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Catalytic Strategies of Self-Cleaving Ribozymes

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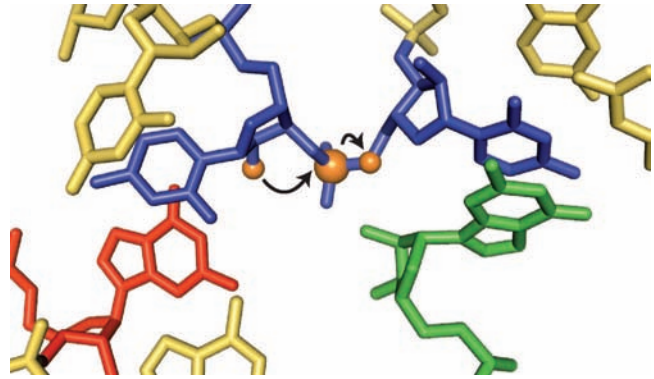
CONSPECTUS

Five naturally occurring nucleolytic ribozymes have been identified: the hammerhead, hairpin, *glmS*, hepatitis delta virus (HDV), and Varkud satellite (VS) ribozymes. All of these RNA enzymes catalyze self-scission of the RNA backbone using a chemical mechanism equivalent to that of RNase A. RNase A uses four basic strategies to promote this reaction: geometric constraints, activation of the nucleophile, transition-state stabilization, and leaving group protonation. In this Account, we discuss the current thinking on how nucleolytic ribozymes harness RNase A's four sources of catalytic power.



The geometry of the phosphodiester cleavage reaction constrains the nucleotides flanking the scissile phosphate so that they are unstacked from a canonical A-form helix and thus require alternative stabilization. Crystal structures and mutational analysis reveal that cross-strand base pairing, along with unconventional stacking and tertiary hydrogen-bonding interactions, work to stabilize the splayed conformation in nucleolytic ribozymes.

Deprotonation of the 2′-OH nucleophile greatly increases its nucleophilicity in the strand scission reaction. Crystal structures of the hammerhead, hairpin, and *glmS* ribozymes reveal the N1 of a G residue within hydrogen-bonding distance of the 2′-OH. In each case, this residue has also been shown to be important for catalysis. In the HDV ribozyme, a hydrated magnesium has been implicated as the general base. Catalysis by the VS ribozyme requires both an A and a G, but the precise role of either has not been elucidated.

Enzymes can lower the energy of a chemical reaction by binding more tightly to the transition state than to the ground states. Comparison of the hairpin ground- and transition-state mimic structures reveal greater hydrogen bonding to the transition-state mimic structure, suggesting transition-state stabilization as a possible catalytic strategy. However, the hydrogen-bonding pattern in the *glmS* ribozyme transition-state mimic structure and the ground-state structures are equivalent.

Protonation of the 5′-O leaving group by a variety of functional groups can promote the cleavage reaction. In the HDV ribozyme, the general acid is a conserved C residue. In the hairpin ribozyme, a G residue has been implicated in protonation of the leaving group. An A in the hammerhead ribozyme probably plays a similar role. In the *glmS* ribozyme, an exogenous cofactor may provide the general acid. This diversity is in contrast to the relatively small number of functional groups that serve as a general base, where at least three of the nucleolytic ribozymes may use the N1 of a G.

Five autolytic ribozymes have been identified that perform the same chemical reaction but have different secondary structures. These include the hammerhead (Figure 1A), the hairpin (Figure 1B), the *glmS* (Figure 1C), the hepatitis delta virus (HDV) (Figure 1D), and the Varkud satellite (VS) ribozymes (Figure 1E).[1−6] The hammerhead, hairpin, HDV, and VS ribozymes aid in processing the products of rolling circle replication of satellite, viral, or virusoid RNA genomes.[1−3,5] The *glmS* ribozyme is also a riboswitch, involved in the control of gene expression and the production of glu-

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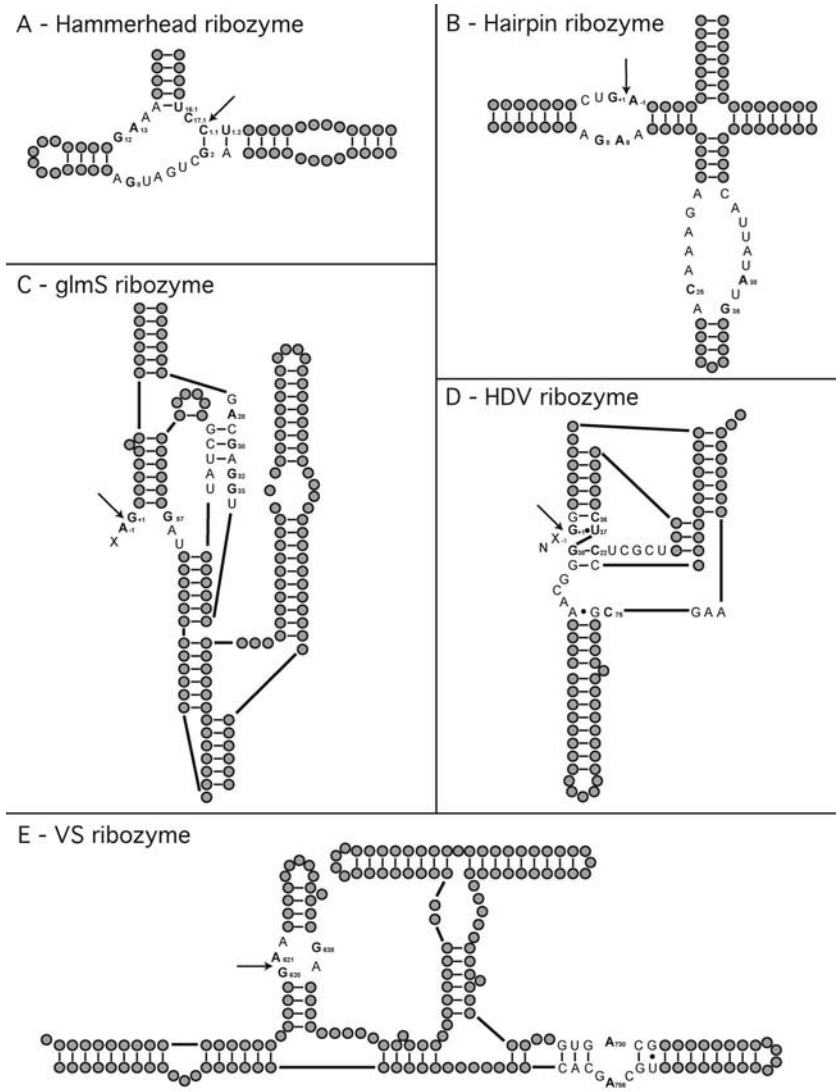

FIGURE 1. Secondary structures of the five nucleolytic ribozymes: (A) hammerhead; (B) hairpin; (C) *glmS*; (D) HDV; (E) VS. Key nucleotides are numbered following the established convention for each ribozyme. Positions occupied with an X can be any of the four nucleotides. The cleavage site is denoted by an arrow.

cosamine-6-phosphate (GlcN6P) in many Gram-positive bacteria.[6] All five of these small, nucleolytic ribozymes catalyze an internal transphosphorylation reaction identical to that of ribonuclease A (RNase A), a trans-acting protein enzyme.[7]

The mechanism of RNase A is the framework upon which ribozyme catalysis has been examined.[7] In a comprehensive review of RNase A, the molecular details of both the structure and catalytic mechanism were described.[7] The strand scission reaction of RNase A and the nucleolytic ribozymes involves attack of the vicinal 2′-OH on the scissile phosphate (Figure 2). Resolution of the transition state results in 2′−3′ cyclic phosphate and free 5′-OH products. The catalytic power of RNase A is derived primarily from four sources:[7] (1) alignment of the nucleophile, scissile phosphate, and leaving group by splaying the nucleotides adjacent to the scissile phosphate; (2) activation of the 2′-OH nucleophile by a general base; (3) increased affinity for the pentacoordinate oxyphosphorane transition state; (4) activation of the 5′-O leaving group by a general acid. The total rate enhancement achieved by RNase A is 3×10^{11} over the background rate of RNA hydrolysis.[7] All of the small ribozymes catalyze the same reaction as RNase A and employ many of the same tactics in promoting chemistry.

The catalytic residues in the RNase A active site have been carefully delineated (Figure 2). In RNase A, the pyrimidine nucleotide 5′ to the scissile phosphate makes hydrogen bonds with main chain and backbone oxygen atoms of Thr45, the side chain that is also responsible for the steric occlusion of a purine nucleotide in this position. Further, the nucleotide 5′ of the scissile phosphate makes stabilizing base-stacking inter-

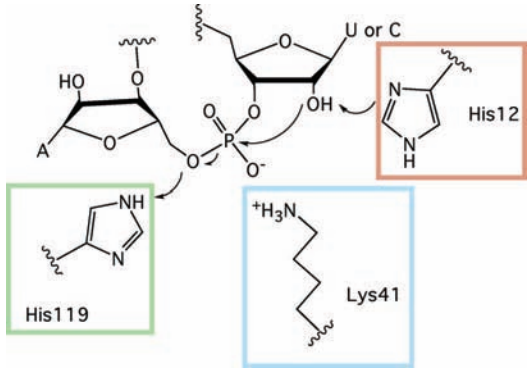

FIGURE 2. The catalytic reaction of RNase A. The general base, His12, is boxed in red. The general acid, His119, is boxed in green. The amino acid responsible for charge stabilization at the transition state, Lys41, is boxed in blue.

actions with Phe120.[8] In classic general acid−base catalysis as is seen in RNase A, the general base that is responsible for deprotonation of the 2′-OH is His12.[7] Lys41 is involved in stabilizing the negatively charged transition state. Mutation of Lys41 to an arginine reduces the activity of RNase A to less than 2% of the wild-type enzyme.[9] The 5′-O leaving group is protonated by His119. Mutation of His119 to alanine leads to an almost 10^4-fold loss in transphosphorylation activity.[10]

Nucleic acids would appear to be at a pronounced disadvantage in their ability to catalyze chemical reactions compared with proteins. RNA lacks a functional group that has a pK_a close to neutrality (like histidine) or one that is positively charged (like lysine). These chemical deficiencies led to an early assumption that most small ribozymes were obligate metalloenzymes.[11] This turns out to be untrue, and while all functional RNAs require positively charged ions to assume a folded structure, high concentrations of monovalent cations or inert polyvalent ions are sufficient for many of the ribozymes to be maximally active.[12,13] The hairpin, hammerhead, and VS ribozymes all appear to use only nucleotides to promote catalysis.[14] However, this does not speak to the role of metal ions in RNA catalysis in a cellular context. Even lacking the chemical diversity of the amino acid side chains, these nucleic acid enzymes catalyze RNA cleavage only 15−500-fold slower than RNase A.[2,15,16] The HDV ribozyme may use a specifically bound magnesium ion as well as nucleotides to catalyze a reaction only about 150-fold slower than RNase A.[17] Finally, the *glmS* ribozyme uses a small molecule cofactor, GlcN6P, as well as nucleotides to achieve chemistry at a rate of about 100 times slower than that of RNase A.[18] Here, we discuss the current state of thinking regarding the strategies used by nucleolytic ribozymes to harness the four sources of catalytic power employed by RNase A.

Active Site Organization

The transphosphorylation reaction promoted by nucleolytic ribozymes has restraints on the geometry of the atoms involved in the bond making and breaking steps. For an S_N2 reaction, the torsion angle between the 2′-OH nucleophile, the scissile phosphate, and the 5′-O leaving group must be 180°. This in-line architecture is never sampled in a canonical, base-stacked A-form helical RNA structure. For the reactive groups to assume an in-line conformation, the nucleotides 5′ and 3′ of the scissile phosphate must be splayed away from the reactive group. Ribozymes provide an alternative set of stabilizing interactions to achieve this unfavorable conformation.

The active site of the hammerhead ribozyme is formed from residues in the central loop of the three-way junction (Figure 1A).[5] This loop contains the cleavage site, an obligate cytosine (C17) 5′ to the scissile phosphate, and many conserved nucleotides. The structure of the full-length hammerhead ribozyme revealed hydrogen-bonding and stacking interactions that lead to the in-line conformation at the cleavage site (Figure 3A).[19] C17 makes a hydrogen bond to A9; mutation of either of these nucleotides is detrimental to catalysis.[19,20] Further, the noncanonical position of C17 is stabilized between U16.1 and G12.[19] The nucleotide 3′ to the cleavage site (C1.1), while not conserved, is involved in a Watson−Crick base pair and stacked between G8 and U1.2.[19] Initial structures of minimal active hammerhead ribozymes displayed geometry inconsistent with biochemical evidence.[21,22] These ground-state structures suggested that a large conformation change was taking place prior to the catalytic step, as two phosphate oxygens that coordinate a single metal were over 20 Å apart in these structures.[23] In the structure of the full-length construct, compaction of the active site brought these two phosphate oxygens to within 4 Å.[19] In this full-length structure, the angle between the 2′-OH nucleophile, scissile phosphate, and 5′-O leaving group is very close to 180°.

The hairpin ribozyme is formed from two helical stacks, with the active site formed in the cleft with nucleotides from both helices (Figure 3B).[2] The nucleotide 5′ of the scissile phosphate, G + 1, is flipped out of its helix and "buried" in a pocket in the other helix, stacking between A26 and A38 (Figure 3B).[24] G + 1 makes a tertiary Watson−Crick base pair with C25, as well as additional hydrogen-bonding contacts to G36 and A38 that seem to be specific for G, explaining the requirement for G at that position.[24] Mutation of G + 1 to any other base resulted in a loss of catalytic activity that was at least partially restored by compensatory mutations at C25.[25]

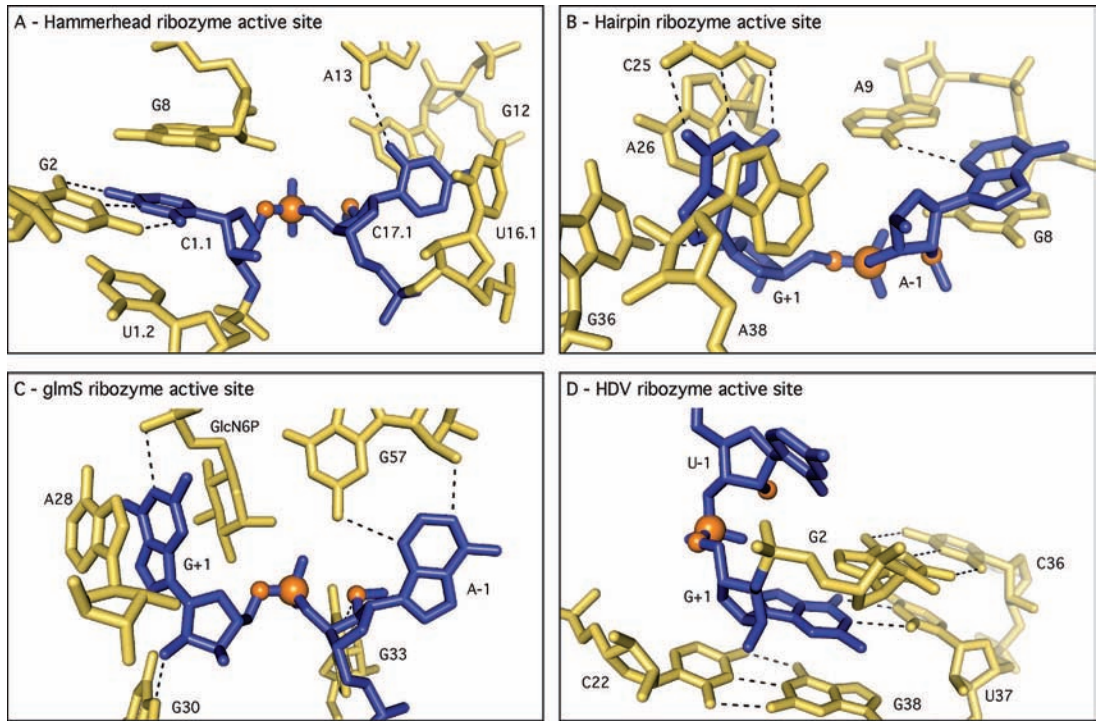

FIGURE 3. Ribozyme active site structures: (A) precleaved *Schistosoma mansoni* hammerhead ribozyme from pdb 2GOZ; (B) precleaved hairpin ribozyme from pdb 1M5K; (C) precleaved *glmS* ribozyme from pdb 2NZ4; (D) U75C inactive mutant HDV ribozyme from pdb 1VC0. The nucleotides flanking the scissile phosphate are in blue. The 2′-OH nucleophile (methylated in many of the structures), the scissile phosphate, and 5′-oxygen leaving group are shown in orange spheres. Hydrogen bonds are shown as dashed lines. This basic coloring scheme is repeated in Figures 4−6. Nucleotides that interact with the reactive atoms through base stacking or hydrogen-bonding interactions are shown in yellow.

The nucleotide 5′ of the cleavage site, A − 1, makes a hydrogen bond to the exocyclic amine of A9 and stacks on top of G8.[24] In structures of hairpin ribozymes that have substitutions at G8, the in-line conformation of the reactive groups is nonideal. Further some of these substitutions also appear to affect the sugar pucker at A − 1.[26] This suggests that G8 plays a key role in organizing the active site for catalysis, along with the additional tertiary contacts from A9, C25, A26, and A38.

The tertiary structure of the *glmS* ribozyme is also formed from two helical stacks, but the active site is formed from an internal loop that forms two pseudoknots (Figure 1C).[4] The nucleotides flanking the scissile phosphate are in a splayed conformation that is stabilized primarily through hydrogen-bonding and stacking interactions (Figure 3C).[27] G + 1 stacks underneath A28 and makes a tertiary hydrogen bond to G30.[27] There seems to be a slight preference for an A 5′ of the cleavage site.[6] In the structures of the *glmS* ribozyme, A − 1 is orientated through hydrogen-bonding interactions with G33 and G57.[27] These noncanonical interactions lead to a in-line conformation for A − 1 and G + 1.

The three-dimensional structure of the HDV ribozyme is that of a double nested pseudoknot that organizes five helical segments (Figure 1D).[1] While several structures of the HDV ribozyme have been solved, none elucidate the full set of interactions made by the active site nucleotides to stabilize the splayed conformation.[28,29] The structure of the HDV product complex showed substantial rearrangements compared with earlier structures of individual helices but did not provide any information about stabilization of the nucleotide prior to the cleavage site.[28,30,31] Details of inactive mutant HDV structures are inconsistent with data regarding the chemical mechanism of the HDV ribozyme.[29,32−34] What does seem to be clear is that the nucleotide 3′ of the scissile phosphate, G + 1, makes a wobble base pair with U37 (Figure 3D).[28,29] The geometry of the wobble pair seems to be required because an A−C wobble is the next most active variant.[35] The G + 1−U37 wobble pair forms the last base pair of the P1 helix, which stacks directly underneath the P1.1 helix, forcing the nucleotide 5′ of the scissile phosphate to sample the in-line geometry required for catalysis.[28,29]

No crystal structure of the VS ribozyme is available, so it is not clear how the inline configuration is achieved in this system. The ribozyme cleaves between a G and an A (Figure 1E).[3] In the structure of an isolated stem−loop construct containing the scissile phosphate, both the G and the A are involved in sheared G−A base pairs.[36] There is some evidence from

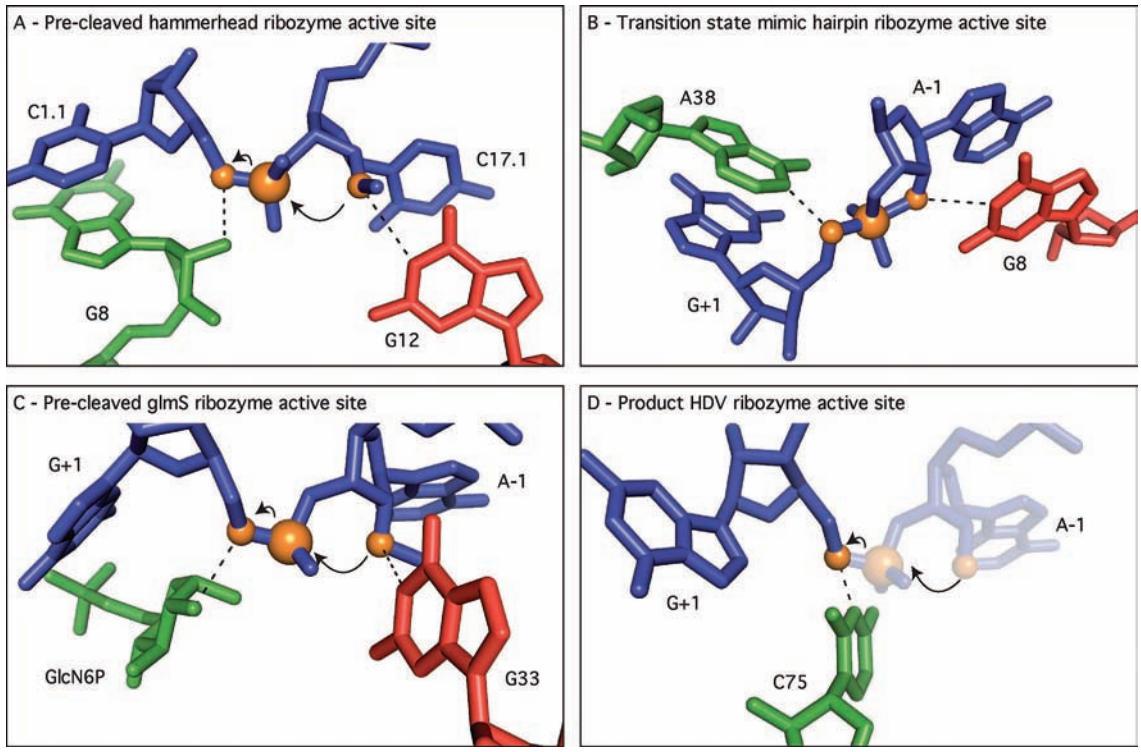

FIGURE 4. Chemical catalysis in the ribozyme active sites: (A) precleaved *Schistosoma mansoni* hammerhead ribozyme from pdb 2GOZ; (B) transition-state mimic bound hairpin ribozyme from pdb 1M5O; (C) precleaved *glmS* ribozyme from pdb 2NZ4; (D) product HDV ribozyme from pdb 1DRZ. Basic coloring scheme is the same as that in Figure 3. Nucleotides proposed to act as general bases are shown in red. Nucleotides or cofactors proposed to act as general acids are shown in blue. The nucleotide 5′ of the cleavage site has been modeled and is slightly transparent. The putative general base, a magnesium ion, is not present in the structure.

covariation analysis that the base pairing in that region becomes rearranged upon formation of an additional tertiary interaction.[37] An NMR structure of a stabilized stem−loop thought to mimic the conformation of the stem−loop in the context of the ribozyme revealed that the sheared G−A base pair containing the G 5′ of the scissile phosphate is preserved, while the second sheared A−G pair is disrupted, and the A is not coplanar with the G.[38]

Activation of the 2′-OH Nucleophile

The transphosphorylation reaction is initiated by attack of the 2′-OH on the scissile phosphate. The reaction is facilitated by, but does not require, deprotonation of the hydroxyl, which increases the nucleophilicity of the group and the reaction rate. The pK_a of the 2′-OH is approximately 12. Guanosine residues in the hairpin, hammerhead, VS, and *glmS* ribozymes and a hydrated Mg^{2+} in the HDV ribozyme have been implicated as the general base.

In the crystal structures of hammerhead, hairpin, and *glmS* ribozymes, a G is within hydrogen-bonding distance of the 2′-OH nucleophile.[19,24,27] This proximity is suggestive of its role in activation, a role for which G seems particularly unsuited, because the N1 is expected to be fully protonated at neutral pH with a pK_a of around 10. This has led to uncertainties about the role of these G's in catalysis. One possibility is that an enol tautomer of the G transiently forms, allowing the N1 to accept a proton from a 2′-OH.[39,40] Another suggestion is that the active site G's are acting to stabilize negative charge that develops at the transition state.[41]

In the active site of the full-length, precleaved hammerhead ribozyme, the N1 of G12 is within hydrogen-bonding distance of the 2′-OH, which has been methylated (Figure 4A).[19] Biochemical analysis of G12 shows a log-linear relationship of activity with pH that does not plateau even at the highest pHs.[39] Mutation of G12 to any of the other three nucleobases leads to a 100−1000-fold decrease in the rate of the reaction.[20] Further, substitution with analogs that perturb the pK_a at N1, such as inosine (∼9), diaminopurine (∼5), and 2-aminopurine (∼4), affected the overall rate of the reaction, with 2-aminopurine being the most detrimental, leading to a rate reduction of $\sim 10^3$.[39] This suggests that the N1 of G is directly participating in the chemical mechanism of the hammerhead reaction.

In crystal structures of the hairpin ribozyme, the N1 of G8 is hydrogen-bonded to the methylated 2′-OH of the nucleophilic residue (Figure 4B).[24] As in RNase A, proton inventory

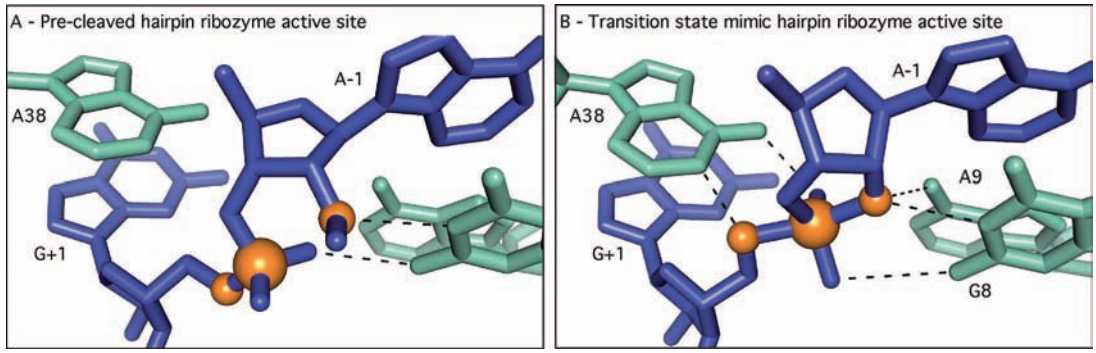

FIGURE 5. Transition-state structural mimic of the hairpin ribozyme: (A) ground-state structure from pdb 1M5K; (B) vanadate-bound transition-state mimic structure from pdb 1M5O. Basic coloring scheme is the same as that in Figure 3. Nucleotides that make hydrogen bonds to any of the reactive atoms or the nonbridging phosphate oxygens are shown in green-blue.

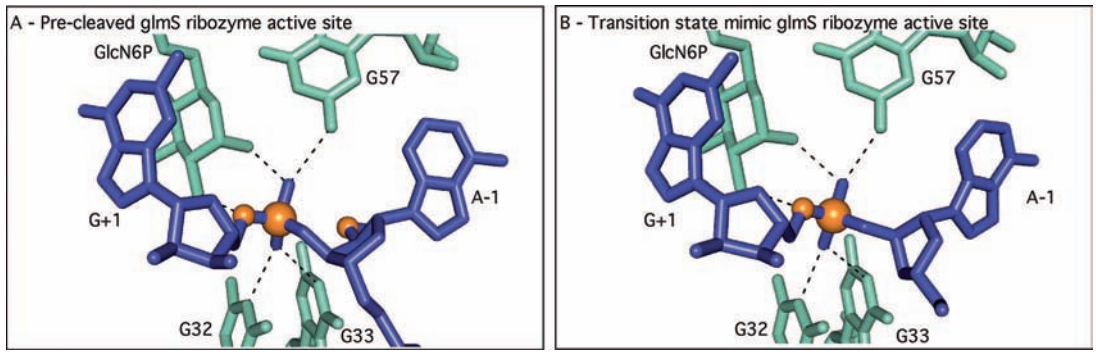

FIGURE 6. Transition-state structural mimic of the *glmS* ribozyme: (A) ground-state structure from pdb 2NZ4; (B) the 2′−5′-linked transition-state mimic structure from pdb 3B4C. Basic coloring scheme is the same as that in Figure 3. Nucleotides that make hydrogen bonds to any of the reactive atoms or the nonbridging phosphate oxygens are shown in green-blue.

experiments suggest that there are two protons "in flight" at the transition state for the rate-limiting step in the reaction.[42] An abasic substitution at G8 results in a $\sim 10^3$ rate reduction.[41] To the extent that it can be measured, this substitution does not appear to affect the pH dependence of the reaction, suggesting that the apparent pK_a results from another source.[41] A mechanism that these data support is one in which G8 specifically interacts with the transition state of the reaction. However, substitution at G8 with inosine shifted the apparent pK_a of the reaction downward slightly, and substitution with diaminopurine at G8 leads to a classic bell-shaped pH profile due to the near-neutral pK_a for diaminopurine at N1, as would be expected if G8 were acting as a general base.[42]

Like the hairpin and hammerhead ribozyme crystal structures, the active site of the *glmS* ribozyme contains a G (G33) whose N1 is within hydrogen-bonding distance of the 2′-OH of A − 1 (Figure 4C).[27,43] Mutation of G33 to any of the other nucleobases results in a 10^3-10^5 reduction in the rate of cleavage in the presence of GlcN6P.[43,44] A recently published crystal structure of a *glmS* ribozyme in which G33 has been mutated to an A reveals an active site in which the only difference is a shift in the position of the nucleobase at position 33.[44] This suggests that G33 is not responsible for positioning the 2′-OH but may be neutralizing charge during the reaction without being directly responsible for deprotonation of the nucleophile.

In the HDV ribozyme, a hydrated Mg^{2+}, acting as a general base, appears to contribute relatively little to catalysis.[33] Mechanistic studies have shown that catalysis in monovalent ions leads to about a 3000-fold reduction in the reaction rate.[45] Of that, ~125-fold is the result of disrupting the structure, and only ~25-fold is from a catalytic contribution.[45] In the absence of magnesium, it has been postulated that a water or hydroxide ion serves the same role, as has been suggested for the His12 to alanine mutant of RNase A.[45] Recent crystal structures of an inactive mutant of the HDV ribozyme show metal ion binding in the active site, although it appears to be bound next to the O5′ leaving group.[29] Comparison of the precursor, mutant, and product forms of the HDV ribozyme reveals that there are significant structural rearrangements during catalysis.[28,29]

Recent biochemical analysis of the VS ribozyme has implicated both an A and a G in acid−base catalysis.[46–48] Substitution of A756 to G results in a $\sim 10^3$-fold reduction in the rate of cleavage, while substitution of G638 to A results in a

$\sim 10^4$-fold reduction in rate.[46–48] In the absence of detailed mechanistic studies or a crystal structure, the precise role of either of these nucleotides remains unknown. Substitution of G638 with inosine (I) reduced the rate of cleavage by a factor of ~50 but also shifted the basic pK_a of the reaction down about 0.2 pH units.[48] This shift is similar to the pK_a difference between the N1s of G and I, but the effect could be indirect.

Transition-State Stabilization

In RNA, there are no functional groups that carry a positive charge at neutral pH. It is unclear whether there is a direct mechanism for charge stabilization in the active sites of ribozymes. Metal ions might play this role, or additional hydrogen bonding may allow the ribozyme to bind to the transition state with greater affinity than to the precursor or product states.

Using vanadate as a mimic for the trigonal pyramidal phosphate, Rupert et al. trapped the hairpin ribozyme in a structure similar to that of the transition state.[24,49] Charge neutralization is not observed, but hydrogen bonding to the transition state is strengthened relative to the ground states (Figure 5).[24,49] In the precursor structure, the hairpin ribozyme makes two hydrogen bonds to the reactive atoms, the 2′-OH nucleophile, scissile phosphate oxygens, or 5′-oxygen leaving group (Figure 5A).[24] In the product state, three hydrogen bonds are made to the reactive atoms.[49] However, in the transition-state structure, five hydrogen bonds are made to the vanadate oxygens (Figure 5B).[49] The hydrogen bonds are made between the reactive atoms and G8, G9, and A38 in the ribozyme, all three of which have been biochemically implicated in catalysis.[24,49]

The structure of a transition-state mimic of the *glmS* ribozyme has also been solved.[44] It is not a vandate-bound structure; a 3′-deoxy A − 1 nucleotide is linked to G1 via a 2′−5′ linkage (Figure 6).[44] This structure is superimposable upon the precleaved form of the ribozyme (Figure 6A).[43,44] The same hydrogen-bonding pattern to the nonbridging phosphate oxygens is present in precleaved ribozyme structure and in the transition-state structure (Figure 6B).[43,44] These data do not suggest how the *glmS* ribozyme stabilizes the transition state over the precleaved or product forms of the ribozyme.

While nucleotides have been predicted to be key for catalysis in the hammerhead, HDV, and VS ribozymes, no structural information exists to indicate whether these are making additional hydrogen bonds in the transition state. In each case, the phosphate and phosphate oxygens are expected to move closer to the nucleotide 3′ of the cleavage site, and this physical shift may allow for additional hydrogen-bonding interactions. However, whether these ribozymes utilize this mechanism for promoting their chemical reactions is yet to be determined.

Protonation of the 5′-Oxygen Leaving Group

The other half of a general acid−base mechanism is a second residue that acts as a proton donor to the leaving group. In the nucleolytic ribozymes, this role may be carried out by a variety of functional groups originating both in nucleotides and in exogenously provided cofactors.

Of the five nucleolytic ribozymes, the HDV ribozyme provides the clearest example of general acid catalysis by a nucleobase.[28,29,32–34] The first crystal structure of the HDV ribozyme was the product form and showed the N3 of C75 within hydrogen-bonding distance of the 5′-O leaving group, predicting that it would act as a general acid (Figure 4D).[28] Crystal structures of the C75U ribozyme showed a rearranged active site with the N3 of U75 close to the 2′-OH of the nucleophile, where it would be a general base.[29] The C75U mutant is inactive, over 10^6-fold down in rate, but activity can be rescued with exogenous imidazole.[50] Mutation of C75 to A slowed the reaction by over 100-fold and also decreased the pK_a of the reaction by ~0.5 pH units, similar to the difference in pK_a's between the N3 of C and N1 of A.[33] In the presence of Mg^{2+}, the reaction rate increased from pH 6 to pH 8 but decreased over the same range in the absence of Mg^{2+}.[33] These data implicate Mg^{2+} as the general base and support the role of C75 as the general acid. Mutations that affect stabilization of the leaving group are predicted to be less disabling in the context of an activated 5′-bridging phosphorothiolate (5′-PS)-linked substrate.[32] The C75U mutant ribozyme is almost completely rescued by the 5′-PS substrate at pH 7.5.[32] These kinetic data implicate the N3 of C75 as the general acid in the HDV reaction.

The crystal structure of the full-length hammerhead ribozyme shows the 2′-OH of G8 within hydrogen-bonding distance of the 5′-O leaving group, with the Watson−Crick face of G8 base paired to C3 (Figure 4A).[19] The 2′-OH of G8 is required for cleavage by the hammerhead ribozyme.[51,52] However, other G8 mutants are also detrimental to catalysis, with the G8C mutant reducing the rate by $\sim 10^5$-fold.[19,20] The double mutation of G8C and C3G at least partially restored activity, resulting in only a ~200-fold decrease in the rate from wild-type.[19] Further, other hammerhead ribozyme constructs are even more tolerant to mutations at these positions, provided that the base pairing between the two nucleotides is

maintained.[53,54] These data suggest that while G8 may be playing the role of the general acid in the cleavage reaction, it uses functional groups common to all RNA nucleotides, such as the 2′-OH as is observed in the crystal structure. This differs from earlier biochemical data where substitution of G8 with analogs implicated the N1 of G as the general acid in the reaction.[39]

In the hairpin ribozyme, A38 has been suggested to be the general acid (Figure 4B). Deletion of the A38 base resulted in 10^4-10^5-fold reduction in catalytic activity.[55] Rescue of this abasic site required a planar heterocycle that had the same Watson−Crick face as an A.[55] These small molecules had pK_a's at N1 (or equivalent) that ranged from 3.8−9.0.[55] By measurement of the pH dependence of the rate of the reaction in the presence of each of the small molecules, it became apparent that the protonation state of A38 was critical. This suggested that at least part of the pH dependence of the hairpin ribozyme arises from A38, which has a calculated pK_a of ~6, similar to that of the acidic pK_a of the natural ribozyme.[55] In crystal structures of hairpin ribozymes, the N1 of A38 is involved in a hydrogen-bonding interaction with the 5′-O leaving group.[24,49]

The proposed catalytic mechanism for the *glmS* ribozyme invokes a novel mechanism for protonation of the leaving group, involving the sugar GlcN6P. In the absence of GlcN6P, the ribozyme is ~10^4-fold less active.[6] Substitution of the cofactor with the closely related Glc6P, in which the C2-amine has been substituted with a hydroxyl, also leads to rate reductions of ~10^4.[56] The pK_a of the C2-amine of GlcN6P is closer to neutral than other RNA functional groups.[56] The pK_a is about 7.8, a value very similar to what is observed for the pK_a of the cleavage reaction.[56] In the crystal structure of the GlcN6P bound *glmS* ribozyme, the C2-amine is hydrogen-bonding to the 5-O leaving group (Figure 4C).[43,57] The location and pK_a of the amine, as well as the effect on the rate of its substitution, suggests that it is responsible for protonation of the leaving group.

The role of A756 in the reaction of the VS ribozyme is still under debate, but it appears to be critical for efficient catalysis.[46,47] Mutation of A756 leads to at least a 300-fold reduction in the rate, while an abasic site at 756 leads to over 10^3-fold loss in catalytic activity.[46] An interference study using pK_a-perturbed nucleotide analogs further implicated A756 in the chemical mechanism of the VS ribozyme.[58] Incorporation of purine or 8-aza-adenosine, both of which have lower pK_a's at N1 than A, resulted in interference at A756 that could be rescue by reducing the pH of the reaction.[58] These results suggest that a protonated base at A756 is important for catalysis. This could be a requirement for A756 to protonate the leaving group or stabilize the transition state, or another less direct role.

Conclusion

Although RNA cleavage by nucleolytic ribozymes is not as efficient as that of RNase A, the catalytic mechanisms do share similarities. The organization of the active sites in ribozymes promotes the reaction by aligning the nucleophile with the scissile phosphate and the leaving group. While some ambiguity remains as to how particular groups are used for catalysis, there is certainly evidence that at least some of the ribozymes are capitalizing on an acid−base mechanism for the reaction. Finally, ribozymes may be binding to the transition state with greater affinity than to the precleaved or product states. By utilizing these four mechanisms for promoting catalysis, nucleolytic ribozymes are able to undergo self-scission in the absence of proteins.

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FOOTNOTES

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