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Two Decades of RNA Catalysis

Review

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Twenty years have passed since the initial discovery of catalytic RNA. Although initial discoveries of ribozymes (RNA enzymes) involved phosphoryl transfer reactions on RNA substrates, our knowledge of the biological repertoire of these enzymes was expanded recently as a result of new evidence suggesting that the RNA component of the ribosome catalyzes peptide bond formation. Ribozymes have posed novel challenges for mechanistic studies, but recent investigations have yielded increasing support for chemical mechanisms involving precisely positioned nucleic acid bases with environmentally perturbed pKa values and metal ions. A continuing challenge for RNA enzymologists is the separation of the structural and chemical effects in interpreting experimental results, a challenge that will be overcome as the intriguing fields of RNA enzymology and structural biology continue to expand.

Introduction

The first discoveries that RNA molecules could perform chemical reactions in the absence of proteins were two decades ago. Following 20 years of active research, mechanisms by which RNA promotes chemistry are coming into focus. The purpose of this review is to summarize the most current views of ribozyme mechanisms.

In 1982-1983, Cech and Altman reported that RNA could catalyze chemical reactions, a discovery that opened a new field of biological research [1, 2]. In two independent RNA molecules, it was found that the RNA performed phosphodiester bond cleavage reactions that were site specific, had rates that were enhanced many orders of magnitude over background rates, and were biologically required. This opened the door to new branches of research: biologically, where is RNA-catalyzed chemistry found? What is the range of reactions accessible to catalysis by RNA? And chemically, how does RNA perform catalysis?

All three branches of ribozyme research are still being enthusiastically explored. Biologically, protein-independent RNA-catalyzed chemistry is found in RNA processing reactions performed by certain organisms and plant viruses. RNA-catalyzed chemistry has also been proposed in two major ribonucleoprotein machines, the ribosome [3, 4], and most recently, the spliceosome [5]. The question of what kinds of chemistry *could* be performed by RNA has been addressed by in vitro selection of active RNAs from huge pools of random sequences, and the RNA chemical repertoire has been expanded from the biologically most prevalent phosphoryl transfer reactions to include aminoacylation and alkylation, among other activities [6, 34]. Finally, the question of *how* RNA performs catalysis has been addressed through classical and neoclassical enzymological methods. While a great deal of investment has been made in formulating RNA catalysis in terms of classical enzymology, the sensitive nature of RNA structure brings new complexities to these experiments. Consideration of RNA catalysis in general [7, 8] and more specific discussions of ribozyme structure and function [9, 10] are among topics of prior reviews. Here, we focus on the current state of proposed mechanisms for four ribozymes that have been the subject of particularly extensive investigations.

RNA-Catalyzed Reactions

Most naturally occurring ribozymes catalyze phosphoryl transfer reactions, specifically by attack of a sugar 2′ or 3′-OH on a phosphodiester linkage. The best-studied ribozymes are separated into two main classes depending on whether the sugar-OH nucleophile attacks its own 3′ phosphodiester (Figure 1A) or if the nucleophile is exogeneous or from a different RNA strand (Figure 1B). Evidence also is mounting that the peptide bond-forming aminoacyl transfer reaction in the ribosome is actually catalyzed by RNA [3, 4], and that reaction is shown in Figure 1C.

There are certain features common to all of the RNA-catalyzed reactions in Figure 1, namely the RNA is required to: (1) activate the nucleophile, (2) stabilize a negatively charged transition state, and (3) stabilize the leaving group. In proteinacious enzymes such as RNase A, these requirements are met by amino acid side chain imidazoles for general acid-base catalysis, and positively charged amines, such as found in lysine. In another host of phosphoryl transfer reactions including polymerases, protein bound metal ions are employed. The question of how RNA, a negatively charged biopolymer with distinct chemical and physical properties and lacking in protonatable groups with pKa values of between 5 and 9, manages these requirements has provoked considerable speculation and experiment.

The most direct parallel with protein-type mechanisms would have RNA catalysis governed by bound metal ions, nucleic acid bases with environmentally perturbed pKas, or a combination of both. Indeed, RNA catalysis requires cations, is usually pH sensitive, and can be highly sensitive to single-atom changes on individual purine or pyrimidine bases. RNA structure, however, is complex and also can be sensitive to pH and single-atom substitutions. Moreover, unlike the case for many protein enzymes, in ribozymes the substrate is generally an integral component of the ground state structure of the molecule. Discriminating structure from chemistry has been by far the most difficult challenge in RNA mechanistic inquiries based on kinetic assays.

In proteins, the mixture of mechanistic enzymology, structure determination, and spectroscopy, in combina-

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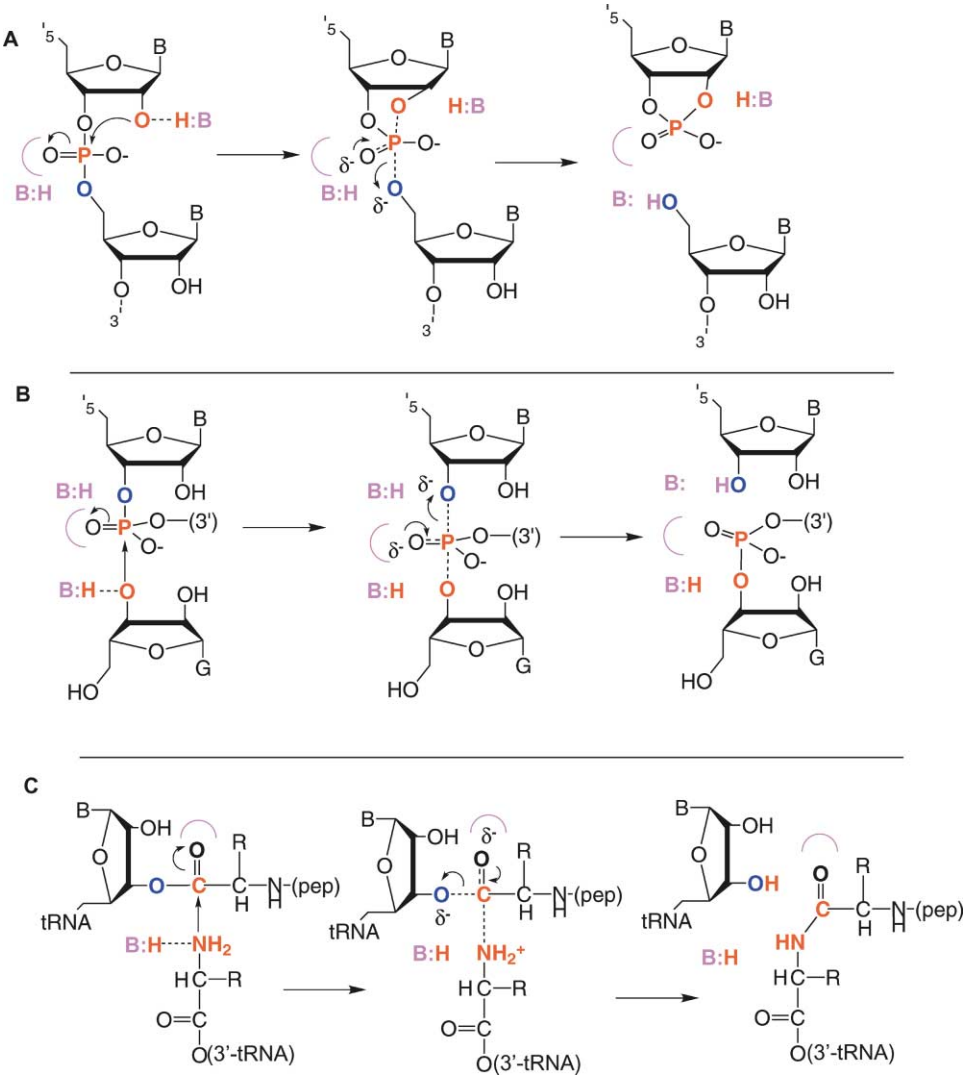

Figure 1. Reactions Catalyzed by Ribozymes

Reactions catalyzed by naturally occurring ribozymes require proton transfers that may be effected by general acid/base chemistry (denoted as A: or B:) or other factors established by the RNA environment (see Figure 4). Neutralization of negatively charged intermediates (pink arcs) may also be important.

(A) Intramolecular phosphoryl transfer reaction catalyzed by one class of ribozymes including the hammerhead, hepatitis delta virus (HDV), hairpin, neurospora VS, and RnaseP RNAs.

(B) The Group I and Group II introns catalyze attack of an extrinsic nucleophile, here shown as a guanosine, at a specific phosphodiester bond.

(C) The ribosome has been proposed to contain an all-RNA active site for peptidyl transfer.

tion with site-directed mutagenesis, has provided substantial information about chemical mechanisms. Each of these tools has been developed for application to the study of ribozymes. This review will focus mainly on the four ribozymes shown in Figure 2, each of which has been characterized by X-ray crystallography and many mechanistic studies. Of these, the hammerhead, hepatitis delta virus (HDV), and hairpin ribozymes catalyze the reactions of Figure 1A and are of the class termed "small" ribozymes. The Group I intron catalyzes the reaction in Figure 1B and is one of the "large" ribozymes. The leadzyme, obtained by in vitro evolution, has also been investigated both mechanistically and structurally [11] but is not included due to space limitations, nor are the important naturally occurring Neurospora VS, RnaseP, or Group II ribozymes [9, 10]. In brief, the most recent reports at this time suggest nucleic acid base-catalyzed reaction mechanisms for the HDV and hairpin ribozymes that involve catalysis by nucleic acid base moieties, whereas direct involvement of metal ions has been proposed for the Group I intron, and mechanisms involving both metals and nucleic acid bases have been proposed for the hammerhead ribozyme. The dominant current evidence for these proposals is summarized below.

The Hammerhead Ribozyme: A Continuing Challenge

The hammerhead ribozyme is a motif found in the genomes of certain plant viruses of ~300+ nucleotides,

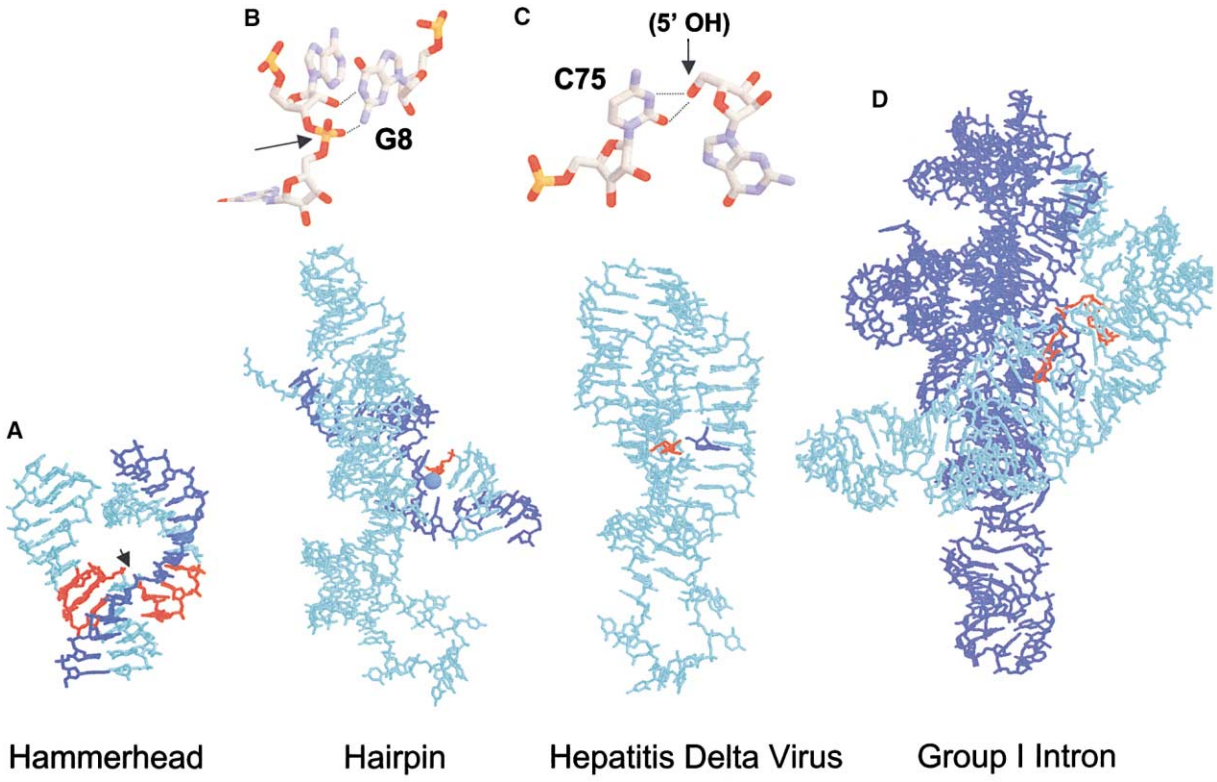

Figure 2. Ribozyme Structures from X-Ray Crystallography

(A) One of several structures of the hammerhead (Protein Data Bank code 1HMH) ribozyme. The "substrate" strand is shown in dark blue, and the site of phosphodiester bond cleavage is indicated by an arrow. The conserved core is shown in red.

(B) The hairpin ribozyme (1M5K) as a four-way junction with the substrate strand shown in dark blue. The active site 2′-OH has been replaced with a 2′-OMe (blue sphere). A conserved base, G8, is shown in red and is within hydrogen bonding distance of the active site 2′-OMe and phosphodiester groups (top).

(C) The Hepatitis Delta Virus (HDV, 1CXO) with 5′-OH product in dark blue. The mechanistically important C75 is shown in red and is within hydrogen bonding distance of the 5′-OH (top).

(D) The Group I intron (1GRZ) with guanosine binding site residues in red. The previously crystallized P4-P6 domain is shown in dark blue.

which performs a single phosphodiester cleavage reaction during rolling circle replication. The motif has been truncated to a minimal active sequence (Figure 3) and separated into "enzyme" and "substrate" strands that enable multiple-turnover reactions. The small size of this motif allows relatively easy synthesis, enabling a variety of studies. The hammerhead was the first ribozyme to be crystallized, and a series of X-ray crystal structures have now been reported [12]. While this ribozyme has been the subject of extensive research, surprising controversies remain regarding its mechanism. As described below, this situation may be due to conformational flexibility in this small motif, and less ambiguity is found for the larger ribozymes described later.

In the presence of cations, the hammerhead ribozyme catalyzes specific cleavage of the phosphodiester bond between nucleotides 17 and 1.1 (Figure 3). Initial X-ray structures of "ground state" hammerhead ribozymes provided woefully little information about the chemical mechanism for RNA cleavage, with the cleavage site sugar 2′ nucleophile directed away from the phosphodiester bond it is to attack and few obvious clues about potential stabilization of the transition state or the 5′-oxygen leaving group. Results from investigating a host of modifications show that hammerhead activity is exquisitely sensitive to modifications of the conserved regions (in red in Figure 3), which also are generally involved in a proposed network of hydrogen bonds and other tertiary interactions in the hammerhead core. At the junction of three RNA helices, many core modifications are likely to inhibit proper folding of the RNA [35], but the effects of some, such as substitutions in G5 and A6, have been difficult to explain based on the initial X-ray structures.

Like all known ribozymes, hammerhead activity is very sensitive to ionic conditions. This sensitivity is in large part due to requirements for RNA structure, making it difficult to tease out specific roles for metals in catalysis. RNA secondary and tertiary structure is stabilized by hydrogen bonding and by hydrophobic and van der Waals interactions and often requires negatively charged phosphodiester groups that are in close proximity to one another. Cations increase the thermodynamic stability of RNA helices, and tertiary structure often has a further and more selective requirement for specific ions such as Mg^{2+}. It is important, when comparing metal-based studies in RNA, to always note conditions of both monovalent (NaCl or KCl) and divalent cations.

In a background of moderate ionic strength ($< \sim$1 M NaCl), the hammerhead requires divalent cations for ac-

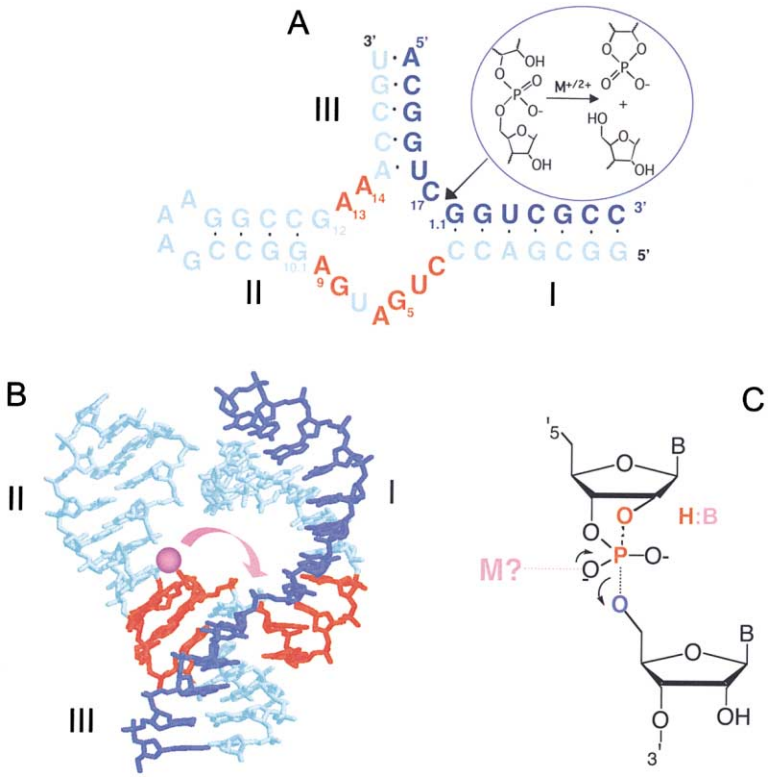

Figure 3. Proposals for Catalysis in the Hammerhead Ribozyme

(A) Hammerhead secondary structure, with conserved core in red.

(B) Hammerhead crystal structure, showing metal binding site at A9 in sphere and arrow denoting putative interaction of that metal ion with the cleavage site, which would require a large conformational change.

(C) Proposed stabilization of negatively charged phosphodiester substrate, or transition state, by metal ions in the hammerhead. As discussed in the text, catalysis by monovalent ions suggests electrostatic stabilization is the main role of cations.

tivity. The required metal ion concentration reflects an apparent affinity for a site relevant to activity, whereas the maximal rate supported by a particular metal reflects its influence on the transition state of the rate-determining step. Both of these properties vary with metal ion for the hammerhead [13], suggesting that at moderate ionic strength the metal ion has an influence on the transition state that is measured in hammerhead activity assays.

The hammerhead exhibits a positive log-linear dependence on pH with a slope close to 1, interpreted to mean that a deprotonation takes place in the rate-limiting step of the reaction. At a given pH and metal ion concentration, the reaction rate also increases as the pKa of aqua ligands to the metal ions decrease [7–9]. This observation has been interpreted to mean that the deprotonation event involves the metal ion itself, intimately involving metal ions with the reaction mechanism, and it has been hypothesized that a metal-hydroxide species could be involved in the rate-limiting step of the reaction, or that the metal ion binds to and lowers the pKa of an ionizable group on the RNA. Researchers expanded on this latter hypothesis by suggesting that if this ionizable group is the sugar 2′-OH nucleophile, the data are then most consistent with direct coordination of the metal ion to that position [14]. Most hammerhead kinetic data have been interpreted by assuming that the rate-limiting step measured in kinetic assays is actually the chemical step of the reaction. This is a critical assumption that, as described below, was recently challenged for the hairpin ribozyme [15]. Most hammerhead activity assays are measured under "single turnover" conditions, where a simple kinetic scheme, such as that described by equation 1 below, with $k1$ assumed much faster than $k2$, is invoked.

$$E + S \underset{}{\overset{k_1}{\rightleftharpoons}} [E:S] \overset{k_2\ (=k_{chem})}{\rightleftharpoons} [E:P] \overset{k_3}{\rightleftharpoons} E + P \qquad (1)$$

If there are rate-limiting conformational changes, however, or even conformational changes with rates approaching those of the chemical step of the reaction, an expanded scheme is more appropriate.

$$E + S \overset{k_1}{\rightleftharpoons} [E:S] \overset{k_2}{\rightleftharpoons} [E:S]^* \overset{k_3\ (=k_{chem})}{\rightleftharpoons} [E:P] \overset{k_4}{\rightleftharpoons} E + P \qquad (2)$$

In the case of the scheme described in Equation 2, the observed rate may be dominated by conformational changes ($k2$) or by chemistry ($k3$), or may be a combination of contributions from both. Alternatively, if a fast equilibrium is established between [E:S] and [E:S]*, the resulting overall rate will reflect any changes that affect the equilibrium population of [E:S]*. The most definitive method of assigning a kinetic rate constant to the chemical step of the reaction would be via kinetic isotope effects (such as O^{18}/O^{16}), which have not been measured for ribozyme reactions. Solvent kinetic isotope effects ($^2H/^1H$) have been measured [16] but do not isolate the location of the isotope effect to a specific site in the RNA. Thus, it is obviously challenging to use these limited techniques to discern whether metal ions are indeed

involved in any of the possible roles described in Figure 1 or are instead required for a specific conformation of the hammerhead as well as other ribozymes. Either way, at moderate ionic strength, metals are required cofactors for the hammerhead reaction.

Phosphorothioate substitutions have been a popular tool for assigning activity-related metal sites in ribozymes [17]. An R_p phosphorothioate substitution (sulfur substitution of the pro-R nonbridging oxygen position) at the cleavage site of the hammerhead decreases Mg^{2+}-dependent rates by a factor of about 10^3–10^5, interpreted to suggest that a Mg^{2+}-oxygen interaction has been inhibited at that site. Mn^{2+} and Cd^{2+} can both rescue this inhibition. Since Mn^{2+} is marginally thiophilic but Cd^{2+} is definitely so, these data strongly suggest that metal ions interact with the cleavage site phosphodiester, thus tying a metal to the active site itself. A second phosphorothioate-sensitive site in the hammerhead ribozyme is the phosphate 5′ to position A9 (Figure 3), which is distant to the active site according to ground state crystal structures.

An R_p E phosphorothioate at A9 also inhibits Mg^{2+}-dependent activity, which can be rescued by addition of Cd^{2+} [17]. Metal rescue of phosphorothioates must be interpreted extremely carefully, however. The larger and more polarizable sulfur atom can cause charge redistribution and conformational changes at the substituted site. Since transition metals in general bind more tightly to nucleic acid bases, switching Mg^{2+} to Mn^{2+} or Cd^{2+} may allow compensation for a conformational change induced by the phosphorothioate. Nevertheless, phosphorothioate "metal rescue" studies often can point to a potential metal site.

For the hammerhead A9 site, ^{31}P NMR has provided direct evidence for Cd coordinating to sulfur, confirming that the rescuing metal actually binds to this site [18]. This is the first metal site directly observed under solution conditions that has been tied to RNA chemical activity, but it is predicted to be at a 20 Å distance from the active site according to the hammerhead X-ray crystal structures. Of note, the same NMR study found Cd^{2+} binding to the cleavage site phosphorothioate comparatively difficult to detect, indicating very weak, if any, binding to the cleavage site under solution conditions. Possible ways to reconcile the results of this NMR study with the cleavage site phosphorothioate inhibition/metal rescue data include: (1) metals do not bind this site in the ground state, or do so at very low population (vide infra); (2) the substitutions (deoxy or 2′-OMe) required to make the substrate inert for the spectroscopic study also inhibit metal binding to the phosphorothioate; (3) metal rescue of the cleavage site phosphorothioate substitution is not due to direct binding at that site. These possibilities are still under investigation.

Two models can account for the effect of the apparently distant A9 site on hammerhead chemistry. A "through RNA" conformational effect may allow the A9 metal site to influence RNA activity as an allosteric effector. This conformational effect may be relatively subtle, since X-ray crystal structures in the presence and absence of soaked-in metal ions show little overall structural change [12]. Another model put forward to explain the critical nature of the hammerhead A9 metal site in activity calls for a large conformational rearrangement of the hammerhead from its ground state crystal structure prior to cleavage [17]. In this model, proposed by Herschlag and coworkers to explain the properties of a doubly substituted hammerhead that is phosphorothioate-labeled at both the A9 and the cleavage site, the same metal ion that binds at the A9 site also contacts the cleavage site phosphodiester pro-R oxygen (Figure 3). In their model, a pretransition state conformational change brings the A9 and cleavage sites into close proximity. This "active" conformation is infrequently populated, which may be one reason for the low apparent reaction rate for this ribozyme. Moreover, the dominantly populated ground state conformation is predicted to support metal binding at the A9 site, but not at the cleavage site, which is consistent with the observations from ^{31}P NMR spectroscopy described above.

The Herschlag hypothesis suggests that, in solution, the hammerhead can achieve a conformation that is not observed in the existing X-ray crystal structures. Ground state or uncleaved ribozyme crystal structures are usually obtained by using a nonreactive substitution of the sugar 2′ nucleophile, either a deoxy or a 2′-OMe group. In addition to structures of noncleavable analogs, Scott and coworkers have reported a series of X-ray structures of a cleavable analog that has a 2′-OH nucleophile and also with a substrate having an extra methyl group on the carbon 5′ to the cleavage site phosphate (a *talo*-5′ methyl modification) that slows the cleavage rate. Conformational rearrangements in the crystal have been trapped with these substrates [19], and four different structures obtained in this way have been proposed to represent a pathway for hammerhead activity. The major conformational rearrangement detected by crystallography is a rotation of the C17 base position, which swings the cleavage site phosphodiester to be in line with the sugar 2′-OH nucleophile and apparently poised for the reaction. A metal ion appears in these structures near the pro-R oxygen of the scissile phosphate. From these data, a pH-dependent conformational change is proposed that might be the rate-limiting step, *k*2 in equation 2 [19].

In the X-ray structure of a trapped form of the hammerhead containing the product 2′-3′ cyclic phosphate of C17, a rearrangement has occurred such that the keto oxygen of G5 is ~3 Å from the C17 2′-O (the nucleophile in the forward reaction), and the exocyclic amine of A6 is located ~3 Å from a nonbridging phosphate oxygen. While neither the purine exocyclic amine nor keto groups are expected to easily fulfill roles as proton donor or acceptor, this is the first observation of proximity of these critical groups to the cleavage site in the hammerhead ribozyme. It is interesting to note that a similar role for a guanine in stabilizing the proposed transition state has been suggested for the hairpin ribozyme (see below).

It is difficult to reconcile hypotheses based on the hammerhead X-ray crystal structure data, which show a modest conformational rearrangement near the active site, with the larger structural change predicted by Herschlag and coworkers for the hammerhead under solution conditions. In the crystals, the ends of hammerhead arms I and II are pinned in place by an adjacent molecule, which would seem to preclude their approach

as required by the Herschlag model. Unless the slow-cleaving substrate used in the crystallographic study promotes a different route for activity, these two proposals will remain distinct and noncomplemetary.

A final, and important, set of results for the hammerhead and other ribozymes that perform the reaction in Figure 1A is the revelation that activity can be supported by very high concentrations of monovalent cations (4 M Li^+ or 4 M NH_4^+) in the absence of divalent ions [16, 20, and references therein]. For the hammerhead ribozyme, the effects of several factors known to affect activity were compared with either 4 M Li^+ or 10 mM Mg^{2+} as supporting cation. The log-linear pH dependence of the single-turnover rate constant is maintained in 4 M Li^+, as are inhibitory effects of several substitutions around the hammerhead sequence, and the maximum rate is within a factor of 10 of that supported by divalent cations. Of interest, the only substitutions that did not have similar inhibitory effects under both supporting cation environments are the phosphorothioate substitutions at A9 and at the cleavage site and base deletions around A9 (G10.1 and C11.1). From these data, it is proposed that the major role of cations in supporting hammerhead ribozyme activity is electrostatic. On top of this, the A9/cleavage site metal(s) apparently provide an additional rate enhancement by a mechanism that remains controversial.

Although the hammerhead ribozyme has been extensively studied, it appears that the current mechanistic impasse cannot be broken without new structural information. Much has been learned from the ground-state X-ray crystal structures, but they do not explain the dire effects that many substitution have on activity, such as those that have been reported at position A9, that are distant from the hammerhead active site. The Herschlag proposal claims that the hammerhead ribozyme is only active in a conformation that is different from the crystal structure and that is infrequently populated in solution, and begs the question as to whether this conformation can be trapped by techniques such as chemical cross-linking or otherwise stabilizing contacts between Stems I and II. Such a construct could enable the requirements for "docking" to be separated from those important in the chemical mechanism. Conversely, hypotheses based on rearrangements observed in the crystallized hammerhead must bear the burden of being confirmed under solution conditions, potentially by solution NMR or other spectroscopic methods. An additional set of tools might be available from structural studies comparing inactive and active hammerhead constructs. For example, high-resolution X-ray or NMR structures of the A9-R_p phosphorothioate hammerhead with and without added Cd^{2+} would allow direct comparison between the ribozyme in an active and inactive state and provide further insight into the elements required for catalysis.

The Hairpin Ribozyme: Conformation May Be Rate Limiting and Evidence for Base Catalysis

Like the hammerhead ribozyme, the hairpin ribozyme performs the reaction in Figure 1A and has a metal ion requirement for activity at moderate ionic strengths. The hairpin ribozyme has two mainly helical regions that fluctuate between a coaxially stacked arrangement and formation of a "docked" complex, with the cleavage site located between them. A 4 helix hairpin construct is more stably docked than a construct with two helices. The dynamics of these large structure changes can complicate kinetic analyses in the hairpin ribozyme.

Recent single-molecule FRET measurements of a 2 helix hairpin ribozyme have provided rate constants for the individual conformational changes that take place during the reaction [15]. These, in particular the rate of *undocking* of the two helices to allow product release, have a dramatic influence on interpretations of solution-state kinetic measurements. In the docked complex, the forward rate of the cleavage reaction is actually slower than that of religation ($K_{eq} \approx 0.5$), and undocking of the two helices is a prerequisite for product release. Kinetic analyses of this particular hairpin construct reveal that the overall rate of the reaction measured under solution conditions of $<1\ min^{-1}$ (pH 7) is a composite of at least four different microscopic rate constants involving both chemistry and conformation. This finding suggests that the previously observed shallow pH dependence of steady-state rates for this ribozyme does not necessarily mean that the rate of the chemical step is pH independent. It should be noted that the measured rate constants are likely to vary with hairpin construct and have been reported for a "two-way junction" motif.

The X-ray crystal structure of a four-way junction hairpin ribozyme with a cleavage site 2′-OMe substitution has been reported [21]. Surprisingly, in this structure the cleavage site 2′-O is positioned in line with the phosphodiester bond to be attacked. Only two metal ions have high population in the structure and they are not close to the cleavage site. Two conserved bases, G8 and A38, are near the 2′ nucleophile and leaving group, respectively, prompting proposals that bases are involved in the reaction mechanism. Removal and many alterations of G8 are not tolerated for hairpin activity [22, 23]. A series of substituted purines are able to reconstitute some activity, the pattern of which suggests that the exocyclic amine and amide functionalities of the Watson-Crick face of G8 are particularly important for function. In the case of a hairpin containing an abasic site at position 8, the pH dependence of activity measured following reconstitution using substituted purines suggested that the bases were protonated in the context of an active hairpin ribozyme [23]. One suggested role for the important substituents of G8 is electrostatic stabilization of the substrate cleavage site phosphodiester through the N2 exocyclic amine and possible positioning or activation of the 2′-OH by the N1 amine (Figure 4) [21–23]. As noted by the authors, however, these studies cannot eliminate the possibility that G8 has a structural rather than catalytic role.

The hairpin ribozyme presents a remarkable opportunity for spectroscopic studies because the X-ray crystal structure suggests that the inhibited form still adopts an "active" conformation. Thus, predictions such as those for G8 may be testable using solution NMR methods on the inhibited ribozyme with some confidence that this structure can be related to the active RNA. It would also be of interest to determine whether either of

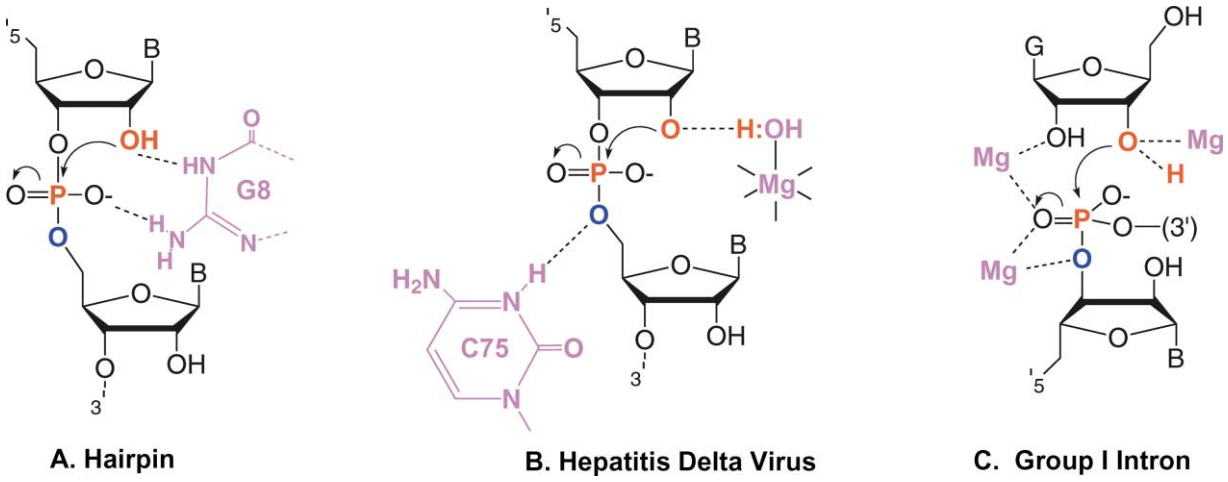

Figure 4. Proposals for Catalytic Mechanisms in the HDV and Group I Intron Ribozymes

(A) In the hairpin ribozyme, the G8 amine or carbonyl groups are proposed to be involved in catalysis.

(B) A protonated cytosine is proposed to donate a proton to the leaving group in the HDV ribozyme, and a metal bound hydroxide may activate the 2′-OH nucleophile.

(C) In the Group I intron, several metal sites are proposed based on kinetic assays of metal-dependent activity following single-atom substitutions.

the metal sites populated in the crystal structure have affinities that match the metal ion requirements for hairpin activity at moderate ionic strength. Like the hammerhead ribozyme, the hairpin is active in high concentrations of monovalent ions. One contrast with the hammerhead is that at moderate ionic strengths, hairpin ribozyme activity can be supported with low concentrations of the ligand exchange-inert ion $Co(NH_3)_6^{3+}$. This result suggests that rather than direct coordination of a metal ion to an RNA functional group, metals instead provide electrostatic or structural roles in the hairpin ribozyme.

The Hepatitis Delta Virus: A Cytosine with a Perturbed pKa

The HDV ribozyme is found in the genomic sequence of a satellite RNA that enhances effects from the Hepatitis B virus. The HDV sequence cleaves site-specifically during rolling circle replication of this RNA via the reaction of Figure 1A. A 72 nucleotide HDV ribozyme has been crystallized in its product form, containing the 5′-OH product following cleavage [24]. Like the Group I intron described below, the active site of the HDV ribozyme is deeply buried in a cleft of this compactly folded RNA. Predicted rate constants for HDV and Group I intron ribozymes are orders of magnitude faster than those for the hairpin and hammerhead and approach those expected from protein enzymes [27], suggesting that these larger RNAs provide a more rigid active site and that the apparent rates are more dominated by the chemical step of the reactions.

Several lines of evidence have now been put forward that suggest that a base with a perturbed pKa value is involved in catalysis by the HDV ribozyme. In the crystal structure, cytosine 75 was found positioned with its N3 within hydrogen bonding distance of the product 5′-OH, suggesting that in the reaction, a protonated cytosine could act as proton donor to the 5′-O leaving group [20]. Subsequent testing of this hypothesis through pH-rate profiles and base substitutions confirmed the importance and pH sensitivity of this position in HDV [25]. Since the HDV ribozyme also has a strict metal ion requirement at moderate ionic strength, current mechanisms invoke C75 acting as a general acid in protonating the leaving group and a potential metal ion acting to deprotonate the nucleophilic 2′-OH [26] (Figure 4B).

Interestingly, attempts to directly measure the pKa of C75 in situ using ^{13}C NMR found that the pKa value was slightly elevated from ~4 to 4.8–5.4, but not to the level of ~7 predicted by kinetic studies [27]. These attempts were made using the *cleaved*, or product-state, construct, and the results suggest that the precleavage structure may be different from that of the product state. Such a suggestion has support from recent FRET studies of the HDV ribozyme that suggest slightly different conformations in the pre- and postcleavage state structures [28]. This in turn suggests that the current crystal structure of the HDV ribozyme more closely resembles the product state than the pretransition ground state for the reaction. Clearly, further information concerning the roles of metal ions or other groups in activating the HDV cleavage site may be available from a structure of the ribozyme in the precleavage state, such as that obtained from a construct containing a noncleavable substrate.

The Group I Intron: A Proposed Metalloribozyme

Models for the mechanism of action of the Group I intron from *Tetrahymena thermophila*, a very large ribozyme that has been extensively studied, have emphasized direct roles for metal ions in the chemical step of the reaction. The Group I intron is studied as a 400 nucleotide sequence with complex secondary and tertiary structure. It catalyzes a series of three reactions with the result that the intron is cut out of its flanking sequences,

which are religated. The Group I and Group II introns most closely resemble RNA splicing activity that occurs in higher-order eukaryotes, catalyzed by the large ribonucleoprotein spliceosome. In its most commonly studied form, the Group I intron is complexed with a short oligonucleotide substrate and an exogenous guanosine whose 3′ hydroxyl is the attacking nucleophile in the first step of the splicing reaction. This reaction can then be measured by formation of a single oligonucleotide product.

A large part of the Group I intron has been crystallized and a structure solved at 5 Å resolution [29] (Figure 2). The current structure lacks the P1 helix that contains the substrate for the first splicing reaction, but contains the proposed binding site for the exogenous guanosine nucleophile (Figure 1B). It is proposed that the Group I structure presents a preorganized active site, and that the size and complexity of this RNA aid in establishing such a relatively rigid cleft.

The Group I intron has a distinct requirement for Mg^{2+} or other divalent metal ions for activity. An all-out search for the sites at which metals might influence chemistry, in the form of metal rescue experiments for a series of individual substitutions around the active site, has yielded a proposal of four active-site ligands and three separate metals [30] (Figure 4C). These proposals are based on Cd, Mn, or Zn rescue of activity following phosphorothioate (nonbridging O→S) and phosphorothiolate (bridging O→S) substitutions, or Mn rescue of a 2′-NH_2 substitution. If activity rescue is due to actual coordination of metals to these groups, as opposed to "correction" of perturbed RNA structure, one might hope to see a trend in activity properties that follows metal-coordination preferences. This trend is observed for the G 2′-NH_2 substitution, for which the apparent Mn^{2+} affinity is increased by ~100 as expected for Mn-amine versus Mn-alcohol coordination [31]. Many sulfur substitutions do not lead to such pleasing results, however, potentially because of larger structural changes.

The constellation of metal ions predicted to be involved in Group I chemistry is unusual and would require precise positioning of the metals with some neutralization of the resulting large positive charge, as is achieved by the acidic residues in active sites of nucleic acid polymerases. Precedent for such metal clusters supported by RNA is found in the "metal ion core" characterized in the X-ray structure of the group I intron P4-P6 subdomain, in which multiple metals are positioned in close proximity (within 5–10 Å) and coordinated through proximal phosphodiester groups. Consistent with this, a potential phosphate ligand to one active site metal in the Group I intron was recently identified through phosphorothioate interference assays [32].

The Ribosome: A Proposed Base-Catalyzed Mechanism

When the first X-ray structure of the 30S ribosomal subunit appeared in 2000, one striking feature of the structure was the location of a transition state analog for peptidyl transfer buried within the RNA component of the ribonucleoprotein [3]. This result strongly supported previous proposals that the catalytic component for ribosome chemistry is based in RNA and not protein. Although hundreds of metal ions are found in the ribosome structures, no metal ions were reported near the putative active site. The observed close proximity between the transition state analog and a highly conserved adenine, A2451 (*E. coli* numbering), suggested this base was important for chemistry, possibly in one of the roles shown in Figure 1C. Since an elevated pKa would be required for the N1 or N3 imine groups of adenine to be facile proton acceptors, a search for bases with elevated pKa values was carried out via pH-dependent dimethylsulfate (DMS) modifications; this search turned up A2451 [4].

However, despite this, the importance of A2451 in peptidyl transfer has been questioned, because mutagenesis and crosslinking of this position have a relatively minor affect on ribosome activity [33]. Assays for peptidyl transfer that are rate limited by chemistry remain under development and will be required to address this mechanism.

The Spliceosome, an Evolving Story

Like the ribosome, the spliceosome is a large ribonucleoprotein machine that carries out a biologically critical reaction with high requirements for fidelity. A recent, tantalizing report has been put forward suggesting chemically active RNA has been observed in a construct of three spliceosomal RNA fragments [5]. It has been proposed that the observed reaction consists of the formation of a triester species, ordinarily very unstable in oligoribonucleotides, between the 2′-OH of a conserved A and a phosphodiester within a conserved trinucleotide distant in primary sequence. However, a very low yield of triester product has been obtained from this curious reaction, making it premature to absolutely conclude that the spliceosome also is a ribozyme, but the possibility is certainly tempting.

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