessity confined the global positions of the distal termini of all three flanking helical stems of the minimal hammerhead (Scott et al. 1996; Murray et al. 2002; Scott 2002), many biochemical experiments designed to probe transition-state interactions and the chemistry of catalysis appeared to be irreconcilable with the crystal structures. For example, the invariant core residues G5, G8, G12, and C3 in the minimal hammerhead ribozyme were each observed to be so fragile that changing even a single exocyclic functional group on any one of these nucleotides results in abolition of catalytic activity; yet few of these appeared to form hydrogen bonds involving the Watson–Crick faces of these nucleotides (McKay 1996). A particularly striking and only recently observed example

Fig. 5 A backbone diagram of a minimal hammerhead ribozyme, in which a shorter strand is the enzyme strand, shown in *blue*, and the longer strand is the substrate strand, shown in magenta

consisted of G8 and G12, which were identified as possible participants in acid–-base catalysis (Han and Burke 2005). After it was demonstrated that the hammerhead ribozyme does not require divalent metal ions for catalysis (Murray et al. 1998a, b), it gradually became apparent that the RNA itself, rather than passively bound divalent metal ions, must play a direct chemical role in any acid–base chemistry within the hammerhead active site. It was, however, completely unclear how G12 and G8 could accomplish this, given the original structures of the minimal hammerhead ribozyme. In addition, the attacking nucleophile in the original structures, i.e., the 2′-OH of C17, was not in a position amenable to in-line attack upon the adjacent scissile phosphate. Perhaps most worrisome were experiments that suggested that the A-9 and scissile phosphates must come within about 4 Å of one another in the transition-state, based upon double phosphorothioate substitution and soft metal ion rescue experiments; the distance between these phosphates in the crystal structure was about 18 Å, with no clear mechanism for close approach if the Stem II and Stem I A-form helices were treated as rigid bodies (Wang et al. 1999). Taken together, these results appeared to suggest that a fairly large-scale conformational change must have taken place to reach the transition-state within the minimal hammerhead ribozyme structure. For these reasons, the two sets of experiments (biochemical vs. crystallographic) appeared not only to be at odds but to be completely and hopelessly irreconcilable, generating a substantial amount of discord in the field (Blount and Uhlenbeck 2005). No compelling evidence for dismissing either set of experimental results was ever made successfully (although some claims to the contrary were made in favor of each).

2.2 Three-Dimensional Structures of Full-Length Hammerhead Ribozymes

A new crystal structure of the full-length hammerhead ribozyme emerged in 2006, 20 years after the hammerhead's discovery (Martick and Scott 2006). This structure includes a set of distal tertiary contacts whose importance was largely unrecognized until 2003, but whose incorporation increases the catalytic prowess by a factor of 1,000. The new crystal structure reveals that this remarkable rate enhancement is a direct consequence of localized yet dramatic active site conformational changes that are stabilized by a comparatively distant set of tertiary interactions. The new structure appears to reconcile twenty years of discord while offering some new insights into RNA structure and catalysis (Nelson and Uhlenbeck 2006).

2.2.1 Schistosomal Hammerhead Structure

The resolution of this vexing conundrum finally came in 2006 with a 2.2 Å resolution crystal structure of the full-length hammerhead ribozyme from *Schistosoma*

Fig. 6 A backbone diagram of a full-length *Schistosomal* hammerhead ribozyme, in which the enzyme strand is shown in *blue*, and the substrate strand is shown in magenta

mansoni (Fig. 6). C17 is now positioned for in-line attack, and the invariant residues C3, G5, G8, and G12 all appear involved in vital interactions relevant to catalysis. Moreover, the A9 and scissile phosphates are observed to be 4.3 Å apart, which is consistent with the idea that, when modified, these phosphates could bind a single thiophilic metal ion. The structure also reveals how two invariant residues, G-12 and G-8, are positioned within the active site – consistent with their previously proposed role in acid–base catalysis. G12 is within hydrogen bonding distance to the 2′-O of C17, the nucleophile in the cleavage reaction, and the ribose of G8 hydrogen bonds to the leaving group 5′-O, while the nucleotide base of G8 forms a Watson–Crick pair with the invariant C3. This arrangement suggests that G12 is the general base in the cleavage reaction, and that the G8 ribose may function as the general acid (Fig. 7). The crystal structure of the full-length hammerhead ribozyme thus clearly addresses the major concerns that appeared irreconcilable with the previous crystal structures (Nelson and Uhlenbeck 2006, 2008).

In addition to the rearranged cleavage site, one of the most prominent features of the full-length hammerhead ribozyme structure is the Stem II loop/Stem I bulge interaction that appears to induce the structural organization of the catalytic core. The loop/bulge interaction is composed of an intricate network of interhelical

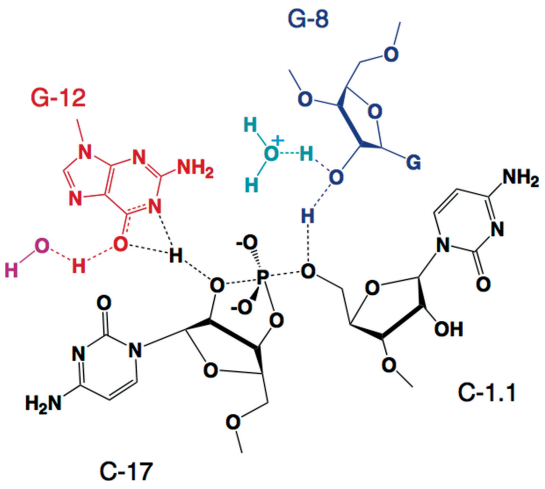

Fig. 7 The disposition of active-site nucleotides in the context of a proposed transition-state structure

noncanonical base pairs and stacks interdigitating Stem II loop into Stem I, kinking Stem I in such a way as to coaxially align its distal helix on top of the Stem II–Stem III coaxial arm. The tertiary contacts between the loop and bulge regions induce structural changes affecting the catalytic core, specifically via the relative underwinding of Stem I. This interaction imparts a severe bend in the distal part of the Stem I helix and a pronounced kink in the backbone of the substrate strand at the cleavage site. These distortions appear to accommodate G-8 and U-7 in the catalytic pocket and in turn stabilize the rearrangement of the augmented Stem II helix that enables G-8 to form the Watson–Crick base pair with C-3 in the catalytic pocket. Concurrently, an overwinding or right-handed twist of Stem II positions the conserved G-12, A-13, and A-14 precisely against the catalytic-site C-17, helping to lock the latter in a catalytically active conformation in which C-17 is oriented for in-line attack (Martick and Scott 2006).

2.2.2 Satellite Viral Hammerhead Ribozyme Structure

The Satellite RNAs all possess a different type of tertiary contact compared to the *Schistosomal* hammerhead. In 2008, crystal structures of an unmodified hammerhead ribozyme derived from the satellite RNA of tobacco ringspot virus (sTRSV) was published (Chi et al. 2008), permitting comparison of the two types of hammerhead (Scott et al. 2009). Briefly, the active site is nearly identical in both types of hammerhead ribozyme, but the tertiary contacts, though imparting the same net structural effect upon the active site, are distinctly different in the two cases.

The two classes of tertiary contacts are shown as secondary structural representations that reflect the tertiary structures in Fig. 8. The only structural feature the two types of tertiary contacts have in common is the presence of an AU Hoogsteen

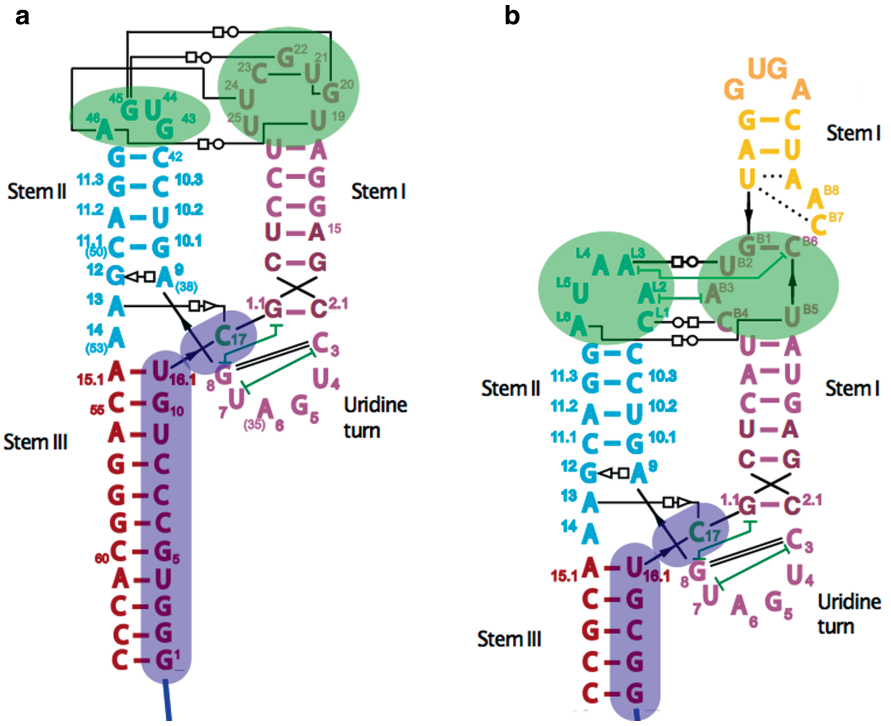

Fig. 8 The secondary structures of two classes of full-length hammerhead ribozymes, with those represented by satellite virus hammerheads shown in **a**, and the Schistosoma-like hammerheads, shown in **b**. The tertiary contacts are highlighted in light green. Little sequence homology in the tertiary contact region in apparent

pair. Remarkably, this apparently conserved interaction escaped detection, even by Eric Westhof's well-trained eyes. The 2003 paper by Khvorova, Westhof, and colleagues (Khvorova et al. 2003) compares 13 natural hammerhead ribozyme sequences, all of the sTRSV class, in an attempt to deduce conserved tertiary interactions. All of these sequences possess GNRA-like tetraloops capping Stem II, where the final A in the sequence is always present. This A makes a Hoogsteen pair with U1.7 in the substrate strand of Stem I. (It also makes a Watson–Crick pair with another U in the 3′-region of the loop capping Stem I in the sTRSV hammerhead, but this U appears not to be conserved even within the second class of hammerheads.) The U that is involved in the Hoogsteen pair interaction is present in 10 of the 13 sequences analyzed by Westhof and colleagues; the other three examples have a C at position 1.7. It is noteworthy that C will participate in the same Hoogsteen pairing interaction if N3 is protonated. If this is indeed the case, it is possible that the C1.7 hammerhead sequences will show decreasing activity at more basic pH values, a property that might be exploitable for control of activity in designer ribozymes.

The structure of the GNRA tetraloop capping Stem II in the sTRSV hammerhead is unusual as well. The GNRA tetraloop, where the 5′ nucleotide is always a G, the second can be any nucleotide (N), the third is a purine (R), and the fourth is always an A, is a canonically stable RNA secondary structural motif. The G pairs with the A via a single hydrogen bond, and the N, R, and A all stack upon one another on the 3′-side of the loop. However, in the context of the sTRSV hammerhead tertiary contact, the structure of the GNRA tetraloop rearranges so that the above-noted interactions disappear and the A rearranges to form the Hoogsteen base pair with U1.7 from Stemloop I.

3 Structure and Mechanism

The cleavage reaction is a phosphodiester isomerization reaction that is initiated by abstraction of the 2′-hydroxyl proton from its 2′-oxygen, which then becomes the attacking nucleophile in an "in-line" or $S_N2(P)$-like reaction, although it is not known whether this proton is removed before or during the chemical step of the hammerhead cleavage reaction. (The cleavage reaction is technically not bimolecular, but behaves in the same way a genuine S_N2 reaction does; it undergoes inversion of configuration subsequent to forming an associative transition-state consisting of a pentacoordinated oxyphosphorane). The attacking and leaving group oxygens will both occupy the two axial positions in the trigonal bipyramidal transition-state structure as is required for an S_N2-like reaction mechanism.

The 5′-product, as a result of this cleavage reaction mechanism, possesses a 2′,3′-cyclic phosphate terminus, and the 3′-product possesses a 5′-OH terminus, as with nonenzymatic alkaline cleavage of RNA. The reaction is therefore, in principle, reversible, as the scissile phosphate remains a phosphodiester, and may thus act as a substrate for hammerhead RNA-mediated ligation without a requirement for ATP or a similar exogenous energy source. The hammerhead ribozyme-catalyzed reaction, unlike the formally identical nonenzymatic alkaline cleavage of RNA, is a highly sequence-specific cleavage reaction with a typical turnover rate of approximately one molecule of substrate per molecule of enzyme per minute at pH 7.5 in 10 mM Mg^{2+} (the so-called "standard reaction conditions" for the minimal hammerhead RNA sequence), depending upon the sequence of the particular hammerhead ribozyme construct measured. This represents an approximately 10,000-fold rate enhancement over the nonenzymatic cleavage of RNA (Stage-Zimmermann and Uhlenbeck 1998).

3.1 Acid–Base Catalysis

Based upon the arrangement of invariant nucleotides in the full-length hammerhead active site, as well as the solvent structure in a combined crystallographic and molecular dynamics investigation, it appears that a specifically bound water molecule

Fig. 9 The satellite RNA of tobacco ringspot virus hammerhead ribozyme. The crystal structure is of a single strand of the RNA, which cleaves slowly due to a G12A mutation that lowers the p*K*a of the general base

(Fig. 9, *light blue*) accepts a proton from G12. G12 must ionize to function as the general base, and the proton is replaced by that from the 2′-OH of C17 (Fig. 9, *black*). The original G12 proton can then be relayed directly to the 2′-OH of G8 to replace a proton that must be donated to the 5′-O leaving group of C1.1 (*black*) as the phosphodiester backbone is cleaved. This mechanism conserves the number of protons during the phosphodiester isomerization. It is testable, in that it predicts that altering the p*K*a of either the purine base at position 12 or the 2′-OH at position 8 will alter the cleavage rate without inducing gross structural perturbations. There are also opportunities for transition-state stabilization of the accumulating negative charges in the pentacoordinated oxyphosphorane. We suggest that either the exocyclic amine of A9 or a divalent cation can perform this function.

3.2 Metal Ions?

The minimal hammerhead ribozyme was originally believed to be dependent upon the presence of divalent metal ions for folding and catalysis (Dahm and Uhlenbeck 1991). However, in the presence of a high concentration of monovalent salt,

including molar concentrations of NH_4^+, the strict requirement for Mg^{2+} may be dispensed with (Murray et al. 1998a, b). Nevertheless, the hammerhead ribozyme appears to be reliant upon Mg^{2+} under in vivo conditions, and is typically assayed in vitro in the presence of 10 mM Mg^{2+}, whereas physiological concentrations of Mg^{2+} are closer to 1 mM. The apparent Km for Mg^{2+} in minimal hammerheads ranges from 10 to 100 mM (Stage-Zimmermann and Uhlenbeck 1998).

Originally, Mg^{2+} was believed to play a direct role in acid–base catalysis in the hammerhead ribozyme (Dahm et al. 1993), and although as early as 1998, it was known that the hammerhead did not require Mg^{2+} for catalysis, it was only with the publication of the full-length hammerhead ribozyme structures, which revealed RNA functional groups positioned for acid/base chemistry, that the participation of Mg^{2+} in acid–base catalysis could be ruled out. An additional potential role for Mg^{2+}, however, is transition-state charge stabilization (Martick et al. 2008a, b; Lee et al. 2007, 2008). For this, any high concentration of positive charge should suffice, so the suggestion that Mg^{2+} or monovalent cations aid in folding as well as transition-state stabilization appears to be the most consistent with all of the data, and accounts for the rather high apparent Km values for Mg^{2+}. What is apparent is that under low ionic strength in vitro assay conditions, the hammerhead ribozyme needs at least ten times the total physiological concentration of Mg^{2+} to cleave efficiently. Therefore, it is very unlikely that the minimal hammerhead sequence will be able to fold and cleave efficiently in vivo, unless folding is assisted by some compensatory mechanism (such as an associated RNA-binding protein).

The full-length hammerhead ribozyme, even under low ionic strength in vitro assay conditions, requires only micromolar concentrations of Mg^{2+} (Khvorova et al. 2003), which is far more consistent with in vivo requirements for activity. It is likely that the requirement for 10–100 mM Mg^{2+} for optimal activity of the minimal hammerhead is partially compensating for the lack of the tertiary contact that stabilizes the active site. Hence, the full-length hammerhead, which includes a naturally occurring tertiary contact, would be by far the most preferable starting point for the design of an in vivo RNA cleaving reagent.

3.3 Substrate Binding and Specificity

One of the most attractive features of the minimal hammerhead ribozyme to those hoping to design a specific RNA cleavage reagent is that almost all of the enzyme–substrate binding specificity can be understood in terms of simple Watson–Crick base-pairing rules. One does not need to know anything about the hammerhead ribozyme's tertiary structure in order to design a hammerhead ribozyme to cleave an RNA substrate of a given sequence. The only sequence restriction that exists, in terms of choosing a target substrate, is that a RUH nucleotide triplet be present. H is the cleavage-site nucleotide. It is typically a C but can be any nucleotide apart from

G. U is uridine. R is either purine, with a preference for G. Because of the tertiary contacts in the full-length hammerhead ribozyme, whose sequence requirements are rather poorly understood, it is less apparent what sequence restrictions might exist for designing a full-length hammerhead ribozyme. For this reason, use of the full-length hammerhead ribozyme in designing cleavage reagents is often avoided in order to circumvent such complications.

Fortunately, comparison of the two full-length hammerhead crystal structures reveals that the only additional conserved nucleotide in the substrate tertiary contact region is U1.7. The remainder of the Stem I contributions to the tertiary contact are all contained within the enzyme strand.

4 Hammerhead Structure, Function, and Design

The above considerations lead quite naturally to explicit requirements for hammerhead ribozyme design.

4.1 Minimal Hammerheads

Minimal hammerhead ribozymes have been employed for many years as antisense RNA cleaving agents in an attempt to target pathological mRNAs and RNA viruses (For a recent review, please see Tedeschi et al. 2009). An advantage of the minimal hammerhead is that the only sequence restriction imposed on the target substrate is at the cleavage site, where a sequence of the type RUH is required (R is either G or A at position 16.2, U must be uracil at position 16.1, H can be anything except G at position 17, the cleavage site nucleotide). The sequences of Stems I and III in a minimal hammerhead enzyme strand, apart from these restrictions, can then be tailored to base-pair with any target sequence. Unfortunately, their practical utility as therapeutic agents has been limited by the apparent need for nonphysiological concentrations of Mg^{2+} and their slow ($\sim$1/min) turnover rates.

4.2 Full-Length Hammerheads

The much greater activity of the full-length hammerhead ribozymes makes them a more attractive alternative as in vivo RNA cleavage agents. In addition, the discovery of naturally occurring full-length hammerheads in mammalian mRNAs that downregulate gene expression (Martick et al. 2008a, b), including some that appear to work intermolecularly, provide a convincing argument that full-length hammerheads should be viable in vivo ribozyme nucleases. But because of the requirement

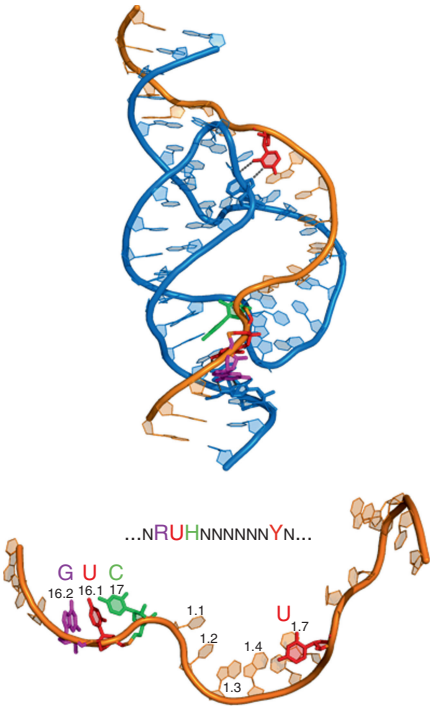

Fig. 10 A schematic representation of the Schistosomal hammerhead ribozyme, with the enzyme strand shown in *blue*, and the substrate strand shown in *orange*. *The restrictions imposed on the sequence are such that any RNA strand with a sequence of the form* ... NRUHNNNNNNYN... can be targeted, although the optimal sequence will have C17 and U1.7. The crystal structures reveal that a U (or possibly C) at position 1.7 is the only additional sequence restriction imposed by the full-length hammerhead tertiary contact region between Stems I and II

for the tertiary contact, whose sequence requirements before now have been rather obscure, full-length hammerhead cleavage agents have not been pursued with vigor.

Comparison of the sTRSV and *Schistosomal* full-length hammerhead crystal structures reveals that only one base-pairing interaction between enzyme and substrate strands in the tertiary contact region is conserved. The AU Hoogsteen pair requires U1.7 to be present (although C might be able to substitute for U at this position; cf. Fig. 10). The remainder of the specific tertiary interactions appears in both cases to lie entirely within the enzyme strands of Stem I and Stem-loop II. Hence the sequence restrictions imposed by the full-length hammerhead on possible RNA targets is simply ...**NRUHNNNNNNYN**... where N is any nucleotide, R can be G or A at position 16.2, U must be uracil at 16.1, H can be any residue at position 17, the cleavage site, although C is preferred, and Y at position 1.7, (i.e., seven nucleotides downstream of the cleavage site), must be U or possibly C.

Examination of the structures therefore strongly suggests that an mRNA sequence that possesses the ...NRUHNNNNNNYN... motif can be targeted and cleaved efficiently by designing a hammerhead enzyme strand complementary to the target sequence. If cleavage is insufficiently efficient, one can subsequently use in vitro selection to further optimize the enzyme sequence by selecting for variants in the tertiary contact region.

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