compelling evidence in favour of this catalytic mechanism [29,30]. It therefore remains possible that most of the catalytic rate enhancement effected by the hairpin ribozyme comes about from distortion of the substrate to align the reactive groups, stabilization of the transition state, or destabilization of the ground state. Understanding the mechanism of action of this ribozyme that does not employ metal ion cofactors remains a challenging problem.

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Comparison of the biochemical and structural features that are shared among small molecules that mediate rescue provides a new perspective on potential mechanisms of hairpin ribozyme catalysis.

**Introduction**

Twenty years after Cech and Altman earned the Nobel Prize for the discovery of catalytic RNA, understanding the mechanisms of catalysis by RNA enzymes remains an intriguing challenge. The significance of this challenge has grown with the recent demonstration that the active site for protein synthesis is composed entirely of RNA [1-3] and evidence that precursor mRNA processing also might be RNA-catalysed [4,5]. Protein synthesis and precursor mRNA splicing are multistep processes that require a variety of cofactors and large, complex RNA-protein structures, so the catalytic chemistry underlying these reactions can be difficult to address experimentally. Mechanisms of catalysis by self-splicing and self-cleaving ribozymes are amenable to detailed study using conventional enzymological methods. The focus of this review is the application of this approach to understanding the catalytic mechanism of one simple self-cleaving RNA known as the hairpin ribozyme.

The hairpin ribozyme catalyses a reversible self-cleavage reaction that generates products with 5’ hydroxy and 2',3’-cyclic phosphate termini [6] (Figure 1). In nature, hairpin ribozyme-mediated self-cleavage and ligation participate in processing intermediates of plant satellite RNA replication. Cleavage occurs through nucleophilic attack of phosphorus by the adjacent 2’ oxygen and proceeds with inversion of configuration, implicating a bimolecular nucleophilic substitution (S₃₂₋) in-line attack mechanism that generates a di-anionic transition state. Ligation occurs through a simple reversal of the cleavage reaction with the 5’ oxygen serving as the nucleophile. Small catalytic RNAs, including the hammerhead, hepatitis delta virus (HDV) and *Neurospora* Varkud satellite ribozymes, all catalyse the same chemical reactions, but adopt different structures and exploit distinct kinetic and catalytic mechanisms.

Several factors might accelerate this reaction in an enzyme active site, including the position and orientation of reactive groups, general acid–base catalysis, electrostatic stabilization of negative charge in the transition state, or destabilization of the ground state [7-9]. RNase A catalyses the same chemical reaction and provides a textbook example of concerted general acid–base catalysis [10]. Two histidine residues in the RNase A active site are responsible for removing a proton from the attacking 2’ oxygen nucleophile and protonating the 5’ oxygen leaving group. An interaction between a positively charged lysine and a phosphoryl oxygen provides electrostatic stabilization as negative charge develops in the transition state.

RNA enzymes seem to have no functional groups as adept as amino acid side chains at catalytic chemistry. Ribonucleosides, at least free in solution, ionize outside the neutral pH range which would appear to make them poor general acid or base catalysts. Furthermore, no RNA functional groups are positively charged at neutral pH like the lysine in the RNase A active site. It once seemed that all catalytic RNAs must recruit metal cation cofactors to provide electrostatic stabilization through interactions with ribose and phosphate oxygens and to mediate proton transfer through metal-bound water. However, the unique pH and metal ion dependencies of hairpin ribozyme catalysis provided an early indication that ribozymes can accomplish catalysis without direct co-ordination of metal cations to phosphate, ribose or water oxygens [11-13]. Together with recent...
evidence from several systems, these results suggest that RNA nucleobases have a previously unrecognized capacity for mediating catalytic chemistry. The crystal structure of the HDV ribozyme self-cleavage product revealed no metal cations in the active site [14]. Instead, the ring N3 of a conserved cytosine lies within hydrogen bonding distance of the 5’ oxygen leaving group, consistent with a role as a general acid catalyst in donating a proton to the 5’ oxyanion leaving group. Elegant enzymological studies support a role for this cytosine in proton transfer [15–17]. The crystal structure of the large ribosomal subunit revealed no proteins within 18 Å of the peptidyl transferase active site, firmly placing the ribosome on the list of naturally occurring ribozymes [2,18]. A conserved adenine with its N3 oriented toward the reactive carbonyl carbon was proposed to mediate proton transfer in peptide bond formation [1,2].

Hairpin ribozyme structure

Two developments have recently made it easier to differentiate the effects of experimental manipulations on catalytic chemistry from effects on the hairpin ribozyme structure. First, a ribozyme variant that assembles in the context of a four-way helical junction was found to display greatly enhanced tertiary structure stability [19–21]. Most studies of hairpin ribozymes have focused on minimal constructs that consist of two helix-loop-helix elements, H1–loop A–H2 and H3–loop B–H4 [6] (Figure 2a). Alterations of most loop nucleotides interfere with activity, evidence that these nucleotides either form critical tertiary interactions or participate directly in catalytic

Figure 2

Consensus secondary structures of the minimal hairpin ribozyme that contain (a) just the essential A and B helix-loop-helix elements and (b) a hairpin ribozyme variant with a more stable tertiary structure [19,20] in which A and B elements form two arms of a four-way helical junction.

The identities of nucleotides indicated in outline font are important for activity; substrate sequences are indicated in bold font; arrows indicate the reactive phosphodiester.
chemistry (outline font, Figure 2). In the functional structure, loop A and B domains dock into a non-coaxial orientation that brings loop nucleotides into proximity. Fluorescence resonance energy transfer studies suggest that only approx. 60% of the minimal ribozyme adopts the functional docked conformation under standard reaction conditions, while approx. 40% remains in an inactive extended conformation [20]. Most modifications of loop nucleotides that block activity do so by disrupting tertiary interactions that are required to stabilize the docked structure [22,23], making it difficult to assess any independent effects of nucleotide modifications on catalytic chemistry. In the hairpin motif found in tobaccoregion virus satellite RNA, the H1-loop A–H2 and H3–loop B–H4 elements comprise two arms of a four-way helical junction [24,25] (Figure 2b). In four-way junction constructs, the docked conformation is favoured over the extended conformation by at least 20-fold under standard conditions [20], making it possible to assess the effects of individual nucleotide modifications on catalysis within an intact structure [19,20,26].

The second critical advance occurred when the crystal structure of a hairpin ribozyme complex with a non-cleavable substrate was solved [27]. The crystal structure showed the 2' oxygen nucleophile in-line with phosphorus and the 5' oxygen leaving group, consistent with the in-line S$_2$2 nucleophilic attack mechanism previously inferred from reaction stereochemistry. Conserved residues G8, A9, A10 and A38, but no metal cations, were found near the reactive phosphate, consistent with mutagenesis and biochemical studies. The in-line phosphate conformation and the agreement with results of structure–function studies strongly argue that the crystal structure represents the ground state of the functional ribozyme. As compelling as this structure is, however, the nature of the catalytic chemistry that takes place within this active site remains far from clear.

**Catalysis does not require direct metal cation co-ordination to phosphate, ribose or water oxygens**

RNAs assemble into compact functional structures only in association with counterions that serve to neutralize negatively charged phosphates. Therefore, distinguishing metal cations that serve as catalytic cofactors from the counterions that provide electrostatic shielding to stabilize functional ribozyme structures presents an experimental challenge. One method used to distinguish cations that associate with RNAs non-specifically from those that participate directly in catalysis relies on changes in metal ion specificity that accompany substitution of specific phosphate or ribose oxygens with sulphur or nitrogen. 'Hard' metal ions, such as Mg$^{2+}$, interact well with oxygen, a 'hard' ligand, but interact poorly with 'soft' ligands, such as sulphur or nitrogen, while 'soft' metal ions, such as Mn$^{2+}$ or Cd$^{2+}$, bind sulphur ligands with higher affinity [28]. A switch in metal cation specificity that accompanies substitution of oxygen by a soft ligand provides evidence of direct (inner sphere) metal cation co-ordination. If direct co-ordination of a metal cation to a phosphoryl oxygen was required for hairpin ribozyme catalysis, substitution of a phosphorothioate for the reactive phosphate would be expected to inhibit cleavage in buffers containing Mg$^{2+}$, but not in buffers containing Mn$^{2+}$ or Cd$^{2+}$.

The hairpin ribozyme cleavage rate constant was 4-fold lower for an R$_s$ phosphothioate linkage and 2-fold higher for an S$_p$ phosphothioate linkage than for a normal phosphate in Mg$^{2+}$ [11–13]. Thus neither diastereomer produced a thio effect of the same order as the 2000–80000-fold inhibition reported for hammerhead cleavage of an R$_p$ phosphothioate linkage in Mg$^{2+}$ [29]. A large thio effect could be underestimated if the chemical step is only partially rate-determining and reactions are limited by a different step, such as a slow conformational change. If the small thio effects that were observed for hairpin ribozyme-mediated cleavage did reflect the loss of Mg$^{2+}$ co-ordination, replacing Mg$^{2+}$ with the thiophilic Mn$^{2+}$ cation should restore full activity. However, R$_S$ and S$_p$ sulphur substitutions had virtually the same effects on cleavage rate constants in reactions with Mn$^{2+}$ as with Mg$^{2+}$. Furthermore, the reverse reaction of ligation of 2',3'-cyclic thiophosphate-substituted 5' cleavage product RNAs displayed similar small effects on ligation rate constants that showed the opposite stereospecificity [12]. Thus thiophosphate substitution experiments do suggest that pro-R$_p$ and pro–S$_p$ phosphoryl oxygens form distinct interactions in the transition state, but provide no evidence that these interactions involve bivalent cations.

Co(NH$_3$)$_6$$^{3+}$ is analogous in geometry and size to hexahydrated Mg$^{2+}$, but associates with RNA only through electrostatic and outer sphere hydrogen bonding and cannot co-ordinate phosphate, ribose, or water oxygens directly [30]. Nonetheless, Co(NH$_3$)$_6$$^{3+}$ promotes hairpin ribozyme
activity even more effectively than Mg$^{2+}$ [11–13]. This finding excludes a catalytic requirement for direct metal cation co-ordination to any ligand. Additional cations, such as NH$_4^+$, polyamines, and aminoglycosides, also support hairpin activity, evidence that a variety of cations can satisfy a structural requirement for counterions and that catalytic chemistry requires no direct, specific cation interactions [31–36].

**Do nucleobases participate directly in catalytic chemistry?**

The lack of essential metal cation cofactors suggests that hairpin ribozymes mediate catalysis exclusively through the use of RNA functional groups. Recent evidence from several other systems also suggests that RNA nucleobases have a previously unrecognized capacity for mediating catalytic chemistry. The crystal structure of the HDV ribozyme self-cleavage product revealed no metal cations in the active site [14]. Instead, a conserved cytosine lies within hydrogen bonding distance of the 5′ oxygen of the reactive phosphate, consistent with a role in donating a proton to the 5′ oxygen leaving group. Elegant enzymological studies support a role for this cytosine in proton transfer [15–17]. The crystal structure of the large ribosomal subunit revealed no proteins within 18 Å of the peptidyl transferase active site, firmly placing the ribosome on the list of naturally occurring ribozymes [2,18]. Again, no metal cations were identified in the active site and a conserved adenine has been proposed to participate directly in catalysis of peptide bond formation [1,2].

To investigate potential roles for active-site nucleobases in hairpin ribozyme catalytic chemistry, we adapted a method that originally was developed to examine proton transfer by amino acid side chains in protein enzyme catalysis. Toney and Kirsch demonstrated that exogenous alkyl amines could rescue an aspartate aminotransferase mutant that had been inactivated by an alanine substitution for an active-site lysine [37,38]. Herschlag and co-workers extended this approach to RNA enzymes by replacing conserved nucleotides of the hammerhead ribozyme with abasic residues and demonstrating that the activity of some abasic variants could be rescued by nucleobases and nucleobase analogues provided in solution [39–41]. In a related approach, Been’s group showed that an HDV ribozyme with a mutation or deletion of the active-site cytosine that had been implicated in proton transfer could be rescued by imidazole derivatives as well as by exogenous nucleobases [15,16].

Unlike conventional site-directed mutagenesis, exogenous nucleobase rescue is not limited by the small number of nucleobases and nucleobase analogues that can be incorporated covalently into RNA through transcription or chemical synthesis; virtually any water-soluble molecule can be tested for its ability to rescue. Thus, structural and biochemical features that influence rescue can be screened rapidly and comprehensively. The nature of the molecules that are able to rescue activity of abasic ribozymes lacking active-site nucleobases and the biochemical features of the rescued reactions can provide insight into catalytic chemistry and critical structural features of the active site. At subsaturating concentrations of the exogenous nucleobase, the apparent second order rate constant for rescue will be proportional to the affinity of the exogenous nucleobase for the rate-determining transition state [40]. If saturating concentrations of a rescuing molecule can be achieved, ground state or binding effects can be distinguished from transition state effects on catalysis from the nucleobase concentration required to achieve saturation and the first order rate constant for reaction of the ribozyme–nucleobase complex.

We prepared a series of abasic hairpin ribozymes that lack the conserved G+1, G8, A9, A10, and G11 nucleobases in the loop A domain (Figure 2b). These abasic substitutions were examined in four-way junction ribozyme constructs to minimize potential complications from tertiary structure destabilization. Abasic substitutions of G+1 or G11 were the most debilitating of the five variants that we tested. G11 forms the first base pair of the H2 helix that flanks loop A, and G+1 forms critical tertiary interactions with loop B that stabilize the docked conformation [6,27]. The loss of activity due to deletion of these nucleobases that seem to perform exclusively structural roles points to a major contribution of positioning and orientation to catalysis. Loss of G8 reduced the cleavage rate constant 350-fold, relative to rate constants of unmodified ribozymes. The N1 ring nitrogen of G8 lies within 2.9 Å of the 2′ oxygen that serves as the nucleophile, suggesting that G8 could activate the nucleophile by accepting a proton from the 2′ hydroxy group [27]. Abasic substitutions of A9 or A10 reduce cleavage rate constants by only 8- or 9-fold. A9 and A10 border a pocket that is large enough to accommodate a water molecule, although it was not occupied by
water in the crystal. One or more of these adenines was proposed to activate bound water to donate a proton to the 5’ oxygen leaving group [27,42].

The observation that cleavage rate constants fall only 8–350-fold suggests that abasic substitutions of G8, A9 or A10 leave active-site architecture largely intact. If abasic substitutions leave a solvent-filled cavity in an otherwise unperturbed active site, certain small molecules might be able to bind specifically in the active site and restore activity. Of over 30 nucleobases, nucleobase analogues, heterocyclic amines and amino acids that were surveyed, we found four that displayed significant rescue of a ribozyme variant, G8dX, that lacks G8. Ribozymes lacking G8 displayed at least 3-fold higher activity when cytosine, isocytosine, 2-aminopyridine or 2,6-diaminopurine was included in reaction mixtures compared with unrescued reactions. Each of these nucleobase analogues shares the guanidinium group that corresponds to the Watson-Crick hydrogen-bonding face of guanine, suggesting that the rescuing nucleobases might bind in the same orientation as the guanine that normally occupies the G8 position.

The N1 ring nitrogen of guanine and the corresponding N3 ring nitrogen of isocytosine should be protonated in the uncharged form at neutral pH, while the N3 ring nitrogen of cytosine should not be protonated at pH values above 4.6, at least in solution [43]. Thus, this limited survey left ambiguous the optimum protonation state of the ring nitrogen that contributes part of the guanidinium group. Higher levels of rescue were observed at lower pH values, evidence that rescue requires protonation. These results suggest that protonation is important for rescue, but do not distinguish whether protonation is required so that cytosine can serve as a proton or hydrogen bond donor or if rescue also requires the positive charge that accompanies protonation. The N3 ring nitrogen of isocytosine is protonated in the neutral form and positive charge localizes to the N1 ring nitrogen at low pH [43]. Nonetheless, isocytosine rescue also increases with decreasing pH. This observation suggests that rescue requires the positive charge that accompanies protonation in addition to the amide form of the ring nitrogen.

Several models might account for the ability of cationic nucleobases to rescue cleavage activity of ribozymes lacking G8. If the exogenous nucleobase stacks between A7 and A9 in the pocket left by the G8 deletion, binding would compensate in part for the loss of G8 by restoring active-site geometry. Exogenous cytosine cannot rescue G8dX activity by mediating general base catalysis because a protonated, cationic nucleobase will not accept a proton from the 2’ oxygen nucleophile. However, a protonated nucleobase might serve as a general acid catalyst by donating a proton to the 5’ oxygen leaving group (Figure 3a). A general acid catalyst also might protonate a phosphoryl oxygen, making the reactive phosphate more susceptible to nucleophilic attack (Figure 3b) in a variation of a mechanism previously proposed for RNase A [44]. Finally, a cationic nucleobase could provide electrostatic stabilization, analogous to the function of Lys41 of RNase A (Figure 3c). The exocyclic amine of the exogenous nucleobase is proposed to hydrogen bond to the pro-R5 oxygen of the reactive phosphate, as does the exocyclic amine of G8. Delocalized positive charge would stabilize hydrogen bonding and counteract negative charge developing in the transition state as five

![Figure 3](https://example.com/figure3.png)

Possible roles for a cationic cytosine in rescue of an abasic ribozyme lacking G8 include (a) protonation of the 5’ oxygen leaving group, (b) protonation of a phosphoryl oxygen, and (c) electrostatic stabilization through hydrogen bonding to phosphoryl oxygens.
electronegative oxygens transiently bond to phosphorus. Future experiments will be required to distinguish between these possibilities and establish how the mechanism of exogenous nucleobase rescue relates to the catalytic mechanism of the unmodified ribozyme.

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