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Rescue of an Abasic Hairpin Ribozyme by Cationic Nucleobases: Evidence for a Novel Mechanism of RNA Catalysis

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Summary

The hairpin ribozyme catalyzes a reversible phosphodiester cleavage reaction. We examined the roles of conserved nucleobases in catalysis using an abasic ribozyme rescue strategy. Loss of the active site G8 nucleobase reduced the cleavage rate constant by 350-fold while loss of A9 and A10 nucleobases reduced activity less than 10-fold. Certain heterocyclic amines restored partial activity when provided in solution to the variant lacking G8. Heterocyclic amines that were capable of rescue shared the exocyclic amine and cyclic amide in common with the Watson-Crick hydrogen bonding face of guanine. In contrast to the shallow pH dependence of unmodified ribozyme activity, rescue activity increased sharply with decreasing pH. These results support a novel model for RNA catalysis in which a cationic nucleobase contributes electrostatic stabilization to negative charge developing in the transition state.

Introduction

Understanding mechanisms of RNA catalysis remains an intriguing challenge, one that has grown in significance since the recent demonstration that the ribosome is an RNA enzyme [1]. Hairpin, hammerhead, hepatitis delta virus (HDV), and *Neurospora* Varkud satellite (VS) ribozymes are small RNA enzymes that catalyze the same reversible RNA self-cleavage reaction that generates 5′ hydroxyl and 2′,3′-cyclic phosphate termini but adopt different structures and exploit distinct kinetic and catalytic mechanisms (Figure 1). A variety of factors could promote this transesterification reaction in the context of an RNA active site; such factors might include positioning and orientation of reactive groups, general acid-base catalysis, electrostatic stabilization of the transition state, or destabilization of the ground state [2–4]. Ribonuclease A catalyzes the same chemical reaction by using histidine and lysine side chains for proton transfer and electrostatic stabilization, respectively [5], and divalent metal cation cofactors are thought to provide these functions for some RNA enzymes [6].

The unique metal cation dependence of hairpin ribozyme reactions gave an early indication that ribozymes can accomplish efficient catalysis by exclusively using RNA functional groups [7]. In contrast to hammerhead, HDV, and VS self-cleaving RNAs that require divalent metal cations such as Mg^{2+} for maximum activity [8–10], hairpin ribozymes remain fully active in buffers with $Co(NH_3)_6^{3+}$ in place of Mg^{2+} [11–13]. The $Co(NH_3)_6^{3+}$ cation is structurally analogous to hexahydrated Mg^{2+} but associates with RNA only through electrostatic and outer-sphere hydrogen bonding [14]. The ability of $Co(NH_3)_6^{3+}$ to support hairpin ribozyme activity excludes a requirement for direct coordination of metal cations to phosphate, ribose, or water oxygens and points to the direct participation of RNA functional groups in catalytic chemistry.

A serious limitation for studies of the hairpin catalytic mechanism has been the difficulty of distinguishing structural from catalytic effects of biochemical modifications. Mutagenesis and modification studies had identified 15 nucleotide bases whose identity is important for activity ([7]; Figure 2). However, it was unclear whether these nucleotides formed critical tertiary interactions or participated directly in catalytic chemistry. Two developments recently transformed the field in this regard. First, hairpin ribozyme variants have been developed with considerably improved stability of the tertiary structure [15]. Initial biochemical studies were carried out with minimal ribozymes that assemble into functional structures through weak interactions between the A and B helix-loop-helix domains [16]. Most modifications that inhibit cleavage of minimal ribozymes do so by interfering with interdomain docking [17–19], obscuring any role of specific functional groups in catalytic chemistry. In nature, the A and B helix-loop-helix domains comprise two arms of a four-way helical junction ([16]; Figure 2). The enhanced tertiary-structure stability characteristic of the 4-way junction construct relative to minimal ribozymes makes it possible to modify active site residues and retain the functional structure [20–22].

The second development that made detailed mechanistic studies feasible was solution of the crystal structure of a hairpin ribozyme complex with an uncleavable substrate analog [23]. Combined with the agreement between biochemical data and functional-group interactions evident in the structure, the extended phosphate conformation, which is consistent with the in-line S_N2 nucleophilic attack mechanism, strongly argues that the crystal structure represents the ground state of the functional ribozyme. Consistent with biochemical studies, no divalent metal cations were identified within the hairpin ribozyme active site. The crystal structure points to G8, A9, A10, and A38 as potential participants in catalysis by virtue of their proximity to the reactive phosphate.

We report the results of our efforts to establish the role of active site nucleobases in hairpin ribozyme catalytic chemistry by using exogenous nucleobase rescue. We adapted a strategy that originally was used to examine proton transfer by amino acid side chains in protein enzyme catalysis. Toney and Kirsch showed that an aspartate aminotransferase mutant that lacked an active site lysine could be rescued by exogenous alkyl amines that restored general base catalysis [24, 25]. In a related

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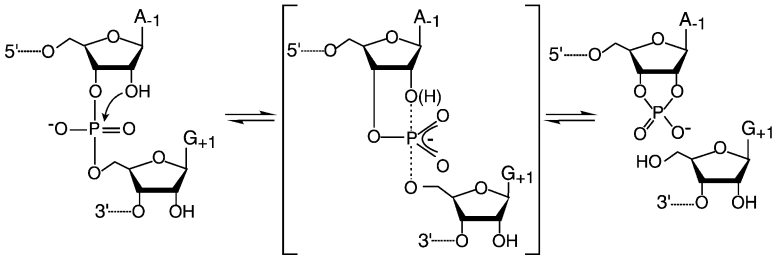


Figure 1. Chemical Mechanism of RNA Cleavage Mediated by the Family of Small Catalytic RNAs that Includes the Hairpin Ribozyme

Cleavage proceeds through an S_N2-type mechanism that involves in-line attack of the 2′ oxygen nucleophile on the adjacent phosphorus. Breaking of the 5′ oxygen-phosphorus bond generates products with 5′ hydroxyl and 2′,3′-cyclic phosphate termini.

approach, Been and coworkers demonstrated that exogenous imidazole and nucleobases could rescue HDV ribozyme mutants lacking an active site cytidine implicated in proton transfer [26, 27]. Herschlag and coworkers investigated RNA structure-function relationships by substituting abasic residues for conserved nucleotides in the hammerhead ribozyme and evaluating rescue by exogenous nucleobases [28–30]. We have combined these approaches to show that certain exogenous nucleobases can restore catalytic activity to a hairpin ribozyme variant lacking an important guanine at position 8. Structural features of the small molecules that are capable of rescue as well as biochemical features of rescued reactions support the novel model that a cationic nucleobase restores catalytic activity through electrostatic stabilization of the electronegative transition state.

Results

Assaying Effects of Nucleotide Modifications on Catalysis

We assessed cleavage activity for a series of ribozyme variants with abasic deoxynucleotides substituted for conserved residues in order to gain a better understanding of the residues involved in catalysis. We used two hairpin ribozyme configurations to facilitate incorporation of abasic residues through chemical synthesis of either the substrate (S) or the substrate binding strand (SB) of loop A (Figure 3). We chose to examine abasic substitutions in hairpin ribozyme variants stabilized by the context of a four-way helical junction to minimize any loss in activity that might result from disruption of interdomain tertiary interactions. The activity of the unmodified R4 ribozyme (Figure 3A) has been character-

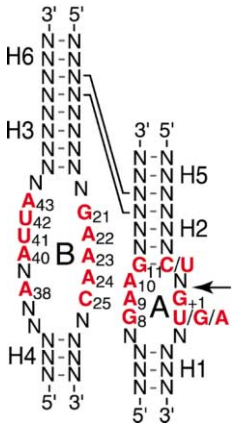
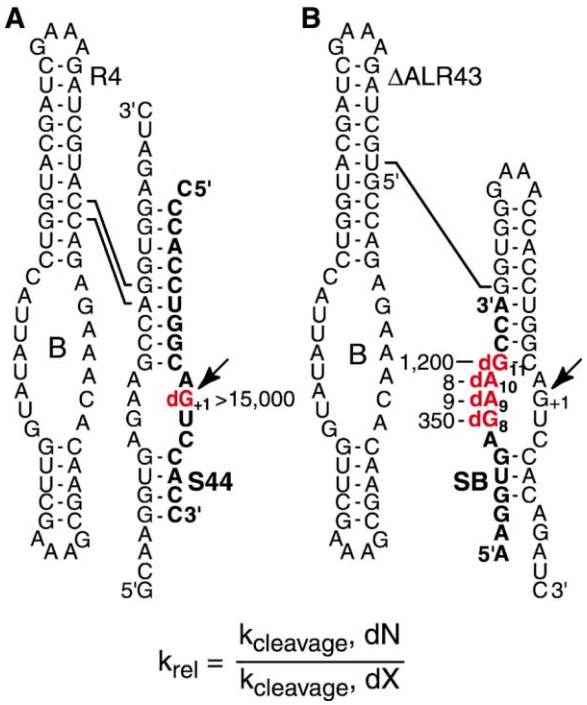


Figure 2. Hairpin Ribozyme Structure

H1 through H6 represent base-paired helices. Nucleotides represented as "N" can be altered without loss of activity if the indicated base pairing is maintained. Nucleotides in red form important tertiary interactions or comprise the active site. The A and B domains associate noncoaxially to form two arms of a four-way helical junction with helices H5 and H6. The arrow indicates the reactive phosphate in loop A.



$$k_{rel} = \frac{k_{cleavage,\ dN}}{k_{cleavage,\ dX}}$$

Figure 3. Abasic Substitutions Interfere with Ribozyme Activity

(A) S44 denotes the substrate (in bold font), which forms a complex with R4, the ribozyme (in regular font). S44dG+1 has deoxyguanosine in place of guanosine at the +1 position, and S44G+1dX has an abasic deoxynucleotide at the +1 position. S43 and S44 RNAs are the same except that S43 derivatives form H1 helices with three instead of four base pairs.

(B) SB (in bold font) denotes the substrate binding strand, which forms a complex with ΔALR43 (regular font). The ΔALR42·SB complex is identical to the ΔALR43·SB complex except that the SB RNA contains C4 in place of G4 so that the ΔALR42·SB complex contains two instead of three base pairs in H1. Abasic deoxynucleotides were substituted for G8 (SBG8dX), A9 (SBA9dX), A10 (SBA10dX), or G11 (SBG11dX). Arrows indicate the reactive phosphate. Values represent the effects of abasic substitutions on cleavage rate constants relative to rate constants measured for RNAs with deoxynucleotide substitutions at the same positions. No cleavage of S44G+1dX was detected after the longest incubations tested.

Table 1. Effects of Nucleotide Modifications on Cleavage Activity

Ribozyme[a]	$k_{cleavage}$ (min^{-1})	K_M (nM)
R4·S43dG+1	0.72 ± 0.04	68 ± 4
R4·S44dG+1	0.21 ± 0.02	22 ± 7
R4·S43G+1dX	<0.000015	–
ΔALR43·SB	0.32 ± 0.03	190 ± 10
ΔALR43·SBdG8	0.52 ± 0.03	720 ± 90
ΔALR42·SBdG8	0.66 ± 0.02	1200 ± 100
ΔALR43·SBG8dX	0.0015 ± 0.0001	–
ΔALR43·SBdA9	0.34 ± 0.02	320 ± 40
ΔALR43·SBA9dX	0.036 ± 0.003	100 ± 5
ΔALR43·SBdA10	0.36 ± 0.05	400 ± 10
ΔALR43·SBA10dX	0.044 ± 0.003	78 ± 32
ΔALR43·SBdG11	0.31 ± 0.01	220 ± 10
ΔALR43·SBG11dX	0.00026 ± 0.00001	–

The mean and range of values were obtained from two or more experiments carried out under standard reaction conditions with 50 mM NaHEPES, 10 mM $MgCl_2$, 0.1 mM EDTA (pH 7.5) at 25°C.
[a] See Figure 3.

ized in detail [22, 31]. Reactions of ΔALR43 combined with unmodified substrate binding strand RNA gave kinetic parameters similar to those of R4-mediated cleavage reactions, evidence that these RNAs assemble into a fully functional ribozyme (Figure 3B, Table 1).

Abasic substitutions could be incorporated into chemically synthesized substrate and substrate binding RNAs only in the form of deoxynucleotides. 2′ hydroxyls of A10 and G11 participate in an interdomain "ribose zipper" hydrogen bonding network [23, 32]. Deoxynucleotide substitutions at these positions strongly interfere with cleavage activity of minimal ribozymes by disrupting weak interdomain interactions [33, 34]. Initial experiments were carried out to determine whether the enhanced tertiary-structure stability conferred by the four-way helical junction compensates for the loss of these 2′ OH hydrogen bond donors. Substrate RNAs with deoxynucleotide substitutions of G+1 cleaved with kinetic parameters similar to those reported for R4 cleavage of all-RNA substrates ([22]; Table 1). Likewise, deoxynucleotide substitutions of G8, A9, A10, or G11 in substrate binding RNAs had little effect on cleavage kinetics (Table 1). Thus, loss of these 2′ hydroxyls had no significant effects on catalysis for these hairpin ribozyme variants that assemble in the context of a four-way helical junction.

Hairpin ribozymes can display reduced cleavage rates when slow dissociation of cleavage products promotes religation so that product dissociation, rather than cleavage, becomes the rate-determining step [22, 35]. It was important to carry out control experiments to confirm that abasic ribozyme reaction kinetics provide a straightforward assay of cleavage activity. Designing abasic ribozymes to generate short 3′ cleavage products that dissociate rapidly ensured that cleavage rate measurements were not complicated by religation of bound products [22]. The 3′ products of S43dG+1 and S44dG+1 cleavage form intermolecular H1 helices with 3 or 4 base pairs, respectively (Figure 3A). The stability contributed by the additional base pair should cause the 3′ product of S44dG+1 cleavage to dissociate ~100-fold more slowly than the corresponding product of S43dG+1 cleavage [31]. The small decrease in the S44dG+1 cleavage rate constant relative to the rate constant for S43dG+1 cleavage could indicate that the 3′ product of S44dG+1 cleavage dissociates at a rate on the same order as the cleavage rate. In every case, however, cleavage rates measured in reactions with abasic ribozymes (below) were considerably lower than those measured for S44dG+1 cleavage, evidence that product dissociation could not be rate determining for abasic ribozyme cleavage. Similarly, substrate binding strand RNAs that assemble into ribozyme complexes with two or three bases pairs in H1 cleave with similar rate constants (Table 1), evidence that the cleavage step and not product dissociation is rate determining for ΔALR43·SB complexes as well. Loss of loop A nucleobases that interact with 3′ cleavage products would most likely weaken product binding and accelerate product dissociation relative to the unmodified ribozyme. Therefore, these control experiments confirm that cleavage rate constants measured for abasic variants monitor the cleavage step in the reaction pathway and are not complicated by slow product dissociation.

Abasic Substitutions Interfere with Activity

All abasic deoxynucleotide substitutions at conserved positions interfered with cleavage activity, as expected. No cleavage was detected even after long incubations of S44G+1dX, the variant with an abasic residue in place of G+1 (Figure 3A, Table 1). An abasic substitution of G11 reduced cleavage activity approximately 10^3-fold (Figure 3B). No functional groups of G+1 or G11 seem to interact directly with the reactive phosphate in the ribozyme active site [23]. Instead, G+1 interacts with conserved nucleotides in loop B that serve to align the A and B domains and position the reactive phosphate in the orientation appropriate for an S_N2 in-line attack. G11 forms the first base pair of H2 and participates in the interdomain ribose zipper [23, 32]. The loss of activity due to abasic substitutions of these nucleotides that seem to perform exclusively structural roles points to a major contribution of positioning and orientation to catalysis.

Abasic substitutions of G8, A9, and A10 have smaller effects on catalysis. The G8dX variant displays 350-fold-reduced activity, and cleavage rate constants of the A9dX and A10dX variants are reduced 9- and 8-fold, respectively (Figure 3B, Table 1). The N1 ring nitrogen of G8 lies within 2.9 Å of the 2′ hydroxyl that gives rise to the 2′ oxyanion nucleophile, leading to the suggestion that G8 acts as a general base catalyst to deprotonate the 2′ hydroxyl [23, 36]. In the crystal structure, A9 and A10 in loop A and A38 in loop B 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 has been proposed to activate bound water to donate a proton to the 5′ oxygen leaving group [23, 37]. Alanine substitutions of the histidines that mediate general acid-base catalysis in the ribonuclease A reaction reduce k_{cat}/K_M by 10^4-fold [38], and deletion or mutation of the cytosine residue that has been implicated in proton transfer reduces HDV ribozyme self-cleavage rates by 10^6-fold [26, 27]. In view of

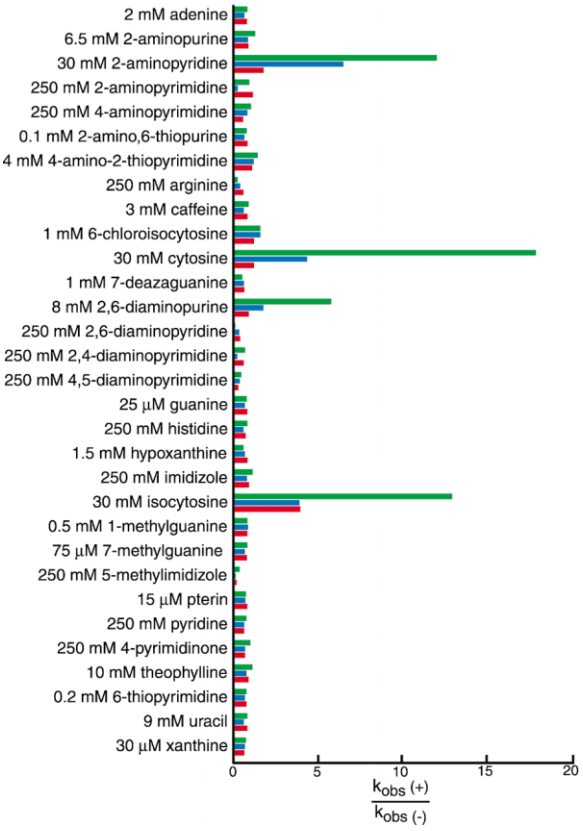


Figure 4. Partial Rescue of G8dX Activity by Exogenous Heterocyclic Amines

Cleavage rates were determined from reactions with the indicated concentrations of small molecules. Bars represent the ratio of cleavage rate constants for reactions with or without the indicated small molecules for reactions at pH 6.8 (green), 7.5 (blue), or 8.6 (red).

the large effects of these mutations, the small effects of abasic substitutions at G8, A9, and A10 might seem to exclude any important role for these nucleobases in catalytic chemistry. However, model experiments assessing general acid-base catalysis by imidazole and morpholine buffers show rate accelerations on the same order as the inhibitory effects of G8, A9, and A10 deletions [39]. Therefore, the magnitude of inhibition is not inconsistent with a role for these nucleobases in proton transfer.

Rescue of an Abasic Substitution at G8 by Exogenous Heterocyclic Amines

If G8, A9, and A10 mediate catalytic chemistry, the fact that rate constants fall only 8- to 350-fold in these abasic variants suggests that base deletions might leave a solvent-filled cavity near the reactive phosphate in a relatively unperturbed active site. This gave us the opportunity to try to identify small molecules that bind specifically in the active site and restore activity. We surveyed 31 nucleobases, nucleobase analogs, heterocyclic amines, and amino acids for their ability to restore activity to the G8dX variant (Figure 4). It is important to note that these molecules vary widely in solubility, with purines being especially insoluble. Therefore, some molecules probably failed to restore activity simply because we could not achieve concentrations that were high enough to support binding. Although guanine would be expected to rescue G8dX, for example, guanine could not be tested at concentrations higher than 25 μM, and no detectable rescue occurred at this concentration. Four heterocyclic amines, cytosine, isocytosine, 2-aminopyridine, and 2,6-diaminopurine, restored partial activity to the G8dX variant (Figures 4 and 5A). At the same concentrations, these heterocyclic amines had no detectable effect on reactions of unmodified ribozymes or ribozymes with other abasic deoxynucleotide substitutions, evidence that rescue of the G8dX variant occurred through specific binding.

Rescuing molecules share functional groups that correspond to the Watson-Crick hydrogen bonding face of guanine (Figure 5A). 4-pyrimidinone retains the keto oxygen corresponding to O6 of guanine but not the exocyclic amine, and it failed to rescue G8dX. Rescue was observed with 2-aminopyridine, which retains the exocyclic amine but not the keto oxygen. Therefore, it seems that the exocyclic amine is important for rescue but the keto oxygen is not. The crystal structure of the active site is consistent with this interpretation. The exocyclic amine of G8 lies within hydrogen bonding distance of the *pro-*R_P nonbridging oxygen of the reactive phosphate, whereas no specific interactions with the keto group are evident [23]. The requirement for the exocyclic amine suggests that the rescuing nucleobases might bind in the same orientation as guanine in the unmodified ribozyme.

Enhanced Rescue by Exogenous Nucleobases at Low pH

The N1 ring nitrogen of guanine and the corresponding N3 ring nitrogen of isocytosine are protonated in the neutral form, whereas the N3 ring nitrogen of cytosine and the N1 ring nitrogen of 2,6-diaminopurine are not protonated in the neutral form, at least for free nucleobases in solution ([40, 41]; Figure 5A). Thus, this survey left the optimum protonation state of this ring nitrogen in question. For each nucleobase, rescue increased at pH 6.8 relative to pH 7.5 or 8.6, evidence that protonation promotes activity (Figure 4). Detailed analysis of the pH dependence of rescue confirmed that rescue increases with decreasing pH for each nucleobase (Figure 5B). Furthermore, fitting pH-rate profiles of cytosine, isocytosine and 2,6-diaminopurine-rescued reactions to the Henderson-Hasselbach equation [42] gave apparent pK_a values that were significantly higher than pK_a values for ionization of these nucleobases in solution (Figures 5A and 5B). This evidence suggests that rescue requires protonation and, conversely, that nucleobase interactions with the ribozyme facilitate 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 ([40]; Figure 5A). Nonetheless, isocytosine rescue also increases with decreasing pH (Figure 5B). This observation suggests that rescue requires the positive charge that accompanies protonation in addition to the amide form of the N3 ring nitrogen. Indeed, the pH-rate profile for isocytosine rescue

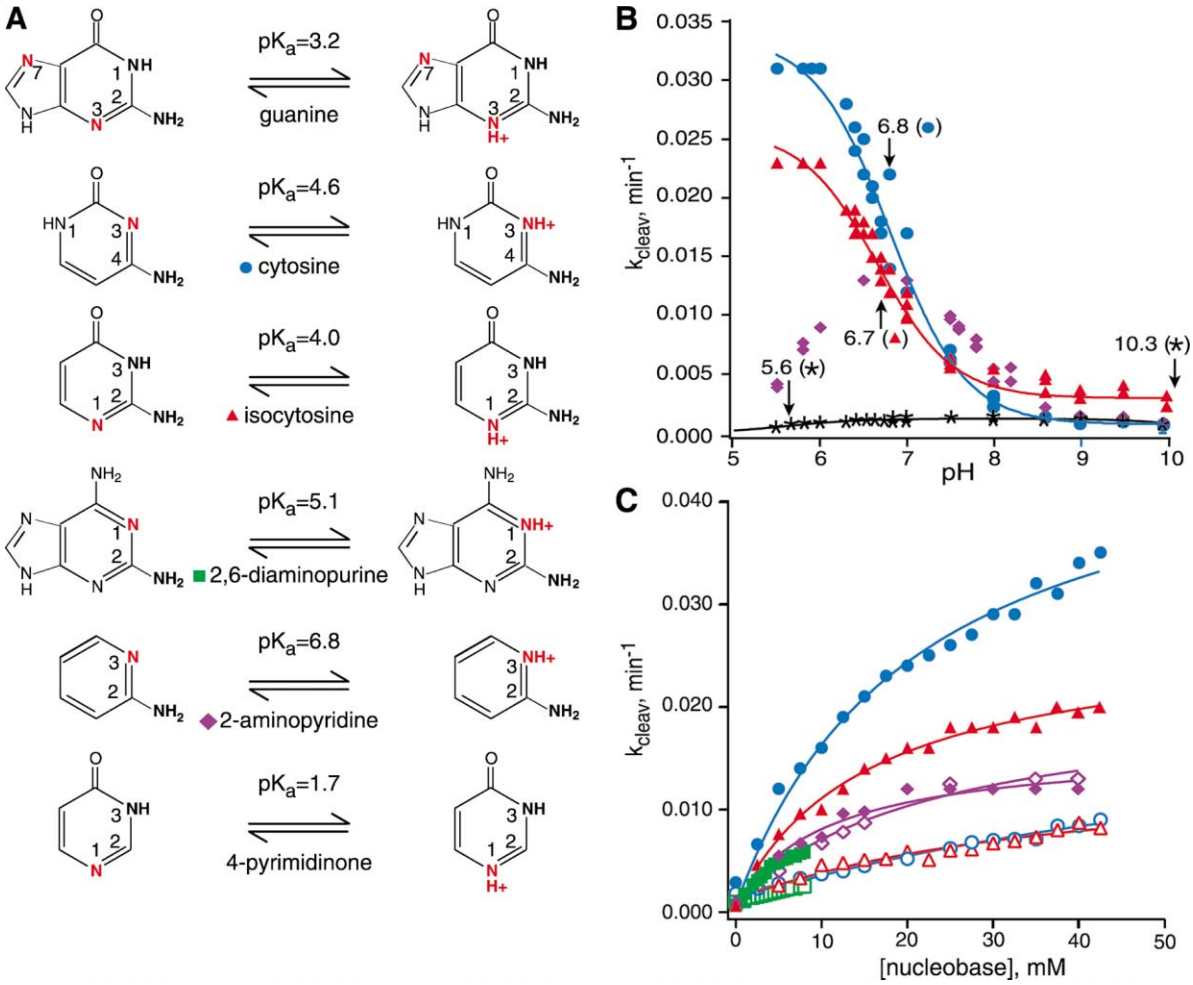


Figure 5. pH Dependence of G8dX Rescue by Exogenous Heterocyclic Amines

(A) Ionization equilibria of nucleobases or nucleobase analogs in solution [40, 41]. Protonation takes place at N7 of guanine at low pH in solution [52], but electron donation by a hydrogen bonded exocyclic amine might stabilize protonation at N3 within the ribozyme active site.

(B) pH-rate profiles of reactions either with cytosine (blue circle), isocytosine (red triangle), and 2-aminopyridine (purple diamond), each at a concentration of 30 mM, or with no added nucleobase (asterisk). Lines represent fits to $k_{obs} = k_{cleave,\,max}/(1 + 10^{(pH - pK_{a,\,app})}) + k_{cleav,min}$ for cytosine and isocytosine or to $k_{obs} = k_{cleave,\,max}/(1 + 10^{(pH - pK_{a,\,app1})} + 10^{(pK_{a,\,app2} - pH)})$ for unrescued reactions. The pH-rate profile for 2-aminopyridine rescue did not fit either equation.

(C) Nucleobase concentration dependence of rescue at pH 6.0 (filled symbols) and pH 7.5 (open symbols). Lines represent fits to $k_{obs} = (k_{unbound} + k_{rescue}[\text{nucleobase}])/(1 + [\text{nucleobase}]/K_d^{base})$ for cytosine (blue circles), isocytosine (red triangles), 2-aminopyridine (purple diamonds), and 2,6-diaminopurine (green squares). For fit computations, $k_{unbound}$ values were fixed at the values of 0.0008 min^{-1} and 0.0015 min^{-1}, which were determined for G8dX reactions in the absence of added nucleobase at pH 6 and 7.5, respectively. Computed fits gave the second-order rate constants $k_{rescue} = k_{bound}/K_d^{base}$, shown in Table 2. Cleavage rate constants declined in reactions with 2-aminopyridine at concentrations above 40 mM (not shown). Therefore, the apparent plateau in cleavage activity between 20 and 40 mM 2-aminopyridine might not reflect saturation of a 2-aminopyridine binding site.

displays a small but reproducible level of rescue at high pH values when the N3 ring nitrogen is in the amide form but isocytosine is not positively charged (Figure 5B). This result supports the idea that the amide form of the N3 ring nitrogen confers some rescue activity consistent with a role for N3 in hydrogen bond donation. However, the observation that low pH promotes isocytosine rescue suggests that positive charge significantly enhances rescue even when the charge localizes to a different ring nitrogen.

In contrast to the pH dependence of cytosine, isocytosine, and 2,6-diaminopurine rescue, pH-rate profiles for 2-aminopyridine rescue reactions were not consistent with the participation of just a single ionization in the rate-determining step. Instead, 2-aminopyridine displayed maximum rescue activity near pH 7 and reduced activity at higher and lower pH values. In contrast to cytosine, isocytosine, and 2,6-diaminopurine, which undergo significant protonation only at pH values below 6 in solution, 2-aminopyridine ionizes with a pK_a value of 6.8 so that a significant fraction will be positively charged at neutral pH (Figure 5A). Cations differ in their ability to stabilize hairpin ribozyme structure [43], and cationic 2-aminopyridine might compete with favorable Mg^{2+} interactions to destabilize ribozyme structure. If so, the abasic variant must be especially susceptible to

Table 2. pH Dependence of Exogenous Nucleobase Rescue

Nucleobase	pH	k_{rescue}[a] ($M^{-1}\cdot min^{-1}$)
Cytosine	6	2.3 ± 0.15
	7.5	0.18 ± 0.01
Isocytosine	6	2.0 ± 0.01
	7.5	0.30 ± 0.04
2-aminopyridine	6	1.5 ± 0.17
	7.5	0.82 ± 0.11
2,6-diaminopurine	6	1.3 ± 0.08
	7.5	0.11 ± 0.03

[a] The second-order rate constant and the standard error for exogenous nucleobase rescue were calculated from the data shown in Figure 5C by least squares fits to $k_{obs} = (k_{unbound} + k_{rescue}[nucleobase])/(1 + [nucleobase]/K_d^{base})$, where $k_{rescue} = k_{bound}/K_d^{base}$.

destabilization because 2-aminopyridine does not interfere with the activity of the unmodified ribozyme at the same concentration at any pH (not shown).

We tried to assess whether protonation promotes binding, catalysis, or both by examining the nucleobase concentration dependence of rescue at high and low pH (Figure 5C, Table 2). If protonation promotes binding, lower concentrations of nucleobase should saturate rescue at low pH, but pH would not affect the rate constant for cleavage within the RNA-nucleobase complex. Conversely, if protonation promotes catalysis and not binding, the catalytic rate constant for rescued cleavage would increase at low pH, but pH would not affect the apparent equilibrium constant for nucleobase binding. Rescued reactions did not display saturation at the highest nucleobase concentrations that could be tested, so these data did not allow independent determinations of equilibrium dissociation constants for nucleobase binding or rate constants for cleavage within the nucleobase bound ternary complex. Nonetheless, values for k_{rescue}, the apparent second-order rate constants for nucleobase rescue, were almost 10-fold higher at pH 6 than at pH 7.5 for cytosine-, isocytosine-, and 2,6-diaminopurine-rescued reactions (Figure 5C, Table 2). If the equilibrium dissociation constant for cytosine binding is assumed to be greater than 20 mM at pH 6, extrapolation to a saturating cytosine concentration indicates that the cleavage rate constant for the protonated cytosine-ribozyme complex is on the order of 0.05 min^{-1}. This value is only 4-fold less than the rate constant of 0.2 min^{-1} measured for the unmodified ΔALR43·SB cleavage reaction at pH 6.

Rescue Specificity

Aminoglycoside antibiotics and polyamines can promote hairpin ribozyme activity at low Mg^{2+} concentrations through nonspecific electrostatic stabilization of ribozyme structure [44]. The requirement for relatively high concentrations of nucleobases raised the concern that rescue occurred through a similar nonspecific electrostatic effect. However, cationic nucleobases did not accelerate cleavage in reactions with the unmodified ribozyme or with G+1dX, A9dX, A10dX, or G11dX variants. Furthermore, a variety of heterocyclic amines failed to accelerate reactions with G8dX at high or low pH (Figure 4). Therefore, rescue of G8dX activity by cytosine, isocytosine, 2,6-diaminopurine, and 2-aminopyridine seems to occur through specific nucleobase binding.

Chemical Mechanism of Rescued Reactions

Control experiments confirmed that loss of G8 caused no fundamental change in reaction chemistry. The G8dX variant cleaves equally well in buffers with 0.25 mM $Co(NH_3)_6^{3+}$ or with 10 mM $MgCl_2$, as do unmodified hairpin ribozymes [11]. Therefore, the G8dX variant has not acquired a requirement for direct coordination of metal cations. Furthermore, 5′ product RNA purified from reactions with the G8dX substrate binding strand undergoes ligation to the same extent and with the same kinetics as 5′ product RNA from reactions with the unmodified substrate binding RNA. Thus, the G8dX variant produces the expected cleavage products with 2′,3′-cyclic phosphate termini and mediates the same cleavage chemistry as unmodified ribozymes.

Discussion

Recent evidence from several systems suggests that RNA nucleobases have a previously unrecognized capacity for mediating catalytic chemistry. The crystal structure identified the ring N3 of a conserved cytosine in the genomic HDV ribozyme as being in a position consistent with a role in proton donation to the 5′ oxyanion leaving group [45]. Elegant enzymological studies reported by the Been and Bevilacqua groups support a role for this cytosine in general acid catalysis [10, 26, 27]. The crystal structure of the large ribosomal subunit revealed no proteins within 18 Å of the active site, firmly placing the ribosome on the list of naturally occurring ribozymes [1]. No metal cations or even phosphates that might serve as metal ligands were found in the peptidyl transferase active site. Instead, N3 of a conserved adenine in 23S rRNA is oriented toward the reactive carbonyl carbon. This adenine was proposed to mediate proton transfer in peptide bond formation [1]. Chemical modification studies demonstrated that the ionization equilibrium of this adenine is shifted toward neutral pH, consistent with the proposed role as a general acid-base catalyst [46], although this notion remains controversial [47].

Cytosine and isocytosine also have been shown to restore partial activity to an HDV ribozyme mutant that lacks the C76 nucleobase that was implicated in proton transfer [27]. However, the biochemical features of rescued reactions differ dramatically for HDV and hairpin ribozymes. In particular, cytosine rescue of HDV ribozymes increases with increasing pH, suggesting that deprotonation rates or equilibria participate in the rate-determining step. Furthermore, imidazole and imidazole derivatives rescue HDV ribozyme mutants lacking C76 [26, 27], but imidazole at concentrations as high as 250 mM gave no detectable rescue of the hairpin G8dX variant (Figure 4). The failure of imidazole to compensate for the loss of G8 does not exclude a role for G8 in proton transfer, but it does suggest that hairpin and HDV ribozymes exploit different catalytic mechanisms.

In the active site structure, the N1 ring nitrogen of G8

lies close enough to accept a proton from the nucleophilic 2′ hydroxyl [23]. Provided that a significant fraction of this guanine exists in the deprotonated state within the active site environment, the proximity of G8 to the 2′ hydroxyl is consistent with a role for G8 in general base catalysis. However, the observation that protonation enhances exogenous nucleobase rescue indicates that rescue does not occur through this mechanism. Exogenous nucleobases cannot rescue G8dX cleavage activity by mediating general base catalysis because a protonated, cationic nucleobase will not accept a proton from the 2′ hydroxyl.

Positioning of a positive charge near the nucleophilic 2′ OH might serve a role similar to that proposed for divalent metal cations in the *Tetrahymena* ribozyme reaction [48, 49]. In *Tetrahymena* ribozyme reactions analogous to 5′ splice site cleavage and exon ligation, metal specificity switches accompany sulfur substitutions of 3′ oxygens of the reactive phosphate or the exogenous guanosine cofactor, respectively. That is, sulfur substitutions led to a loss of activity in reactions with Mg^{2+}, but activity was restored when Mg^{2+} was replaced by more thiophilic Cd^{2+} or Mn^{2+} cations. Although interpretation of the effects of sulfur substitutions on catalytic chemistry can be controversial [6], these results implicate direct interactions between leaving-group oxygens and metal cations in the transition state. Based on the principle of microscopic reversibility, a role for metal cations in leaving-group stabilization also implicates metal cations in activation of the nucleophile. By analogy to the role of metal cations in *Tetrahymena* ribozyme reactions, therefore, a cationic nucleobase might serve to activate the nucleophilic 2′ hydroxyl during hairpin ribozyme cleavage and stabilize the 2′ oxyanion leaving group during ligation.

Alternatively, cationic nucleobases could provide electrostatic stabilization of the electronegative transition state, analogous to the function of Lys41 of ribonuclease A or to the role proposed for divalent metal cations in hammerhead and *Tetrahymena* ribozyme reactions [50]. The G8dX ribozyme variant displayed a loss of activity similar in magnitude to the loss that occurs when Lys41 is replaced by alanine in ribonuclease A [5]. In ribonuclease A catalysis, the cationic ϵ amino group of Lys41 donates a hydrogen bond to the *pro*-R_P nonbridging oxygen of the reactive phosphate [51]. In *Tetrahymena* and hammerhead ribozymes, metal cation specificity switches that accompany substitution of phosphoryl oxygens point to metal cation coordination to *pro*-S_P or *pro*-R_P oxygens, respectively [6].

We propose a model in which the exogenous nucleobase stacks between A7 and A9 in a pocket left by the G8 deletion (Figure 6). Stacking would compensate in part for the loss of G8 by restoring active site geometry. The exocyclic amine of the exogenous nucleobase is proposed to hydrogen bond to the *pro*-R_P oxygen of the reactive phosphate, as does the exocyclic amine of G8 in the unmodified ribozyme [23]. Cytosine and isocytosine undergo protonation at N3 or N1 ring nitrogens, but positive charge would tend to delocalize throughout the conjugated system [52] and contribute electrostatic stabilization to the pentacovalent transition state. Interaction with a cationic nucleobase also could activate the 2′ oxygen nucleophile.

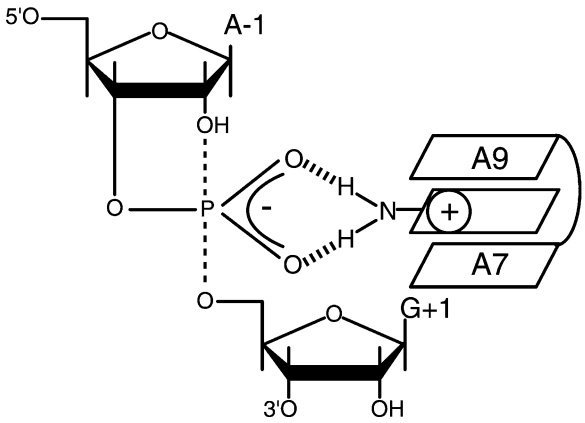


Figure 6. Model of Electrostatic Stabilization by a Cationic Nucleobase

The exogenous nucleobase stacks between A7 and A9 in the pocket left by the G8 deletion and restores active site architecture. The exocyclic amine of the nucleobase is hydrogen bonded to phosphoryl oxygens, an interaction that serves to stabilize negative charge developing in the pentacovalent transition state and to position reactive groups in the orientation appropriate for an S_N2 in-line nucleophilic attack. Interaction between a cationic nucleobase and the 2′ oxygen also might activate the 2′ oxyanion nucleophile.

It is not yet clear whether electrostatic stabilization by cationic nucleobases contributes to catalysis by any unmodified ribozymes. The ionization equilibrium of guanosine would have to shift considerably within the hairpin ribozyme active site in order for any appreciable amount of G8 to be positively charged at neutral pH. Under normal reaction conditions, no naturally occurring ribozymes display increasing activity with decreasing pH, as observed for nucleobase-rescued G8dX reactions. Hammerhead and *Tetrahymena* ribozyme reactions display log-linear pH-rate profiles throughout the neutral pH range, evidence that the rate-determining step requires deprotonation of a basic functional group [53–55]. Hairpin ribozyme cleavage and ligation rate constants increase less than 6-fold across the same pH range, with maximum rates observed near pH 7.5 [11]. Similar shallow pH dependence was observed for VS ribozyme cleavage [56] and for unrescued reactions with the G8dX variant (Figure 5B). If electrostatic stabilization by a cationic nucleobase contributes to catalysis by these ribozymes, therefore, protonation does not participate in the rate-determining step.

In reactions with 1 M NaCl in place of divalent metal cations, the residual, low activity of the genomic HDV ribozyme increases with decreasing pH, consistent with a requirement for protonation of a functional group with a low pK_a [10]. In this case, protonation seems to serve a structural rather than a catalytic role by stabilizing a base triple that requires hydrogen bond donation from the N3 ring nitrogen of cytosine [57]. Through in vitro selection under acidic conditions, one self-cleaving RNA that displays maximum activity at pH 4 and a log-linear pH-rate profile between pH 4 and 6 has been identified [58]. This ribozyme mediates the same reaction chemistry as the hairpin ribozyme and generates products with 2′,3′-cyclic phosphate and 5′ hydroxyl termini, and as

with the hairpin ribozyme, no divalent metal cations are required for optimal activity. Although the sharp increase in activity with decreasing pH is consistent with a requirement for a cationic nucleobase in the mechanism of this unmodified RNA enzyme, further work is needed to establish whether protonation serves a structural or catalytic role.

Significance

We have obtained the first direct evidence for the participation of a cationic nucleobase in electrostatic stabilization of the transition state of the RNA-catalyzed phosphoryl transfer reaction. These results both extend previous work demonstrating that nucleobases can participate in proton transfer steps of RNA-catalyzed cleavage reactions and expand our understanding of the capacity of RNA functional groups to mediate catalytic chemistry. These results might have implications for the mechanism of other RNA-mediated reactions, such as protein synthesis. Like hairpin ribozyme self-cleavage, peptide bond formation also occurs in a 50S ribosomal subunit active site that is composed exclusively of RNA with no evidence of the participation of metal cations.

Experimental Procedures

Preparation of RNAs

RNAs were prepared as described [22]. In brief, R4 and ΔALR43 were prepared through T7 RNA polymerase transcription of linearized plasmids. ΔALR43 was labeled by the incorporation of [α-^{32}P] ATP during transcription. The plasmid template for transcription of ΔALR43 was constructed from pTLR43 [22] by the deletion of sequences that encode the substrate binding strand and the replacement of A15 with guanine as the first nucleotide of the ΔALR43 transcript via QuickChange mutagenesis (Stratagene). RNAs with abasic ribonucleotide substitutions are not yet available commercially, so abasic substitutions were introduced as deoxynucleotides. TriLink BioTechnologies supplied chemically synthesized substrate binding RNAs with abasic deoxynucleotide substitutions. Dharmacon Research supplied unmodified and deoxynucleotide-substituted substrate and substrate binding RNAs as well as the substrate RNA with an abasic deoxynucleotide substitution of G+1. [5′-^{32}P] substrate RNAs were labeled by reaction with T4 polynucleotide kinase and [γ-^{32}P] ATP. After preparative denaturing gel electrophoresis, RNAs were purified as sodium salts by DEAE-650M chromatography (Toyopearl).

Kinetic Analyses

Cleavage reactions were carried out at 25°C under standard conditions with 10 mM $MgCl_2$, 0.1 mM EDTA, and 50 mM NaHEPES (pH 7.5). When an alternate pH is indicated, reactions used 50 mM NaMES (pH 5.5–7.0), NaHEPES (pH 6.8–8), NaTAPS (pH 7.5–9.0), NaCHES (pH 9.0–10.0), or NaCAPS (pH 9.0–10.5) instead of NaHEPES. Kinetics parameters were determined for single turnover reactions under standard conditions, as described [35].

To measure the effects of exogenous small molecules in the neutral pH range (pH 6.5–9), we determined cleavage rate constants from single turnover reactions with 0.1 nM ^{32}P ΔALR43 and SB RNA at two saturating concentrations that corresponded to 10 and 20 times the K_M value and at various concentrations of nucleobase in large excess relative to the RNA. When necessary, reaction buffers were adjusted to the appropriate pH after the addition of exogenous small molecules. Cleavage rates were computed by nonlinear least squares fit to $F = F_\infty (1 - e^{-k_{cleavage,\ obs}t})$ where F is the fraction cleaved at time t and F_∞ is the fraction cleaved at the end of the reaction. Agreement between cleavage rates measured at both SB RNA concentrations confirmed that these RNA concentrations were saturating, so observed cleavage rates reflect the rate constant for cleavage within the ΔALR43·SB complex. Reported values represent the mean of two or more measurements. Saturating RNA concentrations were difficult to achieve at pH extremes, so cleavage rate constants were determined from the SB RNA concentration dependence of observed cleavage rates via Eadie-Hofstee plots, as described [35], for reactions at pH values below 6.5 and above 9.

The apparent second-order rate constant for nucleobase rescue, k_{rescue}, was calculated from nonlinear least squares fit to $k_{obs} = (k_{unbound} + k_{rescue}[\text{nucleobase}])/(1 + [\text{nucleobase}]/K_d^{base})$, where k_{bound} is the rate constant for cleavage within the ternary complex that contains both RNAs and the nucleobase and K_d^{base} is the equilibrium dissociation constant of the nucleobase from the ternary complex [28]. Inability to achieve saturating nucleobase concentrations prevented accurate determinations of K_d^{base} and k_{bound} values. Therefore, our analysis is limited to a comparison of k_{rescue} values that monitor both nucleobase binding and cleavage steps in the rescue reaction pathway. Kinetic parameters obtained from multiple experiments typically varied less than 20%.

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