



ELSEVIER

Nucleobase catalysis in ribozyme mechanism

Philip C Bevilacqua^{1,2} and Rieko Yajima²

RNA performs a wide range of functions in biology including catalysis of chemical reactions. A major goal in the field of ribozyme chemical biology is to understand these functions in molecular terms. There is increasing evidence that ribozymes can use their nucleobases directly in chemical catalysis in a variety of ways. These include hydrogen bonding to the transition state, stabilizing charge development, and transferring protons as general acid–base catalysts. This article highlights recent kinetic, structural, single molecule, and synthetic approaches that have been used to probe the roles of ribozyme nucleobases in phosphodiester bond cleavage.

Addresses

¹ Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, USA

² The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16802, USA

Corresponding author: Bevilacqua, Philip C (pcb@chem.psu.edu)

Current Opinion in Chemical Biology 2006, **10**:455–464

This review comes from a themed issue on Mechanisms
Edited by Carol A Fierke and Dan Herschlag

Available online 28th August 2006

1367-5931/\$ – see front matter
© 2006 Elsevier Ltd. All rights reserved.

DOI 10.1016/j.cbpa.2006.08.014

Introduction

Until the 1980s, all enzymes were assumed to be protein-based. After all, proteins with their chemically diverse side-chains are better suited for chemical catalysis than are other biopolymers. It came as a shock, then, to discover that RNA can also catalyze chemical reactions. In the years following this discovery, the mechanisms of large ribozymes, such as the group I intron and RNase P, were analyzed. Major advances in the mechanisms of these ribozymes have come in recent years including deeper functional and structural understanding of the roles of divalent metal ions in catalysis.

Concurrent with these studies, a mechanistically distinct class of smaller ribozymes (<100 nt in the catalytic core) was discovered in nature. This class includes the hepatitis delta virus (HDV), hairpin, hammerhead, Varkud satellite (VS), and *glmS* ribozymes. In contrast to the larger ribozymes, this class of self-cleaving and self-ligating ribozymes was found to function proficiently in the absence of divalent ions. This suggested that these ribozymes might use their nucleobases to facilitate bond cleavage.

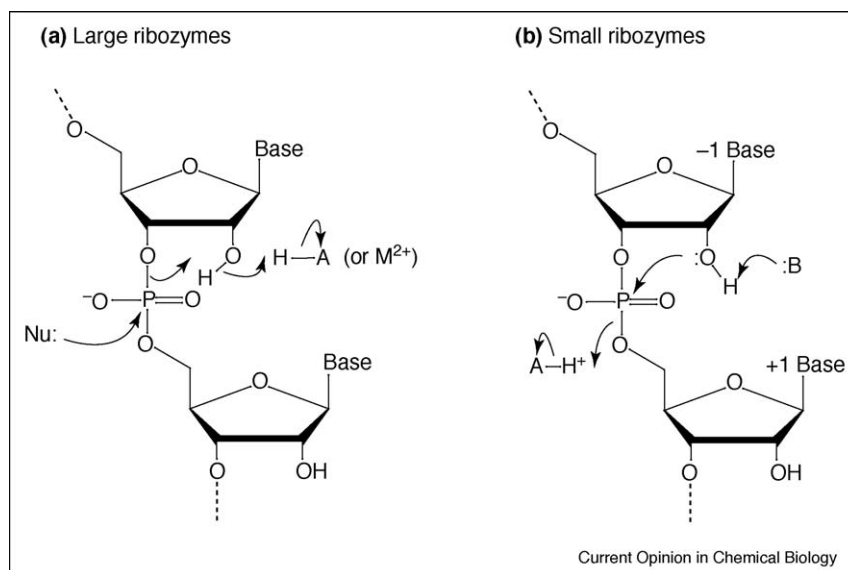
Several reviews detailing the structure and function of RNA enzymes have been written in recent years. These include comprehensive overviews of the field [1,2], structural and folding perspectives [3,4], roles of metal ions in folding and catalysis [5,6], roles of protons in structure and folding [7], model systems of ribozymes [8], and large and small ribozyme viewpoints [9,10]. The present review focuses on a single mechanistic strategy of RNA enzymes: nucleobase catalysis, or the direct involvement of nucleobases in bond making and breaking. This topic merits special attention because of recent advances in the field, its uniqueness in relation to metal ion catalysis, and its similarity to amino acid catalysis by protein enzymes. We discuss advances from kinetic, structural, single molecule and synthetic approaches. Lastly, we attempt to identify key issues that remain unresolved.

Reaction termini suggest unique needs for catalysis

Catalytic RNAs found in nature, with the notable exception of the ribosome, catalyze *trans* or *cis* phosphoryl transfer reactions in which a nucleophile makes an S_N2 attack on a phosphorus center and ejects either the 2′-, 3′- or 5′-bridging oxygen. Ribozyme reactions can be divided into two classes on the basis of the termini generated during self-cleavage reactions. Ribozymes that use an exogenous nucleophile leave 2′,3′-*cis*-diol and 5′-phosphate termini (Figure 1a); the group I intron and RNase P are examples of this class. By contrast, ribozymes that use the vicinal 2′-hydroxyl as the nucleophile leave 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Figure 1b). The first class of ribozymes often needs to bind an exogenous nucleophile and so tends to include larger ribozymes, whereas the second class encompasses the five naturally occurring smaller ribozymes. We focus this review article on the small ribozymes because, as described below, the reaction they catalyze is more reliant on direct proton transfer from a general acid or base. It is helpful to first consider the nature of an uncatalyzed reaction.

A classic approach used in enzymology is to probe the nature of the uncatalyzed reaction. By doing so, one gains perspective on where help is needed and which catalytic devices can best provide it. It has been argued from physical organic studies that the transition state for phosphate diester hydrolysis is synchronous, meaning that it is characterized by partial bond breaking *and* bond making [11]. Brønsted α and β values for general acid–base catalysis are near 0.5 for the HDV ribozyme [12,13], consistent with this notion. As such, there is charge development on both the nucleophile and leaving groups

Figure 1



Cleavage reaction termini for large and small ribozyme reactions. **(a)** The larger ribozymes typically bind an exogenous nucleophile (Nu:) and yield 2',3'-*cis*-diol and 5'-phosphate termini. General acid catalysis can be mediated, directly or indirectly, by the vicinal 2'-hydroxyl. **(b)** The smaller ribozymes use the vicinal 2'-OH as the nucleophile in self-cleavage and yield 2',3'-cyclic phosphate and 5'-hydroxyl termini. General acid catalysis requires participation of a new species, shown here in its functional form as A-H⁺ which could take the form of a protonated nucleobase. By convention, the nucleotide upstream of the scissile phosphate is numbered -1, and the nucleotide containing the scissile phosphate is numbered +1.

in the transition state, suggesting that general acids and bases, as well as properly positioned positive charges, can facilitate rate acceleration. Figure 2 summarizes some of the major strategies that small ribozymes can use to stabilize the transition state for phosphoryl transfer. Similar strategies are used in phosphodiester bond cleavage by RNase A, which leaves the same termini as small ribozymes. RNase A uses two histidines in general acid–base catalysis and a lysine in electrostatic catalysis/transition state hydrogen bonding [14]. Similarities in the mechanisms thus suggest that nucleobases may participate in histidine- and lysine-like roles in ribozymes.

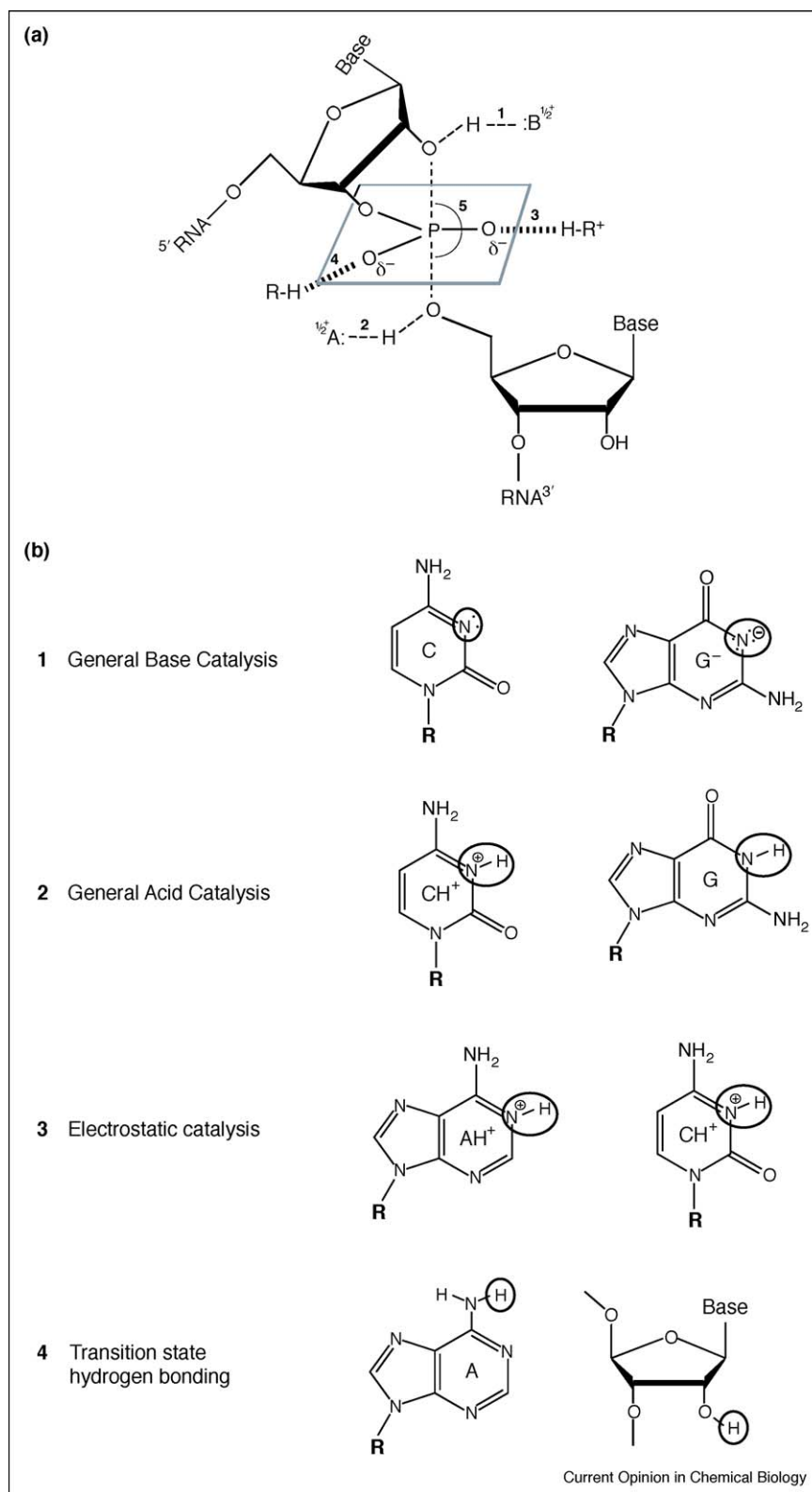
Why might nucleobase catalysis be more important in smaller ribozymes? One important difference between the schemes in Figure 1 is that the leaving group in panel (a) has a vicinal source of somewhat labile protons in the form of the 2'-OH, whereas the leaving group in panel (b) does not. Although the pK_a of the 2'-hydroxyl is in the non-physiological range of 12–15 [7], it can be lowered via coordination to a metal ion or participation in a proton shuttle. Indeed, the vicinal 2'-hydroxyl has been shown to be important in the mechanisms of the group I intron [15] and the ribosome [16]. By contrast, the mechanism in panel (b) lacks a vicinal source of protons and must therefore acquire a proton for the leaving group from another source. One possibility is general acid catalysis by a nucleobase. Indeed, ribozymes that use the mechanism in panel (b) have had, to varying degrees, nucleobase catalysis implicated in their mechanisms.

How general is nucleobase catalysis by ribozymes?

In a seminal study, Murray and co-workers showed that the hammerhead, hairpin, and VS ribozymes are catalytic in the absence of divalent ions, as long as high concentrations of monovalents are present and the pH is kept relatively high [17]. Subsequent studies on the HDV ribozyme confirmed divalent ion-independent activity at low pH [13,18]. In each study, high concentrations of Na₂EDTA were used to sequester polyvalent cations and supply monovalent cations needed to promote RNA folding. Quantitative analysis showed that divalent ions contribute only ~10- to 20-fold to the rate in HDV and hammerhead ribozymes [13,19,20]. This suggested that the primary role for divalent ions is folding and that bond breaking and making is mediated by a non-metal mechanism.

The newly discovered *glmS* ribozyme is part of a riboswitch mechanism that involves binding of glucosamine-6-phosphate, GlcN6P [21]. Like the other small ribozymes, the *glmS* ribozyme leaves 2',3'-cyclic phosphate and 5'-hydroxyl termini. Recently, it has been shown that the *glmS* ribozyme can operate without the direct participation of divalent ions [22^{*}]. The ribozyme has a pseudoknot downstream of its 80 nt core elements that allows it to fold without Mg²⁺ [22^{*},23]. Breaker and co-workers showed that the *glmS* ribozyme is highly active in the presence of EDTA and Co(NH₃)₆³⁺, with the latter being an outer-sphere metal ion complex that is inert to ligand

Figure 2



Transition state model for small ribozyme phosphoryl transfer reactions. In the uncleaved state, A-H is +1 and B is neutral as depicted in Figure 1b. (a) Model assuming a synchronous transition state, as discussed in the text [11]. The bond order of the bonds undergoing making and breaking is thought to be near 1/2. As such, charge development is borne by A and B, the general acid and base for cleavage depicted in Figure 1b.

exchange [22*]. The ribozyme also maintains activity in molar concentrations of KCl used in combination with 25 to 100 mM EDTA. These findings, along with an observed pK_a of 7, support the possible importance of a nucleobase in *glmS* ribozyme catalysis, although it remains possible that the GlcN6P cofactor itself could participate directly in catalysis [21,22*,24,25] (see also Update). In summary, it appears that nucleobase catalysis is quite general, if not ubiquitous, in small, naturally occurring ribozymes. The remainder of this review article is organized according to experimental approaches that have been recently used to probe the role of nucleobases in catalysis, with examples drawn from each of the small ribozymes.

High-resolution structural studies

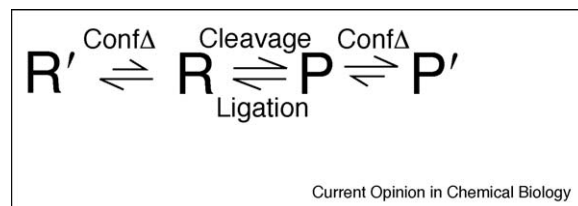
In recent years, several small ribozyme structures have been solved by X-ray crystallography. These reveal complex folds with nucleobases positioned to take on catalytic roles. Figure 3 summarizes a central issue with respect to studying ribozyme mechanism. Four ribozyme states are shown, two of which undergo chemistry in the form of cleavage and ligation, R and P, respectively, and two of which must undergo a conformational change before being able to react, R' and P'. At present, high-resolution structures of hairpin, HDV and hammerhead ribozymes have been solved. A challenge is to determine whether the structures represent R and P, or R' and P'. It is the R and P states that have the potential to tell us how nucleobases contribute to chemical events.

The hairpin ribozyme

The hairpin ribozyme crystal structures are a case wherein the RNA fold is largely consistent with functional data. Three structures were first solved in the Ferre-D'Amare laboratory in post-cleavage, pre-cleavage and transition state (vanadate) conformations using the U1A protein as a crystallization aid [26,27]. These structures were a breakthrough because they provided the first suite of self-consistent pre- and post-cleavage structures of a ribozyme. The structures implicated specific nucleobases in either general acid-base or electrostatic catalysis and have inspired a series of mechanistic studies (detailed below).

More recent structural studies of the hairpin ribozyme come from the Wedekind laboratory, including the crystal structure of a minimal form of the ribozyme [28**]. This work had two important changes. First, it compared the structures of a small catalytic RNA crystallized with and

Figure 3



Four possible states for a ribozyme. Two of these, R and P, are set up to undergo bond breaking (cleavage) and making (ligation) events, whereas the other two states, R' and P', have to undergo conformational changes before being able to react. To understand nucleobase catalysis it is vital to know the structures of R and P. Although R' and P' can be interesting from a regulatory point of view, they may not be directly relevant to chemical catalysis.

without the U1A protein. Second, it tested the structural effects of two gain-of-function mutants. The native structure was found to crystallize in 'exposed' and 'sequestered' conformations. The exposed conformation was similar to the U1A-bound structure, whereas the sequestered conformation differed from the earlier structures [26,27] by up to 13 Å in certain distances. Interestingly, the sequestered conformation was more consistent with certain features of biochemical studies than the exposed conformation. Although the U1A protein does not interfere with the overall fold of the core, it does interact with an important S-turn by symmetry-related interactions. Interestingly, the gain-of-function mutants adopted the sequestered conformation and showed a novel long-range hydrogen bonding network from the S-turn to the O4' of the G+1 nucleotide. Because G+1 contains the scissile phosphate, this new interaction may be of catalytic importance.

Wedekind and co-workers recently solved another suite of structures on the minimal hairpin ribozyme [29*]. In these structures, the catalytically important G8 residue was modified to five different analogs; G8 has been proposed to participate in general base catalysis or electrostatic stabilization. These structures confirmed the importance of the exocyclic amine of G8 in positioning for catalysis and preventing non-productive ground state conformations. In addition, the structure revealed four ordered waters in the active site of the pre-cleavage state, including several near the 2'-hydroxyl of A-1. It remains to be seen whether these waters serve specific acid-base roles in the mechanism, as hypothesized [1] (see also Update).

(Figure Legend Continued) These atoms typically have pK_a values closer to neutrality than secondary or primary alcohols, and are therefore better able to accommodate charge development and avoid unstable intermediates; this is the primary catalytic advantage of general acid-base catalysis. Five means of stabilizing the transition state are depicted and numbered in the figure: (1) general base catalysis (defined in the direction of cleavage); (2) general acid catalysis (defined in the direction of cleavage); (3) electrostatic catalysis; (4) transition state hydrogen bonding; and (5) in-line positioning of the 2'- and 5'-oxygens for S_N2 attack. R is a nitrogen or oxygen atom from a nucleotide and can be positive or neutral depending on the case. (b) Possible sources of the first four means of stabilizing the transition state from the RNA bases and sugar. The unusual anionic guanosine would be consistent with guanosine-mediated general base catalysis in the hairpin [43**,44] and hammerhead [68**] ribozymes. Details are provided in the text.

A recent study from Lambert, Heckman and Burke shows that $\text{Co}(\text{NH}_3)_6^{3+}$ may induce the hairpin ribozyme to fold into a catalytically active conformation that differs from that in Mg^{2+} [30[•]]. The authors used crosslinking and mutagenesis data as input for the MC-Sym modeling program. In contrast to the crystal structure with Mg^{2+} present, in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$, U+2 is bulged out whereas C+3 is stacked on the catalytically important G8. This study suggests that not only can RNA adopt multiple non-functional states, but it can adopt multiple functional ones as well.

The HDV ribozyme

The crystal structure of the HDV ribozyme has been solved by the Doudna laboratory in post- and pre-cleavage states. A structure of the post-cleavage state of the wild-type ribozyme was solved in 1998 [31]. This structure revealed that the N3 of C75 (equivalent to C76 in the closely related antigenomic ribozyme) serves as a hydrogen bond acceptor of the 5'-hydroxyl group of G1, which is the leaving group in the cleavage reaction. On the basis of microscopic reversibility, Nakano, Chadalavada and Bevilacqua suggested that C75 might serve as the general acid in the cleavage step [18]. Mechanistic experiments were consistent with this notion, and they invoked a hydrated magnesium ion (not present in this structure) as the general base [18]. Uncertainty about the mechanism ensued owing to the fact that the rate-pH profiles can also be interpreted in terms of general base catalysis by C75 (see section on pH-rate profiles).

More recently, the Doudna laboratory solved the structure of the HDV ribozyme in a suite of pre-cleavage states [32^{••}]. In the majority of these structures, C75 was mutated to a uracil to prevent self-cleavage, although one structure was solved with a *bona fide* cytosine at position 75 and in the absence of Mg^{2+} . Unfortunately, these structures did not directly reveal the mechanistic role for residue 75 in the reaction: its N3 is positioned 5.5 Å from the nucleophilic 2'-OH of U-1 (general base position) and 4.8 Å from the 5'-bridging oxygen of G1 (general acid position). A hydrated magnesium ion was bound near the 5'-oxygen of G1. Modeling of the crystal structure allowed the nucleophilic 2'-OH to be placed near the N3 of residue 75, but not near the hydrated magnesium ion [32^{••}]. This led the authors to favor a general base model for C75. Recently, Sponer, Walter and co-workers conducted the first molecular dynamics studies on the HDV ribozyme structures [33,34[•]]. They reached a similar mechanistic conclusion as the Doudna laboratory, namely that C75 serves as the general base and hydrated magnesium as the general acid.

The hammerhead ribozyme

Although the hammerhead ribozyme has been studied more extensively than any other small ribozyme, there is still disagreement between biochemical and

crystallographic studies, reviewed in [35]. In the crystal structures, the scissile phosphate is not aligned for $\text{S}_{\text{N}}2$ attack, and nucleobases are not positioned to carry out proton transfer or electrostatic catalysis. Of particular interest is a recent study from Heckman, Lambert and Burke [36[•]]. They constructed a series of hammerhead ribozymes containing sulfur- or bromo-modified nucleobases and subjected them to photocrosslinking. Their results suggest that the two nucleotides flanking the cleavage site stack on two guanosines, G8 and G12, in a transient conformation. This interaction requires a large-scale movement of domain 2, 10–15 Å from the position seen in the crystal structure. Interestingly, one of the cross-linked species retains catalytic activity, which confirms a novel positioning of G8 (see also [66[•],68^{••}] in Update).

Small molecule rescue of RNA catalysis

Another approach for studying the role of nucleobases in RNA catalysis is to rescue inactive ribozymes by small molecule addition. Because histidine with its pK_{a} of neutrality is optimized for general acid-base catalysis, rescue by imidazole or related small molecules is indicative of general acid-base or electrostatic catalysis. There are two general approaches: rescue of an inactivated abasic (ab) ribozyme mutant by addition of a small molecule into the reaction medium, or covalent introduction of an analog into the RNA backbone.

Pioneering studies from Been and co-workers showed that inactive C75U and C75ab HDV ribozymes could be rescued by introduction of exogenous imidazole [37], and that the rate followed a pH dependence characteristic of the pK_{a} of the imidazole derivative [12]. These studies directly implicated C75 in proton transfer, although its role as general acid or base was obscured by kinetic ambiguity. The Been laboratory subsequently conducted Brønsted studies on C76 in the antigenomic ribozyme [12] and C75 in the genomic ribozyme [38[•]]. Importantly, for both ribozymes the dependence of $\log k_{\text{obs, max}}$ on pK_{a} of the imidazole derivative is linear with a slope of 0.5. Dependence of rate on position 75 basicity strongly supports a role for C75/76 in proton transfer. The studies do not exclude an additional electrostatic role for C75/76, especially given that cytosine is cationic when protonated, but if this were its only function then the Brønsted plot would have a slope of zero [38[•]].

Nucleobase rescue has also been applied to the hairpin ribozyme. In a companion set of studies, Fedor and co-workers made abasic substitutions at G8 [39[•]] and A38 [40[•]], which are positioned near the scissile phosphate [26,27]. The authors demonstrated that the rate-pH profiles were similar for the cleavage and ligation reactions. This led to a model wherein G8 serves an electrostatic stabilization role [39[•]] and A38 serves an electrostatic or general acid role [1,40[•]]. The authors

suggest that deprotonating the 2'-OH nucleophile can be facilitated by specific-base catalysis, consistent with recent crystal structures from Wedekind and co-workers [29[•]].

An alternative approach to exogenous nucleobase rescue is to incorporate small molecules directly into the covalent network of the RNA. This approach increases the local concentration of the small molecule at the active site. In early work, Perrin and co-workers applied this method to DNA enzymes [41]. In this study, adenosine was modified with imidazole, and uridine with a cationic amine, and cleavage of an RNA phosphodiester linkage was monitored. They found a pK_a of 7.4 in the absence of divalent ions, consistent with general acid–base and/or electrostatic stabilization.

More recently, Lilley and co-workers synthesized an imidazole phosphoramidite to target imidazole to sites of putative general acid–base catalysis in the VS [42[•]] and hairpin [43^{••}] ribozymes. This approach was adopted because abasic VS and hairpin variants are not rescuable by exogenous imidazole addition. In the first study, A756 in the VS ribozyme was replaced with the imidazole nucleoside [42[•]]. These variants were active, albeit at a reduced rate, for both cleavage and ligation reactions, supporting a possible general acid–base or electrostatic role for A756 in the natural VS ribozyme. In the second study, they used their imidazole nucleoside to probe G8 in the mechanism of the hairpin ribozyme [43^{••}]. A variant in which G8 was covalently substituted with imidazole was reactive. Rate–pH profiles of the G8 imidazole variant were identical for the cleavage and ligation reactions, which was interpreted as being most consistent with a general base role for G8 in cleavage [43^{••},44], although it is consistent with several other mechanisms as well.

Rate–pH profiles and single molecule studies

Analysis of rate–pH profiles is a classical method used to identify the number of protons bound in the transition state. Interpretation of such profiles can be complicated, however, because these data do not identify which protons are being transferred nor where the proton is transferred. As such, multiple mechanisms can account for the observed profiles. Graphical deconvolution has proven to be a useful approach for interpreting the ambiguity underlying ribozyme rate–pH profiles, as discussed by Bevilacqua [44]. It is important to note that rate–pH profiles can be complicated by coupling of catalytic protonation events with RNA conformational changes and acid and alkaline denaturation [45[•]].

Single molecule kinetics on ribozymes have had a major impact on elucidating RNA folding pathways and catalysis, reviewed in [46]. Recent advances have been made in applying these techniques to the hairpin ribozyme.

Zhuang and co-workers showed that a minimal two-way junction hairpin ribozyme folds into four distinct and non-interchanging populations in which coupled docking and cleavage events of individual ribozymes can be monitored [47]. Related mutagenesis studies on the hairpin ribozyme from the Walter laboratory showed that functional groups remote from the active site affect rate constants including those coupled to chemistry [48[•]].

Using a hairpin construct containing the natural four-way junction, the Lilley laboratory also studied the mechanism of the hairpin ribozyme at the single-molecule level [49^{••}]. This construct allows docking to be much faster than cleavage, which enables separation of the rate constants for these two events. Cleavage and ligation reactions were followed as a function of pH and displayed identical rate–pH profiles, similar to what these investigators observed for their imidazole nucleoside experiments [43^{••}].

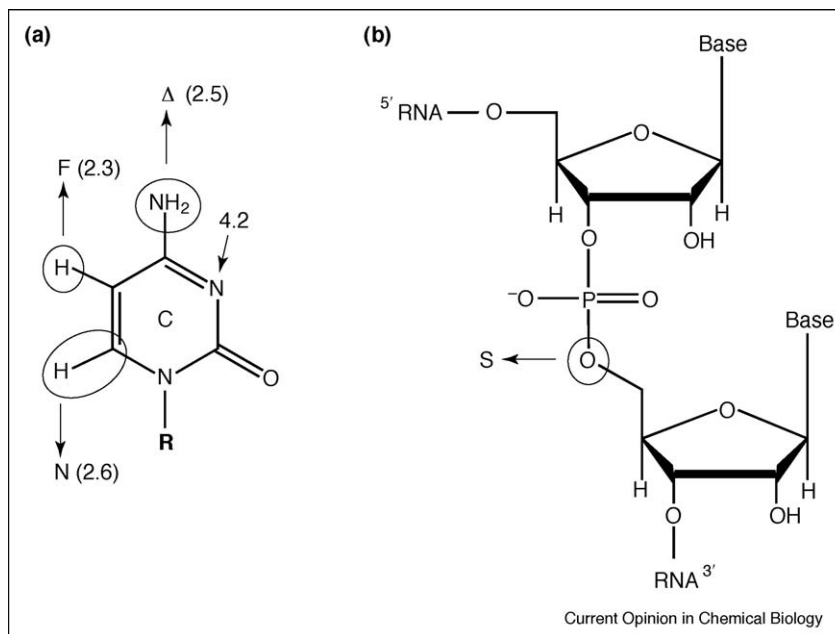
Another recent study that provides insight into nucleobase catalysis from rate–pH profiles comes from Han and Burke on the hammerhead ribozyme [50[•]]. In this work, G8 and G12 of the hammerhead ribozyme were dually substituted with 2,6-diaminopurine, which has an unperturbed pK_a of 5.1. A bell-shaped curve was found for $\log k_{\text{obs}}\text{--pH}$ plots, with two pK_a values near neutrality. Strikingly, G8 and G12 are the same two nucleotides shown by Burke and co-workers to photocrosslink to nucleotides flanking the cleavage site [36[•]]. Overall, these studies support the notion that the hammerhead ribozyme may transiently adopt an active conformation in which two guanines participate in general acid–base catalysis.

Insight from synthetic approaches

Because ribozyme function depends on the precise positioning of atoms, site-directed mutagenesis of one nucleobase for another can have drastic effects on folding as well as catalysis. Functional-group mutagenesis approaches wherein subtle, single-atom modifications are incorporated, tend to be less perturbing overall, allowing one to hone in on the catalytic contributions of key atoms. Chemical biologists have synthesized and incorporated modifications at many positions within the heterocyclic nucleobase, the 2'-OH sugar moiety, and the phosphodiester backbone (Figure 4), reviewed in [51]. Here, we focus on chemical approaches for probing nucleobase protonation in ribozyme mechanism.

Strobel and co-workers prepared a series of pK_a -perturbed cytidine and adenosine nucleotide analogs for use in nucleotide analog interference mapping (NAIM) (e.g., Figure 4a). They used this combinatorial method to probe the functional importance of nucleotide ionization in HDV [52], hairpin [53] and VS [54] ribozyme mechanisms. In the HDV and VS ribozymes, only a single base was found to be sensitive to ionization, C75 in HDV and

Figure 4



Synthetic approaches to studying ribozyme mechanism. **(a)** Examples of cytosine nucleobase analogs with perturbed pK_a values. Changes to cytosine and the accompanying pK_a of the N3 of the nucleotide analog (in parenthesis) are shown [52–54]. Note that the modifications should have relatively minor effects on the structure of the ribozyme into which they are substituted. **(b)** Change in the RNA backbone for the 5'-phosphorothiolate modification [55**].

A756 in VS. Rescue of low pK_a variants at acidic pH supports the importance of protonation of C75 for the HDV cleavage reaction as well as the protonation of A756 for the VS ligation reaction. NAIM studies on the hairpin ribozyme led to the suggestion that ionization of A38 is unimportant for activity [53], although alternative models have been offered [44].

Recently, Das and Piccirilli used a synthetic approach to test the general acid model for C75/76 of the HDV ribozyme [55**,56]. They replaced the 5'-bridging oxygen at G+1 with a sulfur atom (Figure 4b) and carried out ribozyme activity measurements. In contrast to the natural alkoxide leaving group, a thiolate is a good leaving group and therefore does not require assistance by a general acid. The authors showed that the 5'-phosphorothiolate rescued the lethal C75/76U variant, but was otherwise sensitive to ribozyme core mutations. Shapes of rate–pH profiles with various C analogs substituted into position 75/76 with and without the 5'-bridging sulfur strongly supported C75 as the general acid for HDV ribozyme cleavage.

Fast-reacting small ribozymes

We conclude with a consideration of small ribozyme constructs that react exceptionally fast. In the past two years, it has become clear that small ribozymes are capable of reacting much faster than initially thought. The Collins and Pardi laboratories showed that the VS [57*]

and hammerhead [58*] ribozymes react at rates upwards of 10 s^{-1} , while Brown, Chadalavada and Bevilacqua showed that the HDV ribozyme [59*] can react faster than 1 s^{-1} . (See also Update.) These rates, measured by rapid-quench techniques, are on the order of 100-times faster than previously reported and approach those seen in protein enzymes [60,61]. In the case of the hammerhead, inclusion of nucleotides outside of the core, which mediate loop–loop interaction [62,63], was required to accelerate cleavage; the *glmS* ribozyme also requires nucleotides outside of the catalytic core for fast cleavage [22*,23]. For the VS and HDV ribozymes, relatively minor site-directed variations led to accelerated cleavage. Collectively, these data can be explained most simply using the model in Figure 3 wherein RNA has a high intrinsic rate of self-cleavage (R to P) that can be masked because of occupancy of non-active conformers (R' and P'). The fact that the rates among these three ribozymes are similar to those for protein enzymes, and that nucleobase catalysis appears to be important suggests that the observed rates might represent the intrinsic rate of nucleobase catalysis in phosphodiester bond cleavage by RNA [61].

Conclusions and outlook

RNA molecules do not have the functional group specialization of proteins. Nevertheless, RNA molecules have evolved ways to attain remarkable catalytic diversity. The past few years have witnessed important advances in the understanding of nucleobases in catalysis.

Divalent dropout experiments on all five naturally occurring small ribozymes have demonstrated that divalent ions are non-essential for catalysis. Structural studies have revealed potential catalytic positioning of nucleobases and thereby provided inspiration for mechanistic studies. Traditional biochemical kinetics experiments, single molecule studies, and synthetic nucleotide analog introduction have provided new insights into the role of nucleobases in catalysis.

At the same time, there is discrepancy regarding the catalytic roles of specific nucleobases. Indeed, there is presently no clear consensus on the role of nucleobases in the mechanism of any small ribozyme. For the hairpin ribozyme, there is debate as to whether A38 and G8 are the general acid and base in the (cleavage) reaction mechanism, or if they just serve electrostatic roles. The role of water in the mechanism of this (and other) ribozymes is intriguing given recent high-resolution crystal structures, but remains uncertain. In the HDV ribozyme, there is consensus that C75 is involved in proton transfer, but there is debate as to whether it serves as the general acid or base for cleavage. In the hammerhead ribozyme, it appears that crystal structures represent unreactive conformers that have to undergo a major refolding event before catalysis (see Update). New studies implicate G8 and G12 in general acid–base catalysis, but this model awaits structural verification. For the VS and *glmS* ribozymes, high-resolution structural studies of the intact ribozymes are needed.

Resolving these issues will require efforts on several fronts. New atomic resolution structures will be key in deciphering roles of nucleobases in catalysis. Single molecule measurements are likely to play an important role as they provide the opportunity to untangle folding and cleavage events. Improved methods and studies in understanding pK_a shifting of catalytic nucleobases are needed. Clearly, the choice of ribozyme construct can have a profound effect, as revealed by crystallographic, single molecule and rapid-quench kinetic studies. Lastly, as the field continues to move forward, it is possible that theoretical studies will play a greater role in deepening our understanding of these processes.

Update

Recent studies indicate that GlcN6P binds to a prefolded *glmS* ribozyme [64[•]]. The authors suggest that the cofactor may participate directly in the reaction. Molecular dynamics simulations on the hairpin-transition state complex support involvement of G8, A9 and A38 in the cleavage mechanism [65[•]]. In addition, a few water molecules are implicated in the reaction mechanism by virtue of their interactions with catalytic nucleotides and their potential to stabilize charge development. Photocrosslinking carried out on minimal hammerheads [36[•]] has now been conducted on full-length, native hammerhead

constructs [66[•]]. Full-length hammerheads contain the loop–loop interactions [62,63] that led to fast hammerhead catalysis [58[•]]. The crosslinks found for full-length and minimal hammerhead ribozymes are alike, suggesting that their catalytic cores are similar to each other and different from minimal-hammerhead crystal structures. Kinetic studies on a hammerhead stabilized with loop–loop interactions, generated from an *in vitro* selection experiment in this case, reveal exceptional rates of catalysis (up to 50 s⁻¹) in the presence of certain transition metals [67[•]]. The data are consistent with general acid–base catalysis by nucleobases being assisted by a direct or indirect interaction with a metal ion. A spectacular crystal structure of the full-length hammerhead ribozyme has been solved by Martick and Scott [68^{••}]. The structure shows potential general acid–base roles for G8 and G12, as well as other molecular details that accommodate a wealth of biochemical data incompatible with the previous, minimal hammerhead structures [35,36[•],66[•]]. The new structure illustrates how relatively minor alterations in RNA sequence can lead to structural rearrangements with profound functional consequence and insight.

Acknowledgements

We thank National Science Foundation grant MCB-0527102 (PCB), National Institutes of Health grant R01-58709 (PCB), and a post-graduate scholarship from the Natural Science and Engineering Research Council (NSERC) of Canada (RY) for generous support. We also thank Rob Batey, Barbara Golden and members of the Bevilacqua laboratory for helpful discussions and comments on the manuscript.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Fedor MJ, Williamson JR: **The catalytic diversity of RNAs.** *Nat Rev Mol Cell Biol* 2005, **6**:399-412.
2. Doudna JA, Lorsch JR: **Ribozyme catalysis: not different, just worse.** *Nat Struct Mol Biol* 2005, **12**:395-402.
3. Holbrook SR: **RNA structure: the long and the short of it.** *Curr Opin Struct Biol* 2005, **15**:302-308.
4. Lilley DM: **Structure, folding and mechanisms of ribozymes.** *Curr Opin Struct Biol* 2005, **15**:313-323.
5. Woodson SA: **Metal ions and RNA folding: a highly charged topic with a dynamic future.** *Curr Opin Chem Biol* 2005, **9**:104-109.
6. Fedor MJ: **The role of metal ions in RNA catalysis.** *Curr Opin Struct Biol* 2002, **12**:289-295.
7. Bevilacqua PC, Brown TS, Nakano S, Yajima R: **Catalytic roles for proton transfer and protonation in ribozymes.** *Biopolymers* 2004, **73**:90-109.
8. Lonnberg T, Lonnberg H: **Chemical models for ribozyme action.** *Curr Opin Chem Biol* 2005, **9**:665-673.
9. Hougland JL, Piccirilli JA, Forconi M, Lee J, Herschlag D: **How the group I intron works: a case study of RNA structure and function.** In *RNA World*, Edn 3. Edited by Gesteland RF, Cech TR, Atkins JF: Cold Spring Harbor Press; 2006:768.
10. Ke A, Doudna JA: **Catalytic strategies of self-cleaving ribozymes: Relics of an RNA world?** In *RNA World*, Edn 3.

Edited by Gesteland RF, Cech TR, Atkins JF: Cold Spring Harbor Press; 2006:768.

11. Zalatan JG, Herschlag D: **Alkaline phosphatase mono- and diesterase reactions: comparative transition state analysis.** *J Am Chem Soc* 2006, **128**:1293-1303.
 12. Shih IH, Been MD: **Involvement of a cytosine side chain in proton transfer in the rate-determining step of ribozyme self-cleavage.** *Proc Natl Acad Sci USA* 2001, **98**:1489-1494.
 13. Nakano S, Proctor DJ, Bevilacqua PC: **Mechanistic characterization of the HDV genomic ribozyme: assessing the catalytic and structural contributions of divalent metal ions within a multichannel reaction mechanism.** *Biochemistry* 2001, **40**:12022-12038.
 14. Raines RT: **Ribonuclease A.** *Chem Rev* 1998, **98**:1045-1065.
 15. Herschlag D, Eckstein F, Cech TR: **The importance of being ribose at the cleavage site in the Tetrahymena ribozyme reaction.** *Biochemistry* 1993, **32**:8312-8321.
 16. Weinger JS, Parnell KM, Dörner S, Green R, Strobel SA: **Substrate-assisted catalysis of peptide bond formation by the ribosome.** *Nat Struct Mol Biol* 2004, **11**:1101-1106.
 17. Murray JB, Seyhan AA, Walter NG, Burke JM, Scott WG: **The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone.** *Chem Biol* 1998, **5**:587-595.
 18. Nakano S, Chadalavada DM, Bevilacqua PC: **General acid-base catalysis in the mechanism of a hepatitis delta virus ribozyme.** *Science* 2000, **287**:1493-1497.
 19. Curtis EA, Bartel DP: **The hammerhead cleavage reaction in monovalent cations.** *RNA* 2001, **7**:546-552.
 20. O'Rear JL, Wang S, Feig AL, Beigelman L, Uhlenbeck OC, Herschlag D: **Comparison of the hammerhead cleavage reactions stimulated by monovalent and divalent cations.** *RNA* 2001, **7**:537-545.
 21. Winkler WC, Nahvi A, Roth A, Collins JA, Breaker RR: **Control of gene expression by a natural metabolite-responsive ribozyme.** *Nature* 2004, **428**:281-286.
 22. Roth A, Nahvi A, Lee M, Jona I, Breaker RR: **Characteristics of the glmS ribozyme suggest only structural roles for divalent metal ions.** *RNA* 2006, **12**:607-619.
- The newly discovered *glmS* ribozyme is fully active in EDTA and exchange-inert cobalt hexamine suggesting that nucleobases are likely to participate in catalysis.
23. Wilkinson SR, Been MD: **A pseudoknot in the 3' non-core region of the glmS ribozyme enhances self-cleavage activity.** *RNA* 2005, **11**:1788-1794.
 24. McCarthy TJ, Plog MA, Floy SA, Jansen JA, Soukup JK, Soukup GA: **Ligand requirements for glmS ribozyme self-cleavage.** *Chem Biol* 2005, **12**:1221-1226.
 25. Jansen JA, McCarthy TJ, Soukup GA, Soukup JK: **Backbone and nucleobase contacts to glucosamine-6-phosphate in the glmS ribozyme.** *Nat Struct Mol Biol* 2006, **13**:517-523.
 26. Rupert PB, Ferre-D'Amare AR: **Crystal structure of a hairpin ribozyme-inhibitor complex with implications for catalysis.** *Nature* 2001, **410**:780-786.
 27. Rupert PB, Massey AP, Sigurdsson ST, Ferre-D'Amare AR: **Transition state stabilization by a catalytic RNA.** *Science* 2002, **298**:1421-1424.
 28. Alam S, Grum-Tokars V, Krucinska J, Kundracik ML, Wedekind JE: **Conformational heterogeneity at position U37 of an all-RNA hairpin ribozyme with implications for metal binding and the catalytic structure of the S-turn.** *Biochemistry* 2005, **44**:14396-14408.
- Crystal structures of activated hairpin variant ribozymes solved in the absence of the U1A protein reveal U37 in a 'sequestered' conformation, which may assist in positioning of the scissile phosphate for catalysis.
29. Salter J, Krucinska J, Alam S, Grum-Tokars V, Wedekind JE: **Water in the active site of an all-RNA hairpin ribozyme and effects of Gua8 base variants on the geometry of phosphoryl transfer.** *Biochemistry* 2006, **45**:686-700.
- Crystal structure of a hairpin ribozyme wherein ordered water molecules are identified at the active site, suggesting that water may activate the 2'-OH nucleophile in phosphodiester bond cleavage.
30. Lambert D, Heckman JE, Burke JM: **Cation-specific structural accommodation within a catalytic RNA.** *Biochemistry* 2006, **45**:829-838.
- Using biochemical data as constraints, an MC-Sym model of the hairpin ribozyme is generated which differs from previous crystal structures. The MC-Sym model suggests alternate structural roles for U+2 and C+3 in the active site.
31. Ferre-D'Amare AR, Zhou K, Doudna JA: **Crystal structure of a hepatitis delta virus ribozyme.** *Nature* 1998, **395**:567-574.
 32. Ke A, Zhou K, Ding F, Cate JH, Doudna JA: **A conformational switch controls hepatitis delta virus ribozyme catalysis.** *Nature* 2004, **429**:201-205.
- Crystal structures of the pre-cleaved HDV ribozyme with an inactivating C75U mutation. The structure led to a general base mechanism for C75 in self-cleavage.
33. Krasovska MV, Sefcikova J, Spackova N, Sponer J, Walter NG: **Structural dynamics of precursor and product of the RNA enzyme from the hepatitis delta virus as revealed by molecular dynamics simulations.** *J Mol Biol* 2005, **351**:731-748.
 34. Krasovska MV, Sefcikova J, Reblova K, Schneider B, Walter NG, Sponer J: **Cations and hydration in catalytic RNA: molecular dynamics of the hepatitis delta virus ribozyme.** *Biophys J* 2006.
- MD simulations of the entire HDV ribozyme. Results are consistent with C75 and hydrated magnesium as the general base and acid, respectively.
35. Blount KF, Uhlenbeck OC: **The structure-function dilemma of the hammerhead ribozyme.** *Annu Rev Biophys Biomol Struct* 2005, **34**:415-440.
 36. Heckman JE, Lambert D, Burke JM: **Photocrosslinking detects a compact, active structure of the hammerhead ribozyme.** *Biochemistry* 2005, **44**:4148-4156.
- Crosslinking studies with nucleotide analogs suggest that the active conformation of the hammerhead forms only transiently and that G8 and G12 are close to the cleavage site.
37. Perrotta AT, Shih I, Been MD: **Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme.** *Science* 1999, **286**:123-126.
 38. Perrotta AT, Wadkins TS, Been MD: **Chemical rescue, multiple ionizable groups, and general acid-base catalysis in the HDV genomic ribozyme.** *RNA* 2006, **12**:1282-1291.
- Small molecule rescue experiments on the genomic version of the HDV ribozyme give a Brønsted plot with a slope of 0.5, consistent with proton transfer and general acid-base catalysis by C75.
39. Kuzmin YI, Da Costa CP, Fedor MJ: **Role of an active site guanine in hairpin ribozyme catalysis probed by exogenous nucleobase rescue.** *J Mol Biol* 2004, **340**:233-251.
- See annotation to [40*].
40. Kuzmin YI, Da Costa CP, Cottrell JW, Fedor MJ: **Role of an active site adenine in hairpin ribozyme catalysis.** *J Mol Biol* 2005, **349**:989-1010.
- The authors perform exogenous nucleobase rescue studies on G8 and A38 abasic mutants in the hairpin ribozyme. A model is favored in which these residues provide electrostatic catalysis.
41. Perrin DM, Garestier T, Helene C: **Bridging the gap between proteins and nucleic acids: a metal-independent RNaseA mimic with two protein-like functionalities.** *J Am Chem Soc* 2001, **123**:1556-1563.
 42. Zhao ZY, McLeod A, Harusawa S, Araki L, Yamaguchi M, Kurihara T, Lilley DM: **Nucleobase participation in ribozyme catalysis.** *J Am Chem Soc* 2005, **127**:5026-5027.
- Imidazole is introduced into the VS ribozyme backbone covalently at the position of A756. Observation of self-cleavage is consistent with this nucleobase participating in catalysis.
43. Wilson TJ, Ouellet J, Zhao ZY, Harusawa S, Araki L, Kurihara T, Lilley DM: **Nucleobase catalysis in the hairpin ribozyme.** *RNA* 2006, **12**:980-987.

A hairpin ribozyme with an imidazole nucleobase at position 8 shows a bell-shaped rate-pH profile for both the cleavage and ligation reactions, consistent with general acid-base catalysis.

44. Bevilacqua PC: **Mechanistic considerations for general acid-base catalysis by RNA: Revisiting the mechanism of the hairpin ribozyme.** *Biochemistry* 2003, **42**:2259-2265.
45. Moody EM, Lecomte JT, Bevilacqua PC: **Linkage between proton binding and folding in RNA: A thermodynamic framework and its experimental application for investigating pK_a shifting.** *RNA* 2005, **11**:157-172.
- All actions of proton binding on RNA folding are described including unfolded state effects. Simulations and experiments show pK_a shifting as well as acid and alkaline denaturation effects.
46. Zhuang X: **Single-molecule RNA science.** *Annu Rev Biophys Biomol Struct* 2005, **34**:399-414.
47. Zhuang X, Kim H, Pereira MJ, Babcock HP, Walter NG, Chu S: **Correlating structural dynamics and function in single ribozyme molecules.** *Science* 2002, **296**:1473-1476.
48. Rueda D, Bokinsky G, Rhodes MM, Rust MJ, Zhuang X, Walter NG: **Single-molecule enzymology of RNA: essential functional groups impact catalysis from a distance.** *Proc Natl Acad Sci USA* 2004, **101**:10066-10071.
- Single molecule measurements combined with mutagenesis studies on a minimal two-way junction hairpin ribozyme show that functional groups remote from the active site affect rate constants coupled to chemistry.
49. Nahas MK, Wilson TJ, Hohng S, Jarvie K, Lilley DM, Ha T: **Observation of internal cleavage and ligation reactions of a ribozyme.** *Nat Struct Mol Biol* 2004, **11**:1107-1113.
- Single molecule experiments on the natural four-way junction hairpin ribozyme allow rates and pH profiles for docking and cleavage to be separated.
50. Han J, Burke JM: **Model for general acid-base catalysis by the hammerhead ribozyme: pH-activity relationships for G8 and G12 variants at the putative active site.** *Biochemistry* 2005, **44**:7864-7870.
- Rate-pH profile analyses with diaminopurine substituted at G8 and G12 suggest that these G's may participate in general acid-base chemistry in the hammerhead ribozyme.
51. Das SR, Fong R, Piccirilli JA: **Nucleotide analogues to investigate RNA structure and function.** *Curr Opin Chem Biol* 2005, **9**:585-593.
52. Oyeler AK, Kardon JR, Strobel SA: **pK(a) perturbation in genomic hepatitis delta virus ribozyme catalysis evidenced by nucleotide analogue interference mapping.** *Biochemistry* 2002, **41**:3667-3675.
53. Ryder SP, Oyeler AK, Padilla JL, Klostermeier D, Millar DP, Strobel SA: **Investigation of adenosine base ionization in the hairpin ribozyme by nucleotide analog interference mapping.** *RNA* 2001, **7**:1454-1463.
54. Jones FD, Strobel SA: **Ionization of a critical adenosine residue in the neurospora Varkud satellite ribozyme active site.** *Biochemistry* 2003, **42**:4265-4276.
55. Das SR, Piccirilli JA: **General acid catalysis by the hepatitis delta virus ribozyme.** *Nat Chem Biol* 2005, **1**:1-8.
- Rescue of a cleavage-inactive mutant using a hyperactivated leaving group provides evidence for general acid catalysis by cytosine 75 in the HDV ribozyme.
56. Strobel SA: **Ribonucleic general acid.** *Nat Chem Biol* 2005, **1**:5-6.
57. Zamel R, Poon A, Jaikaran D, Andersen A, Olive J, De Abreu D, Collins RA: **Exceptionally fast self-cleavage by a Neurospora Varkud satellite ribozyme.** *Proc Natl Acad Sci USA* 2004, **101**:1467-1472.
- Ultrafast cleavage rates of 10 s⁻¹ in the VS ribozyme support a common mechanism of nucleobase participation in catalysis.
58. Canny MD, Jucker FM, Kellogg E, Khvorova A, Jayasena SD, Pardi A: **Fast cleavage kinetics of a natural hammerhead ribozyme.** *J Am Chem Soc* 2004, **126**:10848-10849.
- Ultrafast cleavage rates of >10 s⁻¹ in the hammerhead ribozyme support a common mechanism of nucleobase participation in catalysis.
59. Brown TS, Chadalavada DM, Bevilacqua PC: **Design of a highly reactive HDV ribozyme sequence uncovers facilitation of RNA folding by alternative pairings and physiological ionic strength.** *J Mol Biol* 2004, **341**:695-712.
- Very fast cleavage rates of 1 s⁻¹ in the HDV ribozyme support a common mechanism of nucleobase participation in catalysis.
60. Emilsson GM, Nakamura S, Roth A, Breaker RR: **Ribozyme speed limits.** *RNA* 2003, **9**:907-918.
61. Breaker RR, Emilsson GM, Lazarev D, Nakamura S, Puskarz IJ, Roth A, Sudarsan N: **A common speed limit for RNA-cleaving ribozymes and deoxyribozymes.** *RNA* 2003, **9**:949-957.
62. Khvorova A, Lescoute A, Westhof E, Jayasena SD: **Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity.** *Nat Struct Biol* 2003, **10**:708-712.
63. De la Peña M, Gago S, Flores R: **Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity.** *EMBO J* 2003, **22**:5561-5570.
64. Hampel KJ, Tinsley MM: **Evidence for preorganization of the glmS ribozyme ligand binding pocket.** *Biochemistry* 2006, **45**:7861-7871.
- Hydroxyl radical footprinting and cross-linking methods were used to show that GlcN6P does not alter the catalytic core of the *glmS* ribozyme. These data suggest that the cofactor may directly participate in the reaction in some way.
65. Park H, Lee S: **Role of solvent dynamics in stabilizing the transition state of RNA hydrolysis by hairpin ribozyme.** *J Chem Theory Comput* 2006, **2**:858-862.
- Molecular dynamics simulations have been carried out on the hairpin-transition state complex and support the involvement of water molecules in the catalytic mechanism.
66. Lambert D, Heckman JE, Burke JM: **Three conserved guanosines approach the reaction site in native and minimal hammerhead ribozymes.** *Biochemistry* 2006, **45**:7140-7147.
- Photocrosslinking studies were conducted on full-length, native hammerhead ribozymes and gave similar results as crosslinking studies on minimal hammerheads. These studies provide support that the reactive conformation of the hammerhead is different from that in minimal-hammerhead crystal structures.
67. Roychowdhury-Saha M, Burke DH: **Extraordinary rates of transition metal ion-mediated ribozyme catalysis.** *RNA* 2006, in press.
- Kinetics experiments on a full-length hammerhead ribozyme with *in vitro* selected tertiary interactions reveal exceptional rates of catalysis (up to 50 s⁻¹) in the presence of transition metal ions. These data suggest that a metal ion may be involved in catalysis, either directly or indirectly.
68. Martick M, Scott WG: **Tertiary contacts distant from the active site prime a ribozyme for catalysis.** *Cell* 2006, **126**:309-320.
- This paper presents the first crystal structure of a full-length hammerhead ribozyme. Many details of the structure are congruent with biochemical data, and a mechanism involving nucleobase catalysis is presented.