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Structure, folding and mechanisms of ribozymes

David MJ Lilley

The past two years have seen exciting developments in RNA catalysis. A completely new ribozyme (possibly two) has come along and several new structures have been determined, including three different group I intron species. Although the origins of catalysis remain incompletely understood, a significant convergence of views has happened in the past year, together with the discovery of new super-fast ribozymes. There is persuasive evidence of general acid-base chemistry in nucleolytic ribozymes, whereas catalysis of peptidyl transfer in the ribosome seems to result largely from orientation and proximity effects. Lastly, important new folding-enhancing elements have been discovered.

Addresses
Cancer Research UK Nucleic Acid Structure Research Group, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, UK

Corresponding author: Lilley, David MJ (d.m.j.lilley@dundee.ac.uk)

Current Opinion in Structural Biology 2005, **15**:313–323

This review comes from a themed issue on
Nucleic acids
Edited by Dinshaw J Patel and Eric Westhof

Available online 24th May 2005

0959-440X/$ – see front matter

DOI 10.1016/j.sbi.2005.05.002

Introduction

Ribozymes are catalytic RNA molecules – RNA species that behave like enzymes. They carry out important roles in all cells and can potentially teach us valuable lessons about the origins of catalytic activity in macromolecules in general.

RNA catalysis may have played a key role during the early evolution of life on this planet [1,2]. If RNA served dual roles as both informational and catalytic RNA molecules, this could solve a tricky 'chicken and egg' problem of how a translation system could be evolved out of nothing. Using *in vitro* selection, many RNA activities that might have been required to support a primitive metabolism have been generated, offering a kind of proof of principle [3–7]. RNA species that catalyse some quite exotic chemical reactions, such as Diels–Alder electrocyclic addition [8–10] and Michael addition [11], have been generated. Allostery has also been engineered into ribozymes [12–15]. The RNA world could have existed around 3.5 billion years ago, but these primitive organisms would have been relatively quickly supplanted by protein-based life.

Nevertheless, RNA catalysis is widespread in the contemporary world and some activities may even be molecular 'fossils' of that period. Over 1500 group I ribozyme sequences are now known. RNaseP catalyses the maturation of tRNA in all kingdoms of life and the peptidyl transferase activity of the ribosome carries out what is arguably the most important reaction in the cell. It is also rather likely that the splicing of mRNA is fundamentally RNA catalysed. Although this has not yet been proved, the similarity to the chemistry of group II intron splicing, the sensitivity of the reaction to phosphorothioate substitution [16] and the observation of intrinsic low-efficiency cross-linking reactions [17,18] all point in that direction, suggesting that the complex between U2 and U6 may be the ribozyme at the heart of the spliceosome.

After a somewhat lean period, new natural ribozymes have been emerging again. Two totally new cleavage activities have emerged in the past year [19,20•], both of which appear to function in the control of gene expression. Several new crystal structures have also appeared during recent months that provide new perspectives on mechanisms. The origins of catalysis have been difficult to pin down with any certainty, are often controversial and sometimes hotly debated. Yet some consensus is perhaps now emerging and we can try to extract some general principles. Some new approaches are having a significant impact in the field. Single-molecule biophysics in particular has seen considerable success in studying the folding and catalysis of ribozymes [21,22,23•,24•], and the fundamental steps of ribozyme action can now be followed by what might be termed single-molecule enzymology [25••].

Discovery of new ribozymes in nature

The riboswitches are a group of control elements in (mainly prokaryotic) mRNA that regulate gene expression by selectively binding a metabolite related to the function of the gene [19]. For the most part, they are natural aptamer species coupled to elements that can switch secondary structure to sequester or release sequences required for transcriptional termination or initiation of translation. However, one of the most recent examples turns out to be a metabolite-responsive nucleolytic ribozyme [26••]. It is present in the mRNA of *glmS*, the *Bacillus subtilis* gene that encodes glucosamine fructose-6-phosphate aminotransferase. The product is terminated by a cyclic 2′3′ phosphate, indicating a

transesterification reaction initiated by nucleophilic attack of the 2′ oxygen in the same manner as most other nucleolytic ribozymes. Interestingly, cleavage is activated 1000-fold by the binding of its effector molecule, glucosamine-6-phosphate. It binds the effector with K_D = 200 μM, inducing site-specific cleavage of the RNA at a rate of 3 min^{-1} in the presence of Mg^{2+} ions. The minimal core sequence of the *glmS* ribozyme has been defined and contains two predicted stem-loop structures; however, there are no structural data available yet.

An intriguing new activity has emerged in human β-globin mRNA, which undergoes co-transcriptional processing. Proudfoot and co-workers [20•] have found that an element in the 3′-flanking region is responsible for RNA cleavage. They have narrowed this down to a 200-nucleotide RNA that undergoes self-cleavage at a particular site in the presence of GTP and Mg^{2+} ions. The kinetics are biphasic, with a faster rate of 1 min^{-1}. The RNA has the potential to form considerable secondary structure, with loops and junctions in abundance, and the cleavage site is contained within a region that exhibits some similarity to the hammerhead ribozyme. However, current indications are that cleavage generates 3′-hydroxyl and 5′-phosphate termini, implying a different chemistry from the standard nucleolytic ribozymes, perhaps akin to RNaseP. So this system requires further investigation before it can be counted amongst the established ribozymes; however, if confirmed, it could be the forerunner of an important new class of functional cellular catalytic RNA species.

Ribozyme structures

X-ray crystal structures have provided much valuable insight into ribozyme mechanisms [27–34] and several new structures have recently become available.

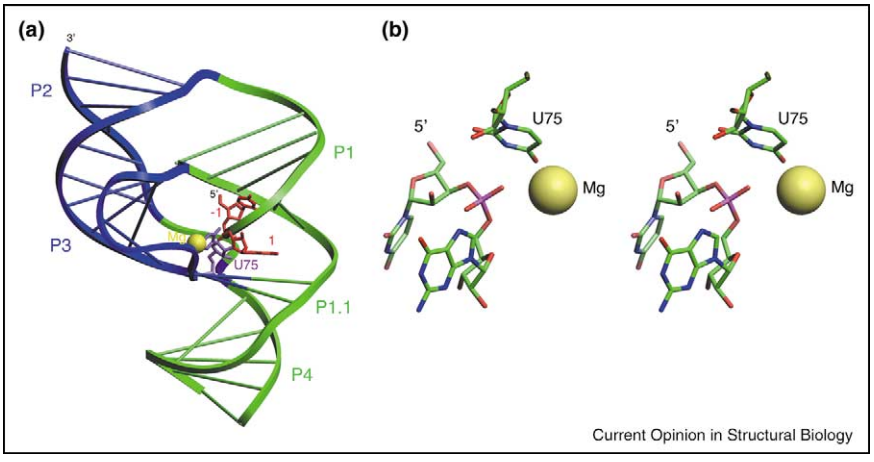
The hepatitis delta virus ribozyme

Ten new crystal structures of the hepatitis delta virus (HDV) ribozyme at different stages have been obtained by Doudna and co-workers [35•] (Figure 1). The overall fold remains unchanged from the original structure determined for the product [32], with the double-pseudoknot fold. However, a structure of the C75U variant now reveals significant local conformational change in the precursor species. The 5′ end of the substrate makes a sharp turn at the scissile phosphate and, in the absence of this, there is movement of the P1 and P3 helices towards the centre, closing the active centre of the ribozyme. It is suggested that the structural rearrangement is coupled to the cleavage activity of the ribozyme and that one role of C75 is to position the substrate for in-line attack. In contrast to the product structure, a divalent metal ion is found coordinated in the active site by outer sphere contacts, where it contacts the 5′-oxygen atom of the leaving phosphate group.

The group I intron ribozyme

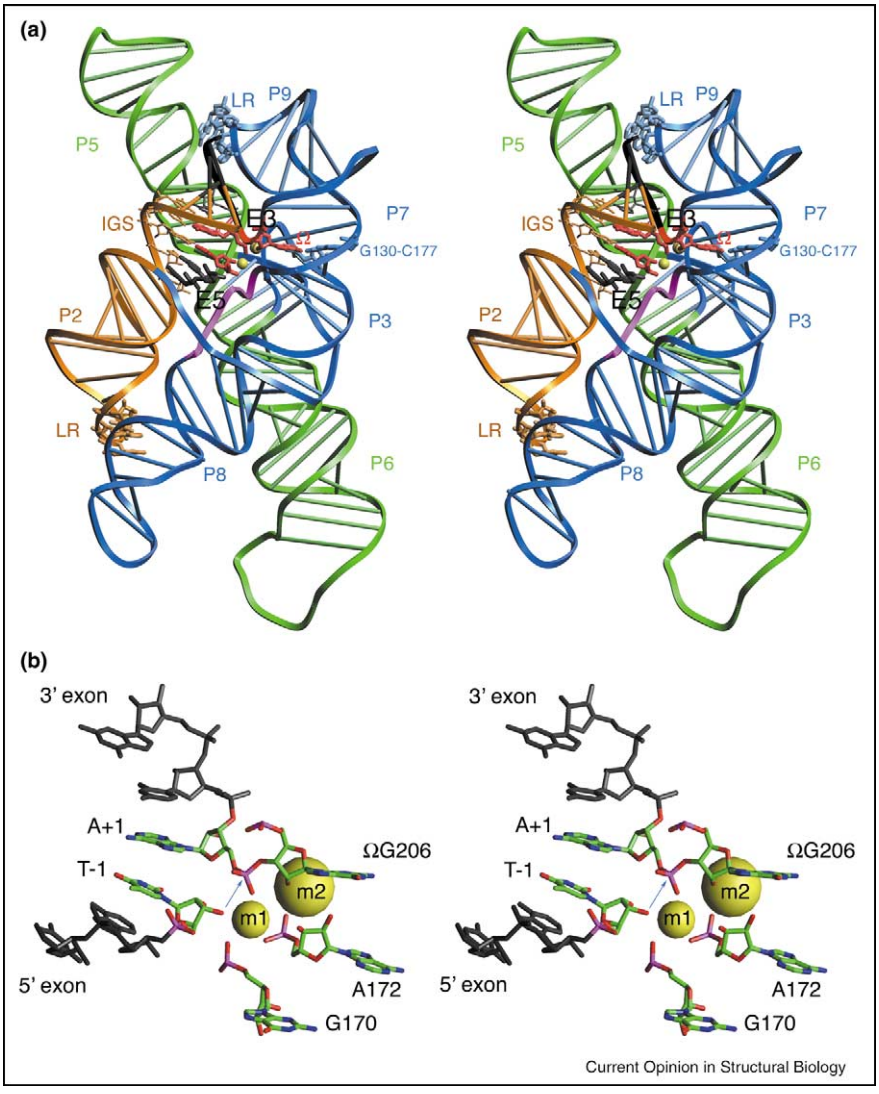
Several new structures have provided perspectives on different stages of the splicing reaction of group I intron ribozymes from different sources. The structure of the *Tetrahymena* ribozyme has been solved at the pre-reaction stage, showing the aporibozyme before the docking of the P1–P2 substrate helix [36], with a new structure at 3.8 Å resolution [37•]. Strobel and co-workers [38••] have presented the structure of the *Azoarcus* ribozyme at 3.1 Å resolution, complete with RNA fragments representing both 5′ and 3′ exons, and poised at the step before the second transesterification reaction that will join the exons (Figure 2). Lastly, Golden *et al.* [39•] have solved a 3.6 Å structure of a relatively elaborated ribozyme of phage origin that is bound to a 4-nucleotide RNA product of the first reaction. The architecture of

Figure 1



Structure of the HDV ribozyme. **(a)** Structure of the complete ribozyme as a C75U mutant, representing the structure before the cleavage reaction [35•]. The Mg^{2+} ion is shown as a yellow sphere and key nucleotides are in stick form. **(b)** Parallel-eye stereoscopic image of the active site, showing the Mg^{2+} ion and the C75U base juxtaposed with the scissile phosphate group.

Figure 2



Structure of the group I intron, complete with fragments of both exons. **(a)** Parallel-eye stereoscopic image of the group I intron ribozyme at the stage preceding the second transesterification reaction [38••]. The P4–P6 domain is coloured green, the P9-P7-P3-P8 domain is blue and P1–P2 is orange. The exons are coloured black, and are labelled E5 (the 5′ exon) and E3 (the 3′ exon). The base pairing between the exons and the IGS, close to the reaction centre, is shown in stick form, with nucleotides T−1 and A+1, which flank the site of reaction, coloured red. The ΩG nucleotide at the 3′ end of the intron (red, stick form) is inserted into the guanine-binding pocket in P7, where it makes a base triple interaction with G130•C177. Tertiary loop–receptor interactions are indicated LR. Two metal ions are shown as yellow spheres. **(b)** Parallel-eye stereoscopic image of the active site, showing the attack of the 3′ hydroxyl of T−1 on the phosphodiester linkage between ΩG206 and A+1. The site is surrounded by phosphate groups that coordinate two metal ions. m1 is assigned as a Mg^{2+} ion. m2 is a K^{+} ion in the crystal, although this is likely to be replaced by Mg^{2+} with full ribonucleotide substitution.

the core of all three ribozymes is remarkably conserved and in good agreement with earlier predictions [40,41]. The overall structure consists of a bundle of three coaxially stacked helical associations comprising the P1 and P2 helices (formed by the substrate–IGS [internal guide sequence] interaction), the P4–P6 helices, and the P9, P7, P3 and P8 helices. These are linked by several tertiary interactions. In the *Azoarcus* structure, the 3′ hydroxyl of the 5′-exon fragment is positioned for in-line attack on the phosphate of the ΩG located at the 3′ end of the intron. In all of the structures, this guanine is snugly held in a pocket in P7, where it makes a triple-base interaction with a C•G pair, exactly as deduced from 15-year-old biochemical experiments [42]. Of course, in the first reaction, this binding site is occupied by the exogenous guanosine molecule that provides the 3′-hydroxyl nucleophile.

On the basis of variation in reaction rate as a function of ligand substitution and metal ion type, three functionally

important ion-binding sites have been proposed in the group I intron [43–45]. For the first transesterification reaction, metal ion M_A is bound to a non-bridging oxygen atom of the scissile phosphate and the 3′ hydroxyl of the 5′ splice site (position –1), M_C is bound to the same non-bridging oxygen atom and the 2′ hydroxyl of the attacking guanosine, while M_B is bound to the 3′ hydroxyl of the attacking guanosine. Metal ions have been found bound at the active sites of all the group I ribozyme structures [37•,38••,39•]. The active centre of the *Azoarcus* ribozyme has a very high density of phosphate groups, which coordinate two metal ions corresponding to M_A and M_C (flanking the scissile phosphate); these have been assigned to Mg^{2+} and K^+ ions, respectively [38••] (Figure 2b). The Mg^{2+} ion is maximally coordinated to phosphate oxygen atoms, with five out of six inner-sphere ligands exchanged. The K^+ ion would be expected to be replaced by Mg^{2+} in the natural ribozyme, which lacks the deoxyribonucleotide substitution of the crystallised complex. A metal ion corresponding to M_A was also observed in the phage ribozyme [39•].

RNaseP

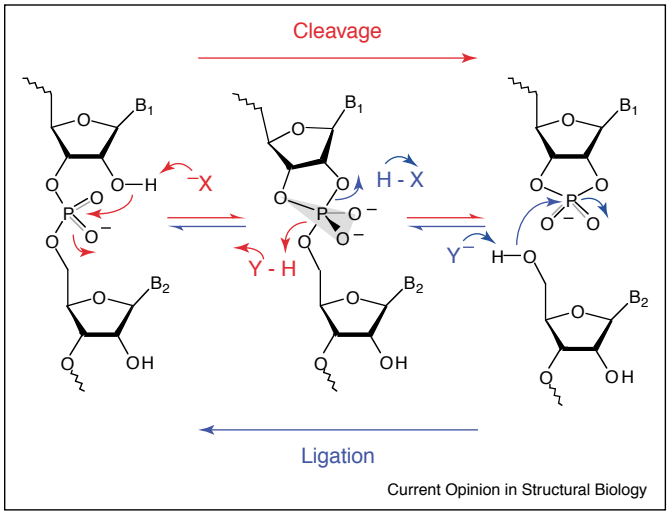
The structure of the all-important RNA component of RNaseP (P-RNA) can be divided into two domains: catalytic and recognition. Unfortunately, there are no high-resolution structural data on the catalytic domain. However, the structures of the recognition domains of the P-RNAs of *B. subtilis* [46•] and *Thermus thermophilus* [47•] RNaseP have been solved by Mondragon and collaborators. In both cases, the RNA adopts a compact fold, organized by a complex helical junction and several tertiary interactions. The overall folds differ, particularly in the nature of the tertiary contacts that stabilize the structures, but the important core regions are structurally conserved. Both contain a prominent four-way junction with an antiparallel geometry. This probably sets up the binding site for the pre-tRNA substrate and the interaction with the catalytic domain of the RNA.

Mechanisms of action and the origins of catalytic activity

Rates of reactions in nucleolytic ribozymes

With the marked exception of peptidyl transfer, the great majority of contemporary ribozymes perform phosphoryl transfer reactions of various kinds: transesterification reactions initiated by nucleophilic attack on a phosphate by the adjacent 2′ hydroxyl (nucleolytic ribozymes), by a remote nucleotide (group II intron) or by the 3′ hydroxyl of exogenous guanosine (group I intron). These closely related reactions are probably subject to a similar group of potential mechanisms for enhancing rates. It is therefore useful to focus on one group as an example — the nucleolytic ribozymes. These bring about cleavage of the phosphodiester backbone by a transesterification reaction, whereby a specific 2′-oxygen atom attacks the adjacent 3′ phosphorus with expulsion of the 5′ oxygen to create a cyclic 2′3′ phosphate (Figure 3). The reverse ligation reaction can also be catalysed, if the correct form of the ribozyme is chosen [48,49•]. Until recently, it was

Figure 3



The chemical mechanism of the nucleolytic ribozymes. The nucleolytic ribozymes undergo cleavage and ligation reactions by an associative reaction, involving in-line nucleophilic attack of the 2′ (cleavage) or 5′ (ligation) oxygen atom on the adjacent phosphorus to generate the dianionic oxyphosphorane high-energy intermediate, which will be closely related to the transition state. General acid-base catalysis is a probable mechanism of catalysis in these ribozymes, with a general base removing the proton from the hydroxyl to generate a much stronger nucleophile and a general acid protonating the oxyanion leaving group. Note that the roles of acid and base are inverted when the reaction direction is reversed. Further catalytic strategies are stabilization of the doubly charged transition state and the use of the local RNA structure to facilitate the trajectory into the transition state. Similar mechanistic considerations will apply to other ribozymes involved in phosphoryl transfer reactions.

commonly observed that the cleavage reaction proceeded at around 1 min^{-1} for most natural ribozymes, reflecting acceleration by a factor of around 10^5 over the intrinsic rate in a flexible dinucleotide at neutral pH [50]. Moreover, single-molecule fluorescence resonance energy transfer (FRET) experiments that can disentangle chemical transformation from undocking processes have shown that cleavage and ligation rates in the hairpin are 1 and 18 min^{-1}, respectively [25••]. It has been suggested that there is some kind of natural 'speed limit' that restricts ribozyme reactions to this rate, at least for a series of selected species [51,52••]. However, it has recently emerged that it is possible to engineer some ribozymes with the result that very much faster rates can be obtained. A novel connection engineered between the VS ribozyme and its substrate resulted in rates of up to 10 s^{-1}, a 100-fold increase compared to previous observations [53••]. Ligation rates of $\geq$1 s^{-1} are also obtainable using a tethered substrate (A McLeod and DMJ Lilley, unpublished). Similarly, using a form of the hammerhead ribozyme with auxiliary loops [54••], cleavage rates of 15 s^{-1} have been measured [55•].

Possible contributions to catalytic rate enhancement

The transesterification reaction of the nucleolytic ribozymes results in inversion of the stereogenic centre at the phosphorus [56–58] and thus proceeds by an associative S_N2 reaction mechanism (Figure 3). In principle, there are several stages at which a catalyst could intervene to accelerate this reaction. General acid-base catalysis could be used to increase nucleophile strength by deprotonation [59] and to protonate the oxyanion leaving group. By the principle of microscopic reversibility, a group acting as an acid or base in the cleavage reaction will of course function as a base or acid, respectively, in the ligation reaction. A common enzymatic strategy is stabilization of the transition state. In this case, the species is dianionic, and should be stabilized by protonation or subject to electrostatic catalysis by juxtaposition of positive charge. Another frequent source of rate enhancement in enzymes comes from manipulating the proximity and orientation of reactants. The transition state of the reaction will closely resemble a trigonal bipyramidal oxyphosphorane, in which there is a colinear arrangement of the 2′-oxygen, 3′-phosphorus and 5′-oxygen atoms. Estimation of the closeness to in-line geometry turns out to be a reasonable predictor of cleavage rates in RNA [60].

Breaker and colleagues [52••] have estimated the maximal rate enhancements available from specific acid-base catalysis by complete deprotonation of the 2′ oxygen, and complete protonation of the non-bridging and 5′ oxygen in the cleavage reaction, over pH ranges in which the effects are operative, each giving values in the range 10^5 to 10^6. By contrast, the effect of constraining the geometry to that of in-line attack is probably worth 10^2 or less. If all these effects were operating independently, a potential rate enhancement of around 10^{19} would be available. In general, the catalytic power of ribozymes is much less than this, and Breaker and co-workers have proposed that many ribozymes are largely restricted to alignment and 2′-oxygen deprotonation over the normally available pH range of 5 to 9 [52••]. Faster ribozymes operating at >1 s^{-1} [53••,55•] imply that further catalytic processes must be operating in these cases.

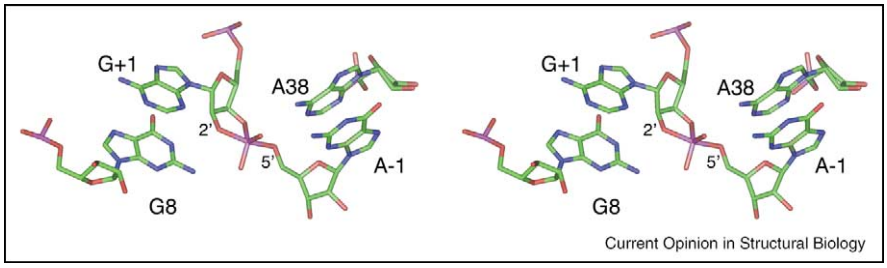
Functional elements in ribozyme catalysis

One of the intriguing aspects of RNA catalysis is the relative paucity of functional groups available compared to protein enzymes. This largely reduces to three potential catalytic resources. First, the RNA structure can be used to position and orient the substrate. Second, metal ions could be used in several ways, in addition to their structural role: as Lewis acids, to provide positive charge; and as a source of water molecules to act in acid-base catalysis. Specific metal ion binding appears to be dispensable for some ribozymes, but ions have been observed bound in the active sites of the HDV [35•] and group I intron ribozymes [38••] recently. Last, the nucleobases may play a direct role in the chemistry, either as general acids and bases, or via electrostatic effects. The natural pK_a values of the nucleobases are far from optimal under normal conditions. For example, the pK_a of adenine N1 is 3.5. Although this means that it is a relatively strong acid, only one molecule in 10 000 will be in the protonated (i.e. acidic) form at pH 7.5. By contrast, although the vast majority of molecules will be unprotonated and could act as a base, it will be weak because it is the conjugate base of a strong acid. However, the pK_a is likely to be perturbed in the context of the active site of a ribozyme and significantly elevated adenine pK_a values have been measured in catalytic RNA species by NMR [61]. Moreover, Bevilacqua [62••] has pointed out that the lower concentrations of species with more extreme pK_a values can be offset, to some degree, by the natural reactivity of these species.

The hairpin ribozyme

Ferré-d'Amaré and co-workers [34] have solved the crystal structure of the hairpin ribozyme containing a pentacoordinate vanadate transition state analogue. The vanadate makes several hydrogen bonds with G8 and A38, suggesting that transition state stabilization is an important aspect of the catalytic mechanism (Figure 4). G8 has previously been suggested to play a direct role in the chemistry of the transesterification reaction [63]; substitution by uridine leads to a marked loss of activity while barely affecting ribozyme folding [64]. These nucleobases are likely to play an important role in general acid-base chemistry (as illustrated in Figure 3), analogous to the two active site histidine residues of RNaseA. Their positions in the crystal structures allow a fairly unequivocal assignment of their potential roles, with G8 acting as the base and A38 as the acid in the cleavage reaction

Figure 4



Parallel-eye stereoscopic image of the active site hairpin ribozyme, as seen in the crystal structure of a transition state analogue. Note that the nucleobases of G8 and A38 are positioned such that they could act as general base and acid, respectively, in the cleavage reaction.

[62••]. Using the junction form of the ribozyme, both the cleavage and ligation activities are dependent on pH. Both rates increase with pH until reaching a plateau around neutrality [25••,65•], corresponding to a pK_a in the region of 5.5–6.5. The simplest interpretation of the cleavage reaction is that the low pH region is the sum of the contributions from A38 (the acid), with a perturbed pK_a of around 6 (so that the concentration of the protonated form is flat at lower pH), and G8 (the base) that becomes protonated at N1 below its pK_a of 10 (illustrated by the simulations in Figure 5). Removal of the base at position 8 (abasic) led to a marked loss of activity, but the same pH dependence [65•]. This supports the contention (although this is not yet proven) that the pK_a of ~6 is due to A38 and, under these conditions presumably something else, perhaps a hydrated metal ion, functions as the base. The predicted reduction of rate above the pK_a of guanine is not accessible experimentally with RNA. However, substitution of G8 by 2,6-diaminopurine or 2-aminopurine (normal pK_a = 5.1 and 3.8, respectively) reduced the pK_a of the base, so that the reduction of the rate with increasing pH was revealed [63]. The role of G8 has been further probed by studying the effects of exogenous bases on the activity of a ribozyme abasic at this position [65•,66]. Activity was restored by high concentrations of cytosine, isocytosine or 2-aminopyridine in the medium. The activity restored by cytosine exhibited bell-shaped pH dependence for both cleavage and ligation, corresponding to pK_a values of 5.6 and 6.4 for cleavage. It is not possible to know which corresponds to A38 and which to the exogenous cytosine from these data. The apparent role of the general acid (A38) in the cleavage reaction argues that the rate of ~1 min^{-1} is not due to limiting the catalytic strategies to alignment and general base catalysis, for this ribozyme at least.

There is presently no evidence of the direct participation of metal ions in the chemistry of the hairpin ribozyme. The ribozyme exhibits good levels of activity in the presence of the substitutionally inert hexammine cobalt (III) ion [67–69] and high concentrations of monovalent metal ions [70]. Moreover, no metals ions have been observed bound in the active site of any of the different crystal structures [33,34]. Internal conversion rates observed by single-molecule enzymology were only weakly dependent on Mg^{2+} ion concentration [25••], perhaps consistent with a minor role in the electrostatic stabilization of the transition state.

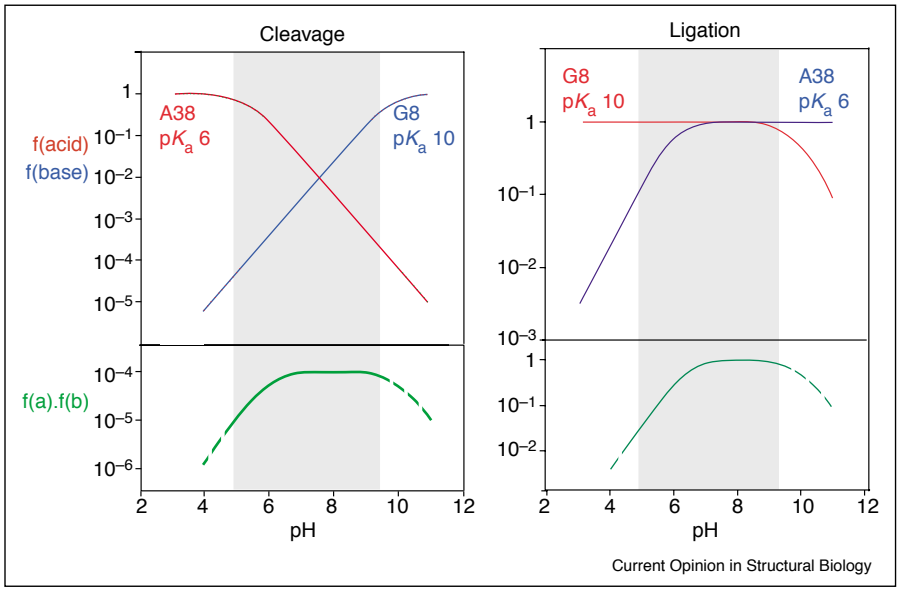
The hepatitis delta virus ribozyme

Crystal structures of the HDV ribozyme [32,35•] show that the substrate lies in a cleft of the RNA, with cytosine 75 (C76 in the anti-genomic HDV ribozyme) adjacent to the scissile phosphate (Figure 1). This suggests a role for the nucleobase in the catalytic chemistry, but assigning the exact role is more difficult than for the hairpin ribozyme. Substitution of the cytosine for uridine led to a marked loss of cleavage activity, but this could be partly restored by the addition of high concentrations of imidazole in the medium [71], providing powerful support for a role of the nucleobase in general acid-base chemistry. The pH dependence of the cleavage reaction is similar to that of the hairpin ribozyme, the rate increasing with pH up to a plateau, corresponding to a pK_a of 6.3. However, in the absence of divalent metal ions, the dependence reversed, with the rate of cleavage increasing with lowering of pH [72]. These data can be understood in terms of general acid-base catalysis, with C75 acting as the acid and a hydrated divalent metal ion as the general base (i.e. performing the role proposed for G8 in the case of the hairpin ribozyme). This requires an elevation in the pK_a of the cytosine from ~4.3 for the free base. As this was not supported by NMR measurements of chemical shift [73], the perturbation may occur only with the development of the transition state.

Peptidyl transferase

The rate of peptide bond formation is accelerated by around 10^7-fold in the context of the ribosome. The crystal structure of the 50S subunit revealed that there is no protein component in the vicinity of the peptidyl transferase centre [74], confirming earlier indications that the reaction is catalysed by the 23S rRNA component [75]. It was noted that N3 of A2451 (*Escherichia coli*

Figure 5



Calculated proportions of protonated and unprotonated forms of a general acid and base with pK_a values of 6 and 10 as a function of pH. For the cleavage reaction, the acid and base have pK_a values of 6 and 10, respectively, and may correspond to A38 (with shifted pK_a) and G8, respectively, whereas the roles are reversed for the ligation reaction. The grey region between pH 5–9 corresponds to the experimentally accessible range at which RNA is stable. Within this range, the fraction of ribozyme in the correct state of protonation for either cleavage or ligation increases with pH up to a plateau around pH 7. This corresponds to the titration of the group with pK_a = 6. The absolute levels of the fractions are very different for the two reactions, although variation in intrinsic reactivity is likely to reduce the significance of this, as discussed in detail by Bevilacqua [62••].

notation) was close to the transition state analogue. It was proposed that this base was responsible for removing a proton from the amino group of the A-site-bound aminoacyl-tRNA in the process of attacking the carbonyl of the growing peptide. It was further proposed that a charge relay system involving G2447 would be responsible for altering the pK_a of this base nitrogen atom. This mechanism has proved controversial and is unlikely to be correct in its original form. Mutational analysis indicated that these nucleotides were not essential for activity [76,77], and a recent study has indicated that the nucleotides closest to the active centre (including A2451) are required for peptide release but not peptide bond formation [78•].

Most recent work has involved the rapid reaction kinetics of a condensation reaction between a dipeptidyl-tRNA in the P-site and puromycin in the A-site, for which conditions can be employed whereby the chemistry appears to be rate limiting [79]. Rodnina and colleagues showed that this reaction was dependent on the titration of a ribosomal group with a pK_a of 7.5 [79], a value that remained unaltered when G2447 was changed to adenine [80•]. Although this might indicate a contribution from acid-base catalysis, it was noted that it could only contribute a relatively small rate enhancement, in the absence of which the residual reaction was still 1000-fold faster than the uncatalysed reaction. Comparison of the temperature dependence of peptide bond formation by the ribosome with that of a related uncatalysed reaction showed that the difference was not enthalpic, but lay in a favourable entropy of activation for the ribosome-catalysed reaction — a difference of almost 60 kJ mol^{-1} [81•]. The ratio of the two rates gave an effective concentration of 10^5 M. A favoured interpretation of these data is that most of the rate enhancement arises from orientation and proximity of the substrates. Green and co-workers [78•] have also concluded that orientation and proximity of the substrates is the major factor in ribosomal rate enhancement. However, a recent study has suggested another factor important in the reaction, that of substrate assistance. Weinger *et al.* [82•] showed that peptide bond formation was reduced one-million-fold by loss of the 2′ hydroxyl of the 3′-terminal adenosine of the P-site tRNA. Although conformational effects probably cannot be excluded, the authors argue that this hydroxyl is poised to remove the amino group proton and could thus serve as a general base in the reaction. The mechanism of peptidyl transferase is likely to remain a hotly debated subject for some time to come — we are unlikely to have heard the last chapter of this story.

RNA folding and catalysis, and the role of auxiliary elements in small RNA species

It is self-evident that there is an intimate connection between RNA folding and catalysis. Catalytic

RNA molecules must be folded into the correct geometry for activity; this normally requires interaction with metal ions to achieve the required structure. Enzymes frequently exploit the energy of substrate binding to contribute to the catalytic process and we have seen that the deformation of local RNA conformation towards in-line geometry could lead to significant acceleration of a transesterification reaction in ribozymes. However, further connections between catalysis and folding have recently emerged. Khvorova *et al.* [54••] observed that versions of the hammerhead ribozyme complete with flanking sequences (such as that from tobacco ringspot viral RNA) were active under physiological conditions, whereas the more conventional minimised sequences were not. The natural sequences contained terminal or internal loops within helices II and I that modelling indicated might interact. This suggested that loop–loop interaction might drive the folding of the ribozyme at relatively low Mg^{2+} ion concentrations by short-circuiting the normal two-stage folding pathway [83]; this was proved to be the case by FRET experiments [84•]. The loops act as 'folding enhancers' and incidentally provide a cautionary tale that illustrates the danger of being too 'scissor happy' when trimming ribozymes down to the smallest unit that is still active. The example of the hammerhead ribozyme is not unique. The hairpin ribozyme is organized around a four-way junction, in the presence of which folding occurs at Mg^{2+} ion concentrations that are reduced by three orders of magnitude [85]. Single-molecule FRET studies have shown that the presence of the four-way junction introduces a discrete intermediate into the folding process, resulting in a 500-fold acceleration of loop–loop docking [24•] compared to the hinged form [21].

The junction also influences the chemistry of the hairpin ribozyme. Fedor [48] showed that the ligation reaction was favoured in this form and new single-molecule data show that the reaction is strongly biased towards ligation, with an internal equilibrium constant of 18 in the presence of 1 mM Mg^{2+} ions [25••]. Similarly, the ligation reaction of the hammerhead ribozyme is favoured if helices II and III are cross-linked [86,87], and the cleavage reaction is greatly accelerated by the addition of the auxiliary loops [55•]. This suggests that the core of the ribozyme is manipulated by the interactions of the remote auxiliary elements, leading to significant perturbation of the chemistry. A more radical rearrangement of the core structure in the transition state has been suggested on the basis of detailed studies of metal ion rescue of multiply phosphorothioate-substituted species [88], but this has to be reconciled with activity observed in ribozymes constrained within crystal lattices [28].

Conclusions and perspectives

The past two years have seen major progress in all aspects of RNA catalysis. Ribozymes are no longer seen as some sleepy backwater of biology, but are known to catalyse some of the most important reactions in cells, including RNA processing, peptide synthesis and probably RNA splicing. And, after a long wait, some brand new ribozymes have come along. Despite the importance of this area, and perhaps what it can teach us about biocatalysis in general, progress in understanding the origins of RNA catalysis has been slow. But the past year or two have seen significant advances and a general consensus on mechanisms is perhaps emerging. The development of super-fast ribozymes coupled with new tools, such as single-molecule approaches, is undoubtedly going to accelerate this process.

Update

The case for A756 acting in the chemistry of the VS ribozyme has been strengthened by an experiment in which the adenosine was replaced synthetically with a novel C-nucleoside containing imidazole in place of the nucleobase [89••]. The modified ribozyme functions in both cleavage and ligation, with rates up to 30-fold below that of the natural species.

Kazantsev and Pace (N Pace, personal communication) have now solved the crystal structure of *Bacillus stearothermophilus* RNaseP RNA at ~3.5 Å. The catalytic core of the ribozyme is formed by long-range interactions in the interhelical regions and some bound metal ions have been identified.

Acknowledgements

I thank Eric Westhof, Marina Rodnina, Tim Wilson and Fritz Eckstein for discussion and comments on the manuscript, and Cancer Research UK for supporting our work on ribozymes in Dundee.

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