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Short Review

Ribozyme Catalysis Revisited: Is Water Involved?

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Enzymatic catalysis by RNA was discovered 25 years ago, yet mechanistic insights are emerging only slowly. Thought to be metalloenzymes at first, some ribozymes proved more versatile than anticipated when shown to utilize their own functional groups for catalysis. Recent evidence suggests that some may also judiciously place structural water molecules to shuttle protons in acid-base catalyzed reactions.

The ''whodunit'' is the most critical and challenging question in any murder story, even if it plays out on the molecular level. In the case of catalysis by RNA, a phenomenon whose discovery revolutionized our understanding of molecular biology 25 years ago, a key question has been ''who'' endows such a chemically monotonous, four-letter biopolymer with the ability to carry out (site-)specific chemistry. A prime example of an unsolved molecular murder mystery is found in the seemingly simple RNA backbone transesterification catalyzed by the five naturally occurring small ribozymes, the hammerhead, hairpin, hepatitis delta virus (HDV), Varkud satellite (VS), and *glmS* ribozymes (Figure 1A) (Lilley, 2004; Winkler et al., 2004; Doudna and Lorsch, 2005; Fedor and Williamson, 2005). Solving this mystery is not only of academic interest in the quest to understand all biological catalysis but also has practical implications for the use of ribozymes in gene therapy (where they can crack down on undesired viral RNA) and biosensor applications (where they can detect the presence of specific biomarkers). Evidence has recently started to arise that the water solvent, i.e., the ''butler,'' may be involved in the case.

Despite the apparent simplicity of their chemical makeup, ribozymes can form complex tertiary structures, as perhaps best exemplified by the ribosome, leading to the intricate placement of potential participants in reaction chemistry (Nissen et al., 2000; Beringer and Rodnina, 2007). When investigations into small ribozyme catalysis were launched, metal cations were obvious suspects as they often catalyze related chemistry in protein-based RNases (Figure 1B). Recent protein examples caught in the act include the tRNase Z family and endonuclease CPSF-73, where a binuclear zinc ion site activates a water molecule to site-specifically hydrolyze the 3′ end of pre-tRNA and pre-mRNA, respectively (Figure 1C) (Vogel et al., 2005; Mandel et al., 2006); and RNase H, where two magnesium ions appear to fulfill a similar role (Yang et al., 2006). Similarly, in the large group I intron ribozymes, an external and an internal if distal 3′-hydroxyl (3′-OH) functional group are thought to be activated as nucleophiles for phosphoryl transfer in the first and second steps of self-splicing, respectively, by two magnesium ions, in analogy to the mechanism employed by DNA and RNA polymerases (Figure 1D) (Stahley and Strobel, 2006; Yang et al., 2006). By comparison, the small ribozymes catalyze a simpler phosphoryl transfer that reversibly interconverts the nearly isoenergetic linear 3′,5′- and cyclic 2′,3′-phosphodiesters in a site-specific and nonhydrolytic reaction (Figure 1A). The similarities in the hydrolytic and nonhydrolytic phosphoryl transfers suggested early on that small ribozymes are metalloenzymes, just like their larger counterparts (Pyle, 1993, 1996). Indeed, experimental (Lott et al., 1998) and theoretical considerations (Leclerc and Karplus, 2006) continue to be invoked to support the notion that small ribozymes judiciously place two magnesium ions to catalyze site-specific chemistry.

Four potential roles may be ascribed to metal ions in the reaction (Emilsson et al., 2003) (Figure 1A): (1) structural stabilization of the in-line nucleophilic attack configuration; (2) deprotonation of the upstream 2′-OH by a general base such as a metal hydroxide; (3) Lewis-acid type stabilization of the developing negative charge in the transition state; and (4) protonation of the leaving group oxyanion by a general acid such as a hydrated metal ion. Due to its highly polyanionic character, RNA attracts nearly stoichiometric counter ion charges under physiologic conditions, with a preference for divalent magnesium ions. What functions do these many divalents have? Nonspecifically as well as site-specifically bound Mg^{2+} ions are crucial in aiding RNA fold into the complex three-dimensional structures required for biological activity (Draper et al., 2005). Most of these divalents bind dynamically quite distant from the RNA active site and so cannot be implicated in catalysis. Circumstantial evidence for a direct catalytic role of a few specific divalents in small ribozyme catalysis was provided by X-ray crystallography, placing metal ions near the catalytic cores of the hammerhead (Scott et al., 1996) and HDV ribozymes (Ke et al., 2004), and by the discovery of small RNA catalysts dependent on specific transition metal ions such as Mn^{2+} (Dange et al., 1990) or Pb^{2+} (Pan and Uhlenbeck, 1992), thought to affect cleavage. In the late 1990s, however, it was discovered that the hammerhead, hairpin, and VS ribozymes, in contrast to their

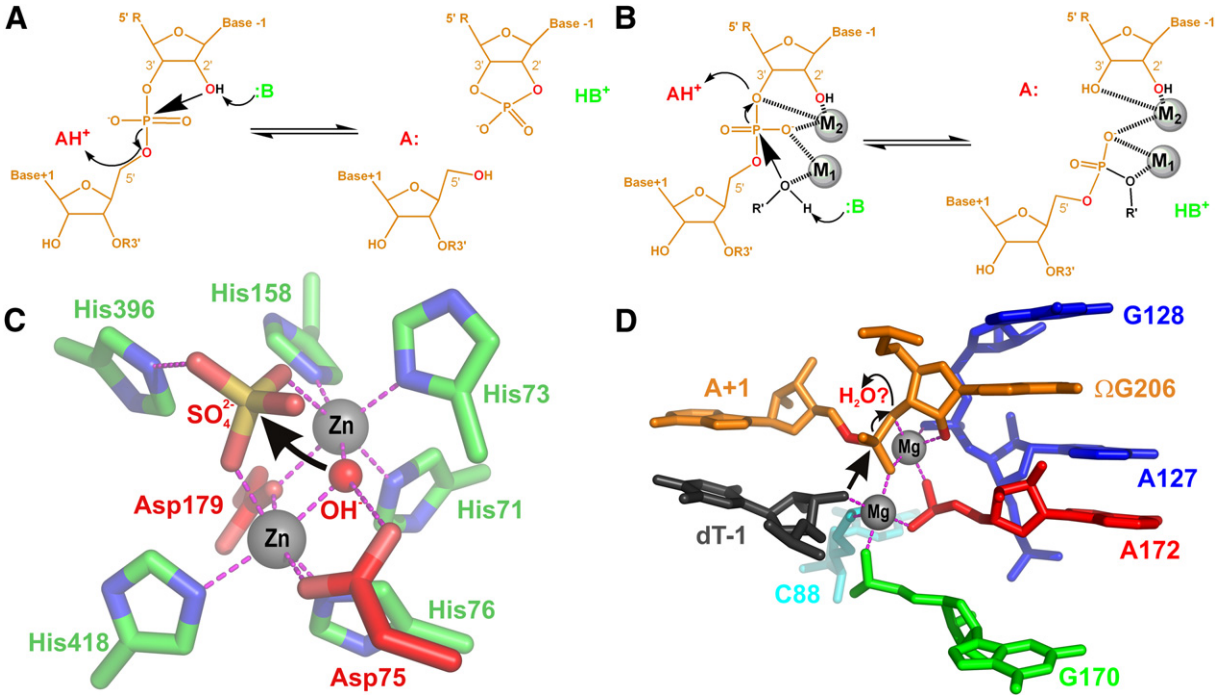

Figure 1. Mechanistic Considerations for Ribozyme Catalysis

(A) Proposed reaction mechanism of phosphoryl transfer in small ribozymes. A base (:B, green) activates a specific 2′-OH as a nucleophile (red) to attack the adjacent scissile phosphate, while the 5′-oxyanion leaving group (red) is protonated by a general acid catalyst (HA^+, red), leading to 2′,3′-cyclic phosphate and 5′-OH product termini (Walter and Burke, 1998; Lilley, 2004; Winkler et al., 2004; Doudna and Lorsch, 2005; Fedor and Williamson, 2005). The color code is maintained throughout both figures.

(B) Proposed reaction mechanism of phosphoryl transfer in large ribozymes such as group I introns. X-ray crystallography supports a model whereby two divalent metal ions (gray spheres, M_1 and M_2) coordinate the scissile phosphate and adjacent ribose (dashed lines), preparing the phosphodiester for nucleophilic attack by a distal 3′-OH, which itself is activated by a general base catalyst (:B, green) (red). Protonation of the 3′-oxyanion leaving group (red) by a general acid catalyst (HA^+, red) completes the reaction cycle (Stahley and Strobel, 2006).

(C) The active site and proposed mechanism of the pre-mRNA 3′ end-processing endonuclease CPSF-73 (Mandel et al., 2006) (PDB ID 2I7V). A dinuclear Zn^{2+}-binding site positions a bridging hydroxide ion (OH^-) for nucleophilic attack (arrow) on the substrate phosphodiester, possibly mimicked by a cocrystallized sulfate (SO_4^{2-}). All other Zn^{2+} and OH^- ligands and their liganding interactions (magenta dashes) are indicated.

(D) The active site and proposed mechanism during the second step of splicing by the *Azoarcus* group I intron ribozyme (Stahley and Strobel, 2005, 2006) (PDB ID 1ZZN). Two Mg^{2+} ions are liganded to the cleavage site (magenta dashes), facilitating nucleophilic attack (arrow) by the 3′-OH of U-1 (in the crystal replaced by a 2′-deoxy-T (dT-1)) on the scissile phosphodiester between ΩG206 and A+1. A water ligand of the second Mg^{2+} ion may serve as a proton source for the 3′-oxyanion leaving group as indicated by arrows.

larger counterparts, undergo catalysis even in the complete absence of divalent metal ions, as long as sufficient monovalent countercharge is provided (Hampel and Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997; Murray et al., 1998). These observations rule out an obligatory role of metal ions in catalysis by these ribozymes and suggest instead that metal ions can support catalysis solely in an indirect, electrostatic mode, presumably through structure stabilization. The same is likely true for the *glmS* ribozyme (Roth et al., 2006), but a partial exception to this rule is the HDV ribozyme, which shows strong preference for divalents over monovalents (Nakano et al., 2000). The HDV ribozyme is thought to employ a hydrated Mg^{2+} ion either as general acid (Perrotta et al., 1999; Ke et al., 2004; Krasovska et al., 2005) or base catalyst in its main reaction channel (Nakano et al., 2000, 2003; Das and Piccirilli, 2005; Liu et al., 2007; Wei et al., 2007), although a minor reaction channel of residual self-cleavage activity persists at molar concentrations of monovalents, in the absence of Mg^{2+} (Nakano et al., 2000, 2001; Wadkins et al., 2001; Perrotta and Been, 2006; Nakano and Bevilacqua, 2007).

With divalents no longer considered indispensable, researchers sought and found new suspects that may affect small ribozyme catalysis: RNA functional groups, in analogy to the histidine side chains 12 and 119 of RNase A, thought to serve as the base and acid catalysts, respectively, in the same reaction (Figure 2A) (Doudna and Lorsch, 2005; Fedor and Williamson, 2005). The apparent pK_as of ribozyme reactions typically differ from those of ionizable functional groups in free nucleobases by several pH units, but it is thought that the latter pK_as can become shifted by the negatively charged RNA environment (Bevilacqua and Yajima, 2006). Structural and mechanistic evidence for RNA functional group involvement was first discovered for the HDV ribozyme (Ferre-D'Amare et al., 1998), where cytosine (C)75 was proposed to complement the hydrated Mg^{2+} ion by serving either as the general

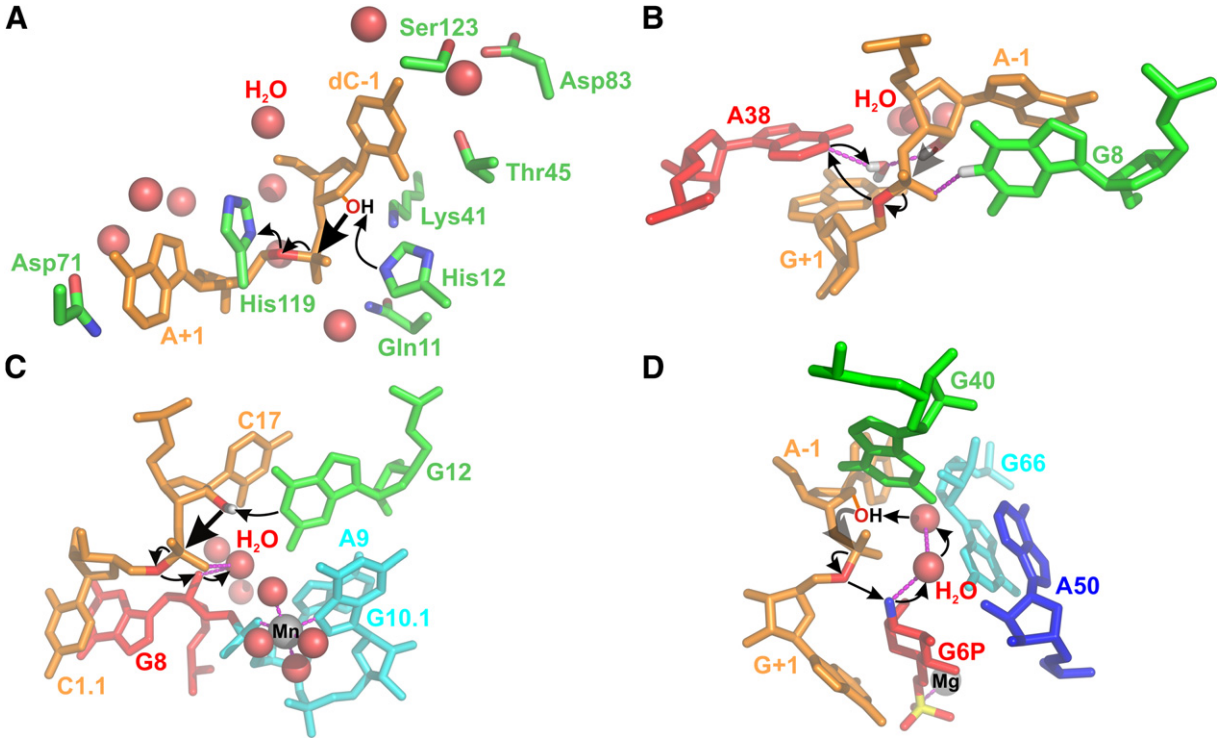

Figure 2. Possible Proton Shuttles Mediated by Water Molecules during RNA Cleavage

(A) The active site and proposed mechanism of RNA cleavage by bovine pancreatic RNase A (Zegers et al., 1994) (PDB ID 1RPG). RNase A in complex with the dinucleotide inhibitor deoxycytidyl-3′,5′-deoxyadenosine shows His12 in proximity to the modeled 2′-OH of dC-1, possibly acting as a general base to activate the 2′-OH for nucleophilic attack (arrows). His119 is positioned to donate a proton to the 5′-oxyanion leaving group. Numerous structural water molecules (red spheres) surround the active site and could potentially aid catalysis by conducting protons to bulk solvent (Meyer, 1992).

(B) The active site and a proposed mechanism for RNA cleavage suggested by MD simulations of the hairpin ribozyme (Rhodes et al., 2006). Upon removal of its catalysis-blocking 2′-O-methyl cap from the ribozyme-inhibitor crystal structure (Rupert and Ferre-D'Amare, 2001), the 2′-OH of A-1 moves into the catalytic core to establish a hydrogen bond (magenta dash) to a water molecule (shown in sticks) that is structurally bound to N1 of A38 in the ribozyme's intradomain cavity. This configuration predicts that N1 of A38 may act both as a remote general base, with the structural water conducting the proton from the 2′-OH nucleophile, and as the general acid that protonates the 5′-oxyanion leaving group (arrows). G8 donates a hydrogen bond to a nonbridging oxygen of the scissile phosphate to help stabilize the transition state. Additional water molecules trapped in the catalytic core are shown as red spheres.

(C) The active site and a proposed mechanism for RNA cleavage by the hammerhead ribozyme (Martick and Scott, 2006) (PDB ID 2OEU). In this scenario, which is most compatible with the crystal structure, a deprotonated G12 abstracts a proton from the 2′-OH of C17 to activate it for nucleophilic attack on the scissile phosphate (please note that C17 is 2′-O methylated in the crystal to suppress catalysis). The 5′-oxyanion leaving group is protonated by the 2′-OH of G8, which replenishes its proton from a nearby structural water molecule (arrows). A Mn^{2+} is found liganded to G10.1 and the A9 phosphate, as well as four water molecules, and may stabilize the developing negative phosphate charge in the transition state electrostatically. Additional water molecules are shown as red spheres.

(D) The active site and a proposed mechanism for RNA cleavage by the *glmS* ribozyme (Klein and Ferre-D'Amare, 2006) (PDB ID 2H0Z). In this complex with the inhibitor ligand glucose-6-phosphate (G6P), a 2-amino group modeled onto G6P (blue) would be in a position to remotely abstract a proton through two structural water molecules from the (modeled) 2′-OH of A-1, which then nucleophilically attacks the scissile phosphate; the 5′-oxyanion leaving group is protonated by the 2-amino group of the ligand to close the circuit (arrows). A Mg^{2+} ion mediates binding to the phosphate group of the ligand. In another model G40 plays the role of general base catalyst (Klein and Ferre-D'Amare, 2006; Cochrane et al., 2007).

base (Perrotta et al., 1999) or acid (Nakano et al., 2000). Once a precedent was set, analogous suggestions were made for the hairpin ribozyme (Pinard et al., 2001) and the ribosome (Muth et al., 2000; Nissen et al., 2000), although the latter hypothesis could not be substantiated (Muth et al., 2001; Rodnina et al., 2007). For hairpin ribozyme catalysis, the protonation state of G8 was found to be important (Pinard et al., 2001). In a crystal structure of a ribozyme-inhibitor complex, G8 is found close to the 2′-OH of A-1, the functional group that needs to be deprotonated during the reaction (Rupert and Ferre-D'Amare, 2001). Of several models consistent with this observation, the notion that G8 stabilizes the transition state by electrostatic coordination and/or hydrogen bonding is supported by exogenous nucleobase rescue experiments (Kuzmin et al., 2004), X-ray crystallography of a transition state analog (Rupert et al., 2002), and molecular dynamics (MD) simulations of the active ribozyme-substrate complex, where G8 is observed to hydrogen bond with a nonbridging oxygen of the scissile phosphate (Figure 2B) (Rhodes et al., 2006). Furthermore, A38 is located adjacent to the 5′-oxyanion leaving group of the scissile phosphate

(Figure 2B) (Rupert and Ferre-D'Amare, 2001; Rhodes et al., 2006; Salter et al., 2006), suggesting that it may act as the general acid (or possibly base for the 2′-OH). All of these assignments remain controversial, however, as nucleotide analog interference mapping and exogenous nucleobase rescue experiments instead support a model in which A38 stabilizes the transition state electrostatically (Ryder et al., 2001; Kuzmin et al., 2005) and G8 serves in general acid-base catalysis (Wilson et al., 2006).

Are there other suspects that may affect catalytic function in small ribozymes? An omnipresent participant in all of biology is water, the universal solvent of life. Water dissolves more substances than any other fluid due to its unique physicochemical properties, among them the capacity to form very strong hydrogen bonds combined with fast rotational diffusion, leading to rapid reorientation of hydrogen bonds. In addition, water as a polyprotic acid is equally likely to be in protonated (H_3O^+) and unprotonated (OH^-) states at neutral (near-physiologic) pH, conducts protons by fast tunneling through chains of aligned water molecules (during the so-called Grotthus conduction mechanism), and is present at high concentrations (around 55 M) in all biological systems. Structurally ordered water molecules can be found associated with RNA in high-resolution crystal structures of tRNA (Westhof, 1988) as well as MD simulations of RNA (Auffinger and Westhof, 2000). Water is thought to contribute to the hydrophobic collapse of a folding RNA (Sorin et al., 2005) and to the stabilization of a 2′-O-methylated over an unmodified RNA helix (Auffinger and Westhof, 2001). It is also invoked in catalysis of the HDV ribozyme (Nakano et al., 2000) as well as group I intron ribozymes (Figure 1D) (Stahley and Strobel, 2006) through proton transfers involving the inner solvation sphere of the catalytic magnesium ions. Yet until recently water was rarely observed in crystal structures of ribozymes, mostly due to the difficulty of assigning the residual electron densities observed at moderate resolutions to water molecules rather than small ions associated with the RNA. This "invisibility" of water kept it largely out of the sights of ribozyme researchers. This contrasts with the attention that structurally ordered (low-B factor) water molecules in protein enzymes and proton pumps have attracted, where they were suggested (Meyer, 1992; Frank et al., 2004; Wang et al., 2007) and observed (Garczarek and Gerwert, 2006) to participate in proton shuttling through "relays" and "wires." RNase A, for example, harbors several chains of conserved structural water molecules radiating from its catalytic core (Figure 2A) (Zegers et al., 1994) that have been proposed to serve as proton shuttles to bulk water at the enzyme's surface (Meyer, 1992). Water wires are thought particularly suitable to conduct protons by the Grotthus mechanism, as the initial proton transfer can occur at neutral pH in under 150 fs (Mohammed et al., 2005) and is followed by proton transfer to other water molecules within 30 fs (Geissler et al., 2001; Garczarek and Gerwert, 2006), keeping each water molecule in a wire uncharged during most of the process.

The hairpin ribozyme exemplifies the mounting evidence for an extended functionality of water solvent in RNA catalysis (Park and Lee, 2006; Rhodes et al., 2006; Salter et al., 2006; Torelli et al., 2007). During MD simulation several water molecules were found to become trapped in the solvent-protected catalytic core for many consecutive nanoseconds as an inherent part of the RNA structure, only occasionally exchanging with bulk solvent (Figure 2B) (by comparison, typical water residency times on the outside of RNA last only fractions of nanoseconds [Auffinger and Westhof, 2001; Rhodes et al., 2006]). These water molecules are also observed in higher-resolution crystal structures (Salter et al., 2006; Torelli et al., 2007) and appear to participate in extensive hydrogen bonding networks similar to those observed in protein enzymes (Meyer, 1992). These networks form the basis for correlated motions that amplify local chemical modifications into functionally relevant structural rearrangements throughout the catalytic core (Rhodes et al., 2006). In addition, the trapped water molecules line up in a hydrogen-bonded chain, reminiscent of the "proton wire" found in protein enzymes (Frank et al., 2004). Coincidentally, a central water molecule in this wire accepts a hydrogen bond from the 2′-OH of A-1, engaging the proton that needs to be removed during catalysis (Figure 2B). The same water is also found to frequently donate a hydrogen bond to N1 of A38 (Rhodes et al., 2006), opening the possibility of a mechanism in which a proton is first shuttled from the 2′-OH of A-1 through the water molecule to N1 of A38 and then onto the 5′-oxyanion leaving group in a concerted mechanism (Figure 2B). In this fashion, a water-assisted A38 could serve both as general base and acid in the reaction. Additionally, or alternatively, the trapped water molecules may stabilize the enhanced negative phosphate charge in the transition state as suggested by MD simulation and X-ray crystallography (Park and Lee, 2006; Torelli et al., 2007).

Higher-resolution electron density maps from X-ray crystallography have recently begun to detect water molecules in the catalytic cores of other ribozymes, including the hammerhead (Figure 2C) (Martick and Scott, 2006) and *glmS* ribozymes (Figure 2D) (Klein and Ferre-D'Amare, 2006) as well as the ribosome (Schmeing et al., 2005). In the hammerhead ribozyme, for example, a protonated water molecule has been proposed to serve as a proton source in a proton relay involving the 2′-OH of G8 as general acid catalyst (Figure 2C) (Martick and Scott, 2006). In the case of the *glmS* ribozyme, a proposal has been made that the external glucosamine-6-phosphate ligand acts as general acid catalyst as well as, through a proton wire involving two structural water molecules, remote general base catalyst (Figure 2D). Notably, the underlying crystal structure contains a 2′-deoxy modification at the cleavage site (similar to the crystal structure of RNase A shown in Figure 2A) and lacks the amino functionality of the ligand (Klein and Ferre-D'Amare, 2006); it is known that such minor chemical modifications can lead to RNA conformational changes (Rueda et al., 2004;

Rhodes et al., 2006). A more recent crystal structure carrying a cleavage site 2′-O-methyl modification and the native glucosamine-6-phosphate ligand does not display these water molecules and thus predicts direct general base catalysis by G40 (Figure 2D; G33 in the authors' numbering scheme) (Cochrane et al., 2007).

Is there proof for whether or not structural water molecules are active participants in ribozyme catalysis? Of course any proton shifted to or from a hydrated metal ion or an RNA functional group will reside in bulk water sooner or later; that is, water molecules from the ubiquitous solvent will for sure indirectly be involved in any of the plausible mechanisms discussed above. But the open "whodunit" mystery is whether or not strategically placed, long-residency water molecules play a more direct role by, for example, shuttling protons during catalysis. The jury is still out, but water is an intriguing suspect to join the lineup with metal ions and RNA functional groups as potential catalytic effectors. In light of the molecular heterogeneities observed in RNA by single-molecule techniques (Zhuang, 2005) it is even plausible that different ribozyme molecules in an ensemble follow alternative reaction pathways. Clearly, additional experimental approaches such as neutron scattering (Zaccai, 1986), NMR (Newby and Greenbaum, 2002), and Fourier transform infrared (FTIR) spectroscopy (Garczarek and Gerwert, 2006) are needed to further pinpoint the locations and roles of water molecules in RNA catalysis. Studies on RNA will undoubtedly be guided by the extensive prior work on protein enzymes. It is tempting to speculate that evolution has found ways to exploit highly abundant solvent molecules with unique physicochemical properties to enhance the catalytic capabilities of both protein and RNA. Given our limited understanding of even the bulk properties of water (Leetmaa et al., 2006), it may well be that isolated structural water molecules bound to the surface of a biopolymer become quite unique chemical reagents (Buch et al., 2007).

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