RNA structure: crystal clear?

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Structured RNAs play an essential role in chromosome maintenance, RNA processing, protein biosynthesis, and protein transport. To understand RNA function in these diverse biological systems, the rules for RNA folding and recognition must be learned. Recent crystal structures of hammerhead ribozymes, a group I intron domain, and RNA duplexes provide new insights into the principles of RNA folding and function.

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Abbreviations

FRET	fluorescence resonance energy transfer
P	base-paired

Introduction

Many RNAs fold into complex 3D shapes that define their biological activity, yet the structural basis for RNA folding and catalysis remains largely unknown. The past two years have seen a turning point in RNA crystallography with the determination of crystal structures of hammerhead ribozymes and a large domain of the *Tetrahymena* self-splicing intron. Together with recent RNA duplex structures solved at high resolution, these structures of large RNAs provide new snapshots of RNA folding, from the fine detail of RNA hydration and metal binding to the large scale packing of helices that is thought to occur in self-splicing introns, the ribosome, and the spliceosome.

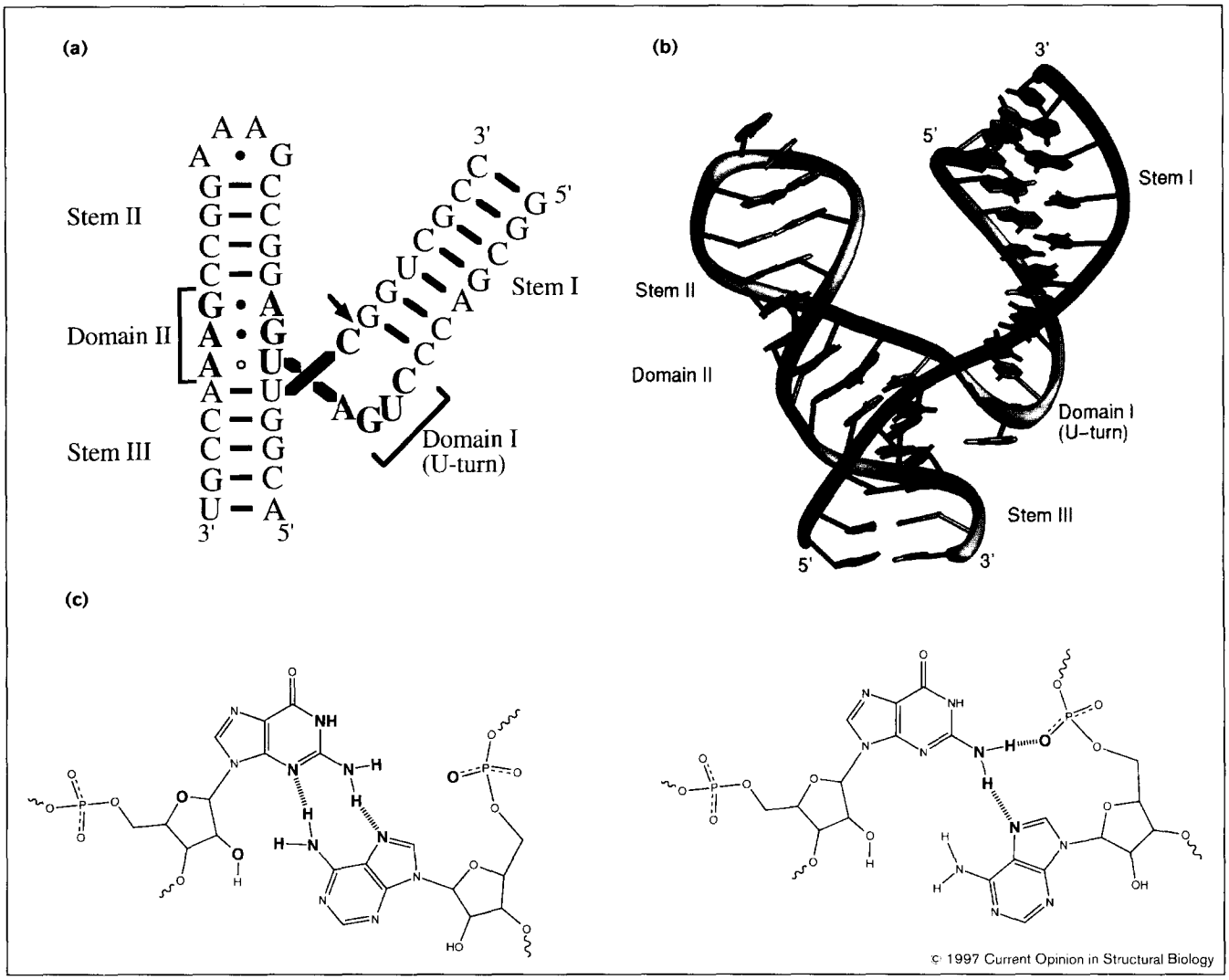
The hammerhead ribozyme

The hammerhead motif is a self-cleaving RNA found in small pathogenic RNAs of plants that allows multimeric genomes to process during rolling circle replication [1]. Unlike ribozymes such as self-splicing introns and the catalytic RNA subunit of RNase P, the hammerhead domain is small, consisting of three helices flanking a core of phylogenetically conserved nucleotides (Fig. 1a) [2]. Cleavage occurs via the nucleophilic attack of the 2′-hydroxyl of a specific nucleotide within the core on its adjacent phosphodiester bond to produce 2′,3′-cyclic phosphate and 5′-hydroxyl termini [3]. Normally a single-turnover catalyst, the hammerhead is readily made into a multiple-turnover enzyme by separating the strand containing the cleavage site from the rest of the core (Fig. 1a) [2,3]. These designs have proven useful for crystallization by allowing the substitution of the substrate strand with an all-DNA strand [4], or with an RNA strand modified at the cleavage site by a 2′-O-methyl group [5••]. The crystal structures of these hammerhead–inhibitor complexes reveal the overall geometry of the ribozyme but raise new questions concerning the catalytic mechanism.

In three dimensions, the hammerhead is shaped like a wishbone or γ, with Stems I and II forming the arms, and Stem III and the core forming the base (Fig. 1b) [4,5••]. This fold is seen in both inhibitor complexes despite different RNA backbone connectivities, substrate strand identity, crystallization conditions, and crystal packing arrangements. Whereas the three stems are all A-form helices, the structure of the central core is created, in part, by noncanonical pairings of the phylogenetically conserved nucleotide bases. Stems II and III sandwich two sheared G·A base pairs and an A·U base pair to form one long pseudocontinuous helix from which Stem I and the catalytic site emanate (Fig. 1b,c). The highly conserved sequence CUGA between Stems I and II forms a tight turn nearly identical in conformation to the uridine turn previously seen in the X-ray crystal structure of yeast phenylalanine transfer RNA [6,7]. The cytosine at the cleavage site between Stems I and III is positioned near the CUGA cleft by interactions with the cytosine and adenosine of that sequence. This proximity led Klug and coworkers [5••] to propose that the uridine turn, called Domain I by McKay's group [4], constitutes the catalytic pocket of the ribozyme.

Since the hammerhead–inhibitor complexes do not position the scissile bond correctly for catalysis, these crystal structures probably represent the ground state of the ribozyme [4,5••]. This has led to speculation that the hammerhead ribozyme may undergo a conformational change in order for cleavage to occur [8]. Eckstein and coworkers [9] have used fluorescence resonance energy transfer (FRET) data to build a 3D model of the hammerhead ribozyme that is similar to the X-ray models, except that the helical groove of stem I facing stem II differs. To distinguish between the solution and X-ray models, an elegant set of disulfide cross-linking experiments has been carried out [10•]. When stems I and II are cross-linked in conformations that exclude either the FRET or X-ray models, only the ribozyme cross-linked in a manner consistent with the X-ray structures is active. In addition, gel electrophoresis and transient electric birefringence have shown that the three stems are roughly coplanar and do not rearrange significantly after cleavage [11]. On the basis of these data, the cleavage reaction probably does not require a large global change in conformation of the ribozyme.

Figure 1



The hammerhead ribozyme. **(a)** Secondary structure of a canonical hammerhead ribozyme construct [2], drawn in the 'wishbone' format [5••]. The conserved core is in bold letters, with an arrow pointing to the cleavage site. **(b)** Tertiary structure of the hammerhead–DNA inhibitor complex [4] in the same orientation as the secondary structure. The DNA strand is shown in darker gray. **(c)** Conformation of a sheared G·A base pair [4,32,33]. Note that the base-pairing pattern differs slightly depending on context. Left, as seen in tandem sheared G·As, in the 5′-GAAA-3′ internal loop, and in the tRNA(Ser)-aminoacyl tRNA synthetase cocrystal structure [4,5••,40••,61]. Right, as seen in GAAA tetraloops [5••,29••,32,33]. Atoms in bold letters are either sometimes or always involved in RNA–RNA hydrogen bonds. Hydrogen bonds shown by thick dashes occur in all examples.

Recently, new crystal structures of a hammerhead ribozyme complexed with a cleavable substrate have been determined, adding new insight into local rearrangements in the catalytic pocket [12••]. Unlike the previous hammerhead ribozymes, this construct is active in the crystal lattice, allowing the authors to 'trap' an intermediate in the reaction pathway. The major changes from the previous structures only involve a repositioning of the substrate nucleotides in the catalytic pocket. In the trapped structure, a divalent metal ion is bound to the pro-R_p oxygen of the phosphate involved in the cleavage reaction, as previously proposed [13–15]. The scissile bond, however, is still not positioned for an in-line attack mechanism.

Although Scott *et al.* [12••] propose a new model for the transition-state structure, some key questions remain unanswered. First, what are the actual positions of the metals during catalysis? Second, what is the role of G5 in the CUGA U-turn in the catalytic pocket? Biochemical studies have clearly shown that all of the Watson–Crick base functionalities of G5 and its 2′-OH are critical for catalysis [16], yet none of the crystal structures reveals a clear role for this nucleotide. In the new structures, weak

density interpreted as a divalent metal ion appears next to this guanosine, which is consistent with uranium-induced cleavage at that site [17•], but no function for this metal ion has been shown.

Finally, the tandem sheared G·A base pairs seen in the crystal structures seem to be incompatible with the available biochemical data (Fig. 1b,c) [18]. Functional group modification studies initially led to the prediction that the G·A base pairs could not be in the sheared conformation [19]. Besides the clear need to distinguish ground state and transition state structure stabilities, other factors probably need to be considered when relating the biochemical data to the crystal structures. For example, the thermodynamic stability and even the base-pairing conformations of tandem G·A pairs change dramatically depending on their context [20,21,22•]. In addition, the modified RNAs may have many alternate conformations that are not easily detected in the biochemical experiments [23•].

The P4–P6 domain of the *Tetrahymena* ribozyme

Group I introns, defined by a conserved catalytic core and reaction pathway (Fig. 2a), splice precursor RNAs to form mature rRNAs, tRNAs, or mRNAs [24,25]. Half of the conserved core in the *Tetrahymena thermophila* intron resides in an independently folding domain consisting of the base-paired (P) regions P4 through P6 (P4–P6; Fig. 2) [26]. The P4–P6 domain alone folds into a structure whose chemical protection pattern is very similar to that seen for the P4–P6 region of the intact intron [26,27]. The crystal structure of this domain, a 160-nucleotide RNA, reveals several new facets of RNA secondary and tertiary folding and shows for the first time the kind of helical packing that is thought to occur in large ribozymes and RNA–protein complexes.

In the 2.8 Å crystal structure of the P4–P6 domain, a sharp bend allows stacked helices of the conserved core to pack alongside helices of an extension (helices P5a, P5b and P5c, or P5abc) that is important for folding and catalytic efficiency (Fig. 2b) [28,29••]. Two specific tertiary interactions clamp the two halves of the domain together: an adenosine-rich corkscrew plugs into the minor groove of helix P4; and a GAAA tetraloop binds to a conserved 11-nucleotide internal loop [30•], termed the tetraloop receptor. The A-rich bulge (Fig. 2) coordinates two Mg^{2+} ions via its phosphate oxygens, allowing the backbone to invert and the bases to flip out. The adenosines make numerous tertiary contacts that bridge the core helices with the helices in the P5abc extension. From biochemical evidence, these interactions are crucial to the stability of the entire domain [26,29••,31]. The other half of the clamp, equally important to the packing of helices P5abc against the core (although not to the folding of the P5abc subdomain), involves a GAAA tetraloop in the same conformation as seen previously [32,33]. The tetraloop receptor, a motif seen in many RNAs [30•], has a widened minor groove that docks with the tetraloop in a highly specific manner [29••,31].

The ribose 2′-hydroxyl group is involved in a common motif that occurs in both clamp interactions between the helical stacks. Pairs of riboses form an interhelical 'ribose zipper'—a major component of the packing interactions. McKay and coworkers [33] also observed packing that involves pairs of 2′-hydroxyl contacts between a GAAA loop and the Stem II minor groove of another hammerhead molecule in the crystal lattice. In a group II intron, riboses likely to be involved in a ribose zipper each contribute 2 kcal mol^{-1} of binding energy via their 2′-OH groups [34•]. The number of ribose zippers seen so far suggests that this is a common way to pack RNA helices together.

One unexpected motif seen in the P4–P6 domain structure mediates both intramolecular and intermolecular interactions. At three separate locations in the 160-nucleotide domain, adjacent adenosines in the sequence lie side-by-side and form a pseudo base pair within a helix [35••]. This AA platform opens the minor groove for base stacking or base pairing with nucleotides from a noncontiguous RNA strand. The platform motif has a distinctive chemical modification signature which may enable its detection in other structured RNAs ([26,35••]; however, see [36•]). The ability of this motif to facilitate higher order folding provides one explanation for the abundance of adenosine residues in internal loops of many RNAs.

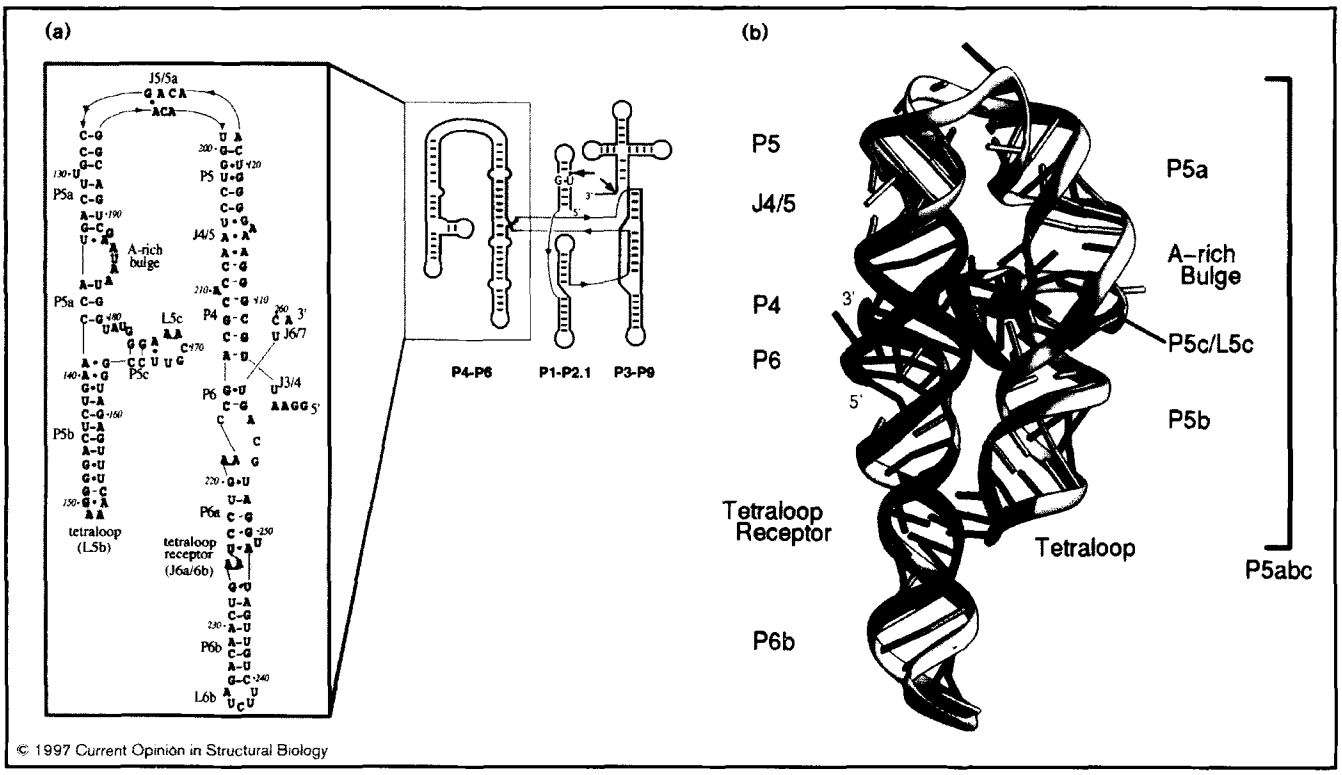
Most of the contacts that stabilize the P4–P6 domain structure, as well as the packing in the crystal lattice, involve the wide and shallow minor groove as opposed to the deep and narrow major groove. However, osmium (III) hexammine, the compound used to determine the RNA structure, binds at three locations in the major groove where nonstandard base pairs create pockets of negative electrostatic potential [37••]. In two cases, the heavy atoms occupy sites normally bound by magnesium in the native RNA [37••]. One of the motifs involved, tandem G·U wobble pairs, occurs frequently in ribosomal RNAs, suggesting a mechanism for metal binding in the ribosome [38].

Duplex structures

Although the hammerhead and P4–P6 domain crystal structures provide many new insights into RNA tertiary folding and catalysis, these structures have been solved at modest resolution (2.6–3.1 Å). Some recently solved RNA duplex structures are at higher resolution, which allows a detailed look at RNA metal binding, mismatch base pairing, base bulging, helix packing, and hydration.

Most structured RNAs require divalent metal ions for folding, and ribozymes generally need them for catalysis [39]. Several divalent ions have been located in

Figure 2



The *Tetrahymena* group I intron P4–P6 domain. **(a)** Secondary structure of the *Tetrahymena* group I intron [25,62]. Elements of the conserved catalytic core are shaded in the right panel. The P4–P6 domain is enlarged in the left panel, with the clamp regions (see text) highlighted by shading. **(b)** Tertiary structure of the P4–P6 domain [29••]. The orientation is rotated 180° around the vertical axis of the secondary structure to emphasize the A-rich bulge. Segments involved in the clamp between the helical stacks are shown in dark gray. J, joining region; L, loop; P base-paired region. P5a, P5b, P5c, helices of the extension.

the hammerhead and P4–P6 domain crystal structures [4,5••,12••,29••,37••], but their functional significance remains unclear. One divalent metal ion binding site seen in the hammerhead structures has also recently been found in the crystal structure of a duplex containing sheared G·A and asymmetric A·A base pairs [40••]. The site occurs at a C·G pair followed by the sheared G·A pair. Interestingly, tandem G·A mismatches have been found near the active sites of a lead-dependent ribozyme and a RNA ligase ribozyme, and they occur frequently in ribosomal RNA [41–43]. Thus, this motif may turn out to be a common way to position divalent metal ions within an RNA structure.

Noncanonical or mismatch base pairs are common in RNA [44]. Internal loops of rRNA often contain a high proportion of adenosines [44]. In addition to the AA platform and tandem sheared pairs discussed above, many other pairing patterns are likely to occur [20,21,22•,45•,46]. One possible example has been seen in a symmetric duplex containing 5′-GAAA-3′ surrounded by Watson–Crick pairs [40••]. In this structure, tandem asymmetric A·A pairs are sandwiched between sheared G·A pairs. Another common motif that occurs in rRNA involves tandem U·U pairs [44]. Although three duplex structures contain these, the structures of the U·U tandems and the effect of flanking sequences on them vary [47••–49••]. Two of the duplexes, which have internal U·U tandems, form U·U wobble pairs [47••,49••]. Interestingly, the number of hydrogen bonds between the U·U wobble pairs changes depending on the flanking base pairs. This pattern is consistent with effects seen in thermodynamic studies [45•], but crystal packing forces may also effect the base pair geometry. In the third example, the U·U tandems form at the end of a duplex in an intermolecular contact [48••]. These U·U tandems form unusual Hoogsteen pairs, in which the N3–H and O4 of one uridine hydrogen bond to the O4 and C5–H of the other. While it is not clear whether tandem Hoogsteen U·U pairs could form in the middle of a duplex region, these types of U·U pairs might occur at the end of a helix. More importantly, the structure provides clear examples (at 1.4 Å resolution) of CH–O hydrogen bonds in base pairs and provides a model for U·Ψ base pairs in RNA.

RNA packing and hydration play important roles in RNA function, as highlighted in recent experiments involving large entropic contributions to ΔG [50,51]. One recent structure of an RNA–DNA chimera duplex highlights the variability induced in A-form helix geometry and helical packing due to a single looped-out adenosine

[52•]. In addition, the conformation of the looped-out adenosine provides some insight into why the backbone of some looped-out bases is susceptible to Mg^{2+}-induced hydrolysis. Two high-resolution structures reveal in detail the pattern of hydration of G·C base pairs [48••,53,54••], while a lower resolution structure sheds new light on the hydration of A·U pairs ([55•]; see also [56]). Two groups play key roles in the hydration patterns: the 2′-OH; and the pro-R_p phosphate oxygen (O1P). As 2′-OH groups play important roles in RNA packing [29••,33,34•,47••,57–59,60•], the heavy involvement of the 2′-OH group in hydration is a major factor to consider in thermodynamic studies of RNA–RNA interactions.

Conclusions

Although the new RNA crystal structures provide many insights into RNA folding and catalysis, exciting challenges lie ahead. The hammerhead catalytic center is now the best understood of numerous ribozyme active sites; the others remain mysteries. Furthermore, the structures and roles of RNA in telomerase, the ribosome and signal recognition particle, and in the spliceosome remain to be tackled by crystallographers. Careful biochemical experiments that have been critical in solving and understanding the hammerhead and P4–P6 domain structures will be the key for structural studies of these other RNAs.

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