Ribozymes: A Distinct Class of Metalloenzymes

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Ribozymes are an important new class of metalloenzymes that have an unlikely feature: they are made entirely of ribonucleic acid (RNA). Metal ions are essential for efficient chemical catalysis by ribozymes and are often required for the stabilization of ribozyme structure. Most ribozymes catalyze reactions at phosphorus centers through one of two major mechanistic pathways, and reaction has been observed at carbon centers. Creative experiments have revealed the position of metal ions in the active site of two ribozymes. The exploitation of variable metal geometry and reactivity has expanded ribozyme chemistry and has facilitated the application of in vitro selection for the creation of novel ribozymes.

The discovery that RNA molecules can function as enzymes revolutionized the understanding of chemistry in biological systems. Before that finding, it was assumed that protein enzymes were the sole catalytic workers of the cell and that nucleic acids provided only genetic information and structural scaffolding. It was believed that DNA is transcribed into RNA, which is then translated into protein, and that all the intermediate catalytic steps are carried out by protein enzymes. However, about a decade ago it was shown that RNA molecules can catalyze chemical transformations on themselves and on other RNA molecules (*1, 2*). Since these original discoveries, it has become apparent that reactions by catalytic RNA molecules (ribozymes) are fundamental to the biochemical function of many organisms (*3–7*). One additional component has consistently been found to be essential for efficient RNA catalysis: divalent metal cations. Unlike protein enzymes, which do not all require metals for activity, ribozymes are always metalloenzymes. There are at least two distinct functions for metals in ribozymes: Divalent cations are required for chemistry and often aid in the structural stabilization of folded RNA.

The RNA molecule is an extended polyanion that is not easily packed into catalytically active forms without substantial charge neutralization between its interacting strands. Cations neutralize the charge in folded RNA molecules, and there is evidence that divalent metal ions can bind to specific sites within complex RNAs (Fig. 1). By interacting with nearby functional groups, these ions may bridge separate strands and stabilize folded "pockets" (*8–10*). In this capacity, metal ions represent a form of tertiary interaction, reminiscent of Zn^{2+} ions that stabilize the DNA binding domains of Zn transcription factors (*11*). Numerous models can be used to explain the function of divalent cations in RNA structure. However, the dual role of these cations in screening negative charge and binding to specific sites complicates our ability to understand their precise contribution to RNA folding.

Ribozymes are generally known for their ability to bind another strand of RNA and specifically cleave the phosphodiester backbone through one of two mechanisms (Fig. 2) (*12–14*). Certain ribozymes also catalyze the cleavage of DNA (*15, 16*), the polymerization of RNA monomer units (*17*), the replication of RNA strands (*18*), the opening of 2′-3′-cyclic phosphate rings (*19*), and reactions with phosphate monoesters (*20*). Ribozyme-catalyzed reactions at carbon centers have been observed (*21, 22*) and ribozymes with new kinds of catalytic reactivity are being created through techniques of in vitro selection and evolution (*23*). Collectively, this work demonstrates that ribozymes have a vast catalytic repertoire.

Central to the development of this chemistry is a concerted effort to define the function of metal ions in ribozyme catalysis. There have been a number of theoretical papers (*24, 25*) and several reviews on this subject (*26, 27*). The role of metal ions in RNA catalysis is expanding along with ribozyme chemistry as different metals are used to modulate macromolecular reactivity. The variations in metal coordination number, ionic radius, electrophilicity, and oxidation state may be exploited to generate novel RNA enzymes that can catalyze new chemical transformations.

The RNA molecule contains a number of ligands for specific metal coordination. Predominant are the phosphate oxygens, 2′-hydroxyls, base carbonyls, and transition-state oxyanions, which preferentially coordinate to hard alkaline earth metals such as Mg^{2+} (*28*). The natural affinity of RNA for Mg^{2+} may be the reason why so many ribozymes depend on this ion for structure and function. The Mg^{2+} ion usually coordinates six ligands in an octahedral geometry, whereas coordination numbers greater than six are common for Ca^{2+}. The Mg^{2+} ion has a low affinity for nitrogen ligands

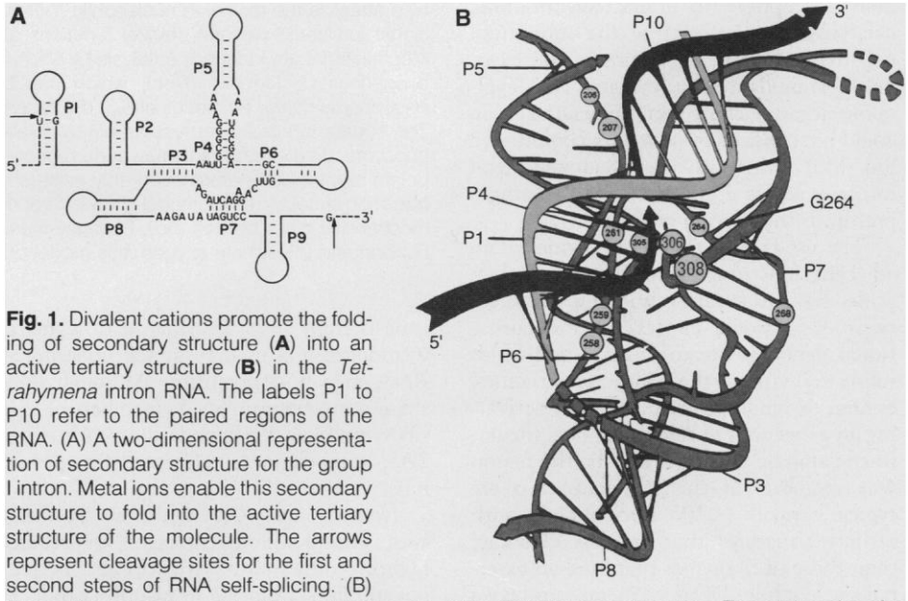

Fig. 1. Divalent cations promote the folding of secondary structure (**A**) into an active tertiary structure (**B**) in the *Tetrahymena* intron RNA. The labels P1 to P10 refer to the paired regions of the RNA. (A) A two-dimensional representation of secondary structure for the group I intron. Metal ions enable this secondary structure to fold into the active tertiary structure of the molecule. The arrows represent cleavage sites for the first and second steps of RNA self-splicing. (B) Three-dimensional model of the catalytic core of the *Tetrahymena* ribozyme. The active site of a group I intron was modeled with distance constraints from many different tertiary interactions, including the phylogenetic covariation of conserved nucleotides (*87*). Circles represent probable sites of divalent metal ion coordination to the phosphate backbone (*64*). These sites are believed to include metal ions important to the structure of and catalysis by the ribozyme. Circled numbers correspond to positions in the *Tetrahymena* ribozyme; G264 is the binding site for the guanosine nucleophile.

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and almost no ability to coordinate sulfur. Transition metals such as Mn^{2+} have more relaxed ligand specificities, coordinating to ribonucleotide oxygens as well as ring nitrogens of the bases. Like Zn^{2+} and Cd^{2+}, Mn^{2+} also forms stable bonds to sulfur (*29*). Sulfur coordination is relevant to certain modified bases and to enzymological studies that use sulfur derivatives of nucleic acids.

This article describes the participation of metal ions in many different types of ribozyme chemistry. Ribozymes, like protein metalloenzymes, fall into distinct mechanistic classes, and a wealth of information has illuminated the function of metal ions in each of these groups. Known ribozyme reactions will be presented with a perspective on the future of ribozyme chemistry.

Attack on Phosphorus by an Internal Nucleophile

Ribozymes that activate an internal nucleophile use a distinctive reaction mechanism to catalyze the cleavage of RNA phosphodiester linkages (Fig. 2A). Reactions by this ribozyme class involve the intramolecular attack of an adjacent 2′-OH group on phosphorus, generating 2′-3′-cyclic phosphate and 5′-OH products. This mechanism defines at least five ways that metal ions can potentiate an RNA cleavage reaction. A metal-coordinated hydroxyl group may act as a general base, deprotonating the 2′-OH group (M1) (*8*). Direct metal ion coordination to the phosphoryl oxygen (M2) may render the phosphorus center more susceptible to attack (electrophilic catalysis) or may stabilize the substantial negative charge of oxyanions in the trigonal-bipyramidal transition state. The developing negative charge on the leaving group could be stabilized by a directly coordinated ion (M3). Alternatively, a hydrated metal ion may act as a general acid, donating a proton to the leaving group (M4).

The use by this class of ribozymes of an adjacent functional group as the nucleophile has important structural ramifications. One would expect fewer organizational demands on an enzyme that binds substrates without the additional structural burden of binding, orienting, and activating an exogenous nucleophile. This streamlined catalytic approach may be the reason why members of this ribozyme class are typically small (<100 nucleotides), with tertiary structures that are less elaborate than those of ribozymes that bind an external nucleophile. Divalent metal ions play a particularly important role in the chemistry rather than in the folding of several ribozymes that activate an internal nucleophile. The small size and high sequence selectivity of ribozymes such as the hammerhead make them promising agents for gene therapy (*30*), in which a ribozyme is introduced and used to attack undesirable RNA in a host organism. Although they share a mechanism (m_A), members of this ribozyme class use metals differently (Fig. 2A), with distinct preferences for specific ions.

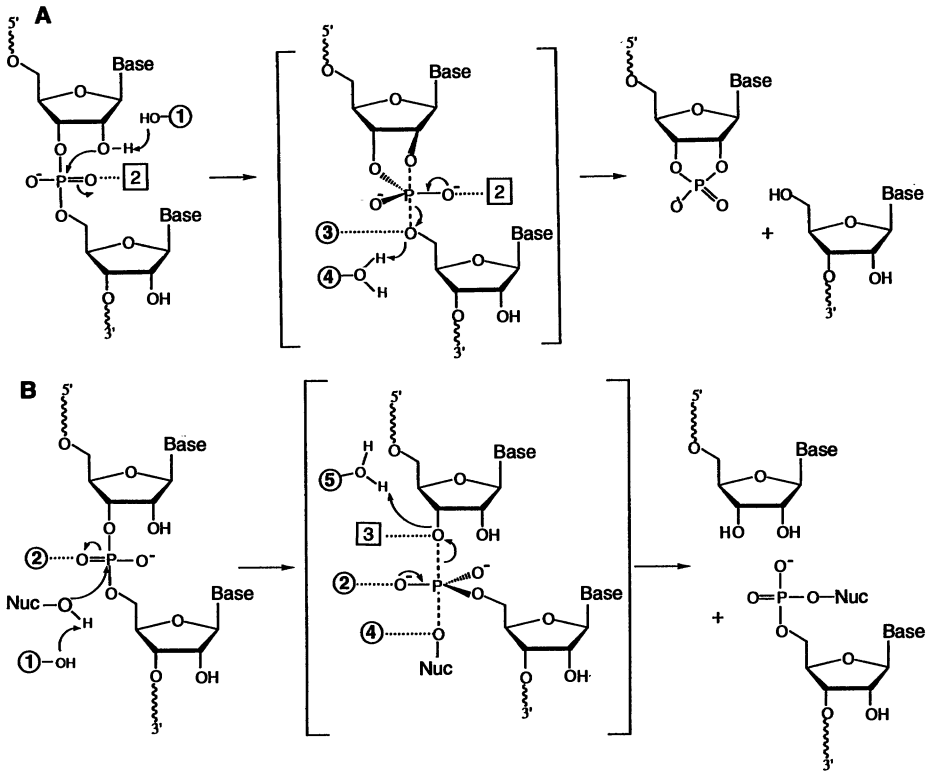

Fig. 2. The two mechanistic classes of ribozyme phosphodiester cleavage. Circles represent divalent cations and several ways that they may participate in catalysis; squares represent sites directly supported by experimental data. Although most of the ions are shown participating in the transition state of the reaction, they may also contribute to ground-state stabilization and substrate binding (represented by M2, the square labeled 2). (**A**) Mechanism A (m_A), observed in metal-catalyzed RNA hydrolysis and RNA cleavage by the hammerhead, hairpin, hepatitis delta, Neurospora, and tRNA ribozymes. Although chirality is not shown, the attack of the 2′-OH group proceeds by an in-line S_N2 nucleophilic displacement with inversion of the configuration at phosphorus (*42*). The M2 square represents a divalent cation that promotes catalysis by the hammerhead ribozyme. In addition to coordinating the nonbridging phosphate oxygen, M2 may also stabilize the oxyanion nucleophile. Although four discrete ions are represented for clarity, a single ion could perform several functions at once (such as M2 and M4, or M1 and M2). (**B**) Mechanism B (m_B) found in catalysis by ribonuclease P, group I, and group II intron ribozymes. The exogenous nucleophile (Nuc), which can be water or a hydroxyl functionality, attacks the phosphorus center by means of S_N2 displacement (*88*). Stereochemistry of attack is not indicated. The square labeled 3 represents M3, a divalent ion that promotes catalysis by the *Tetrahymena* ribozyme. Single metal ions may perform more than one of the five functions shown, and additional points of metal ion participation may exist (such as interaction of M3 with the 2′-OH group in the transition state). A recent model shows M3 and M4 as the two metals necessary for catalysis by this mechanistic class of RNA (*60*). Because these two metals are also poised for interaction with the nonbridging phosphate oxygen, this model does not require the presence of M2.

Nonenzymatic RNA hydrolysis by metal ions. Phosphodiester linkages are readily hydrolyzed by multivalent cations in aqueous solution, resulting in reaction products typical of m_A. This effect is especially pronounced at high pH for Mg^{2+}, whereas other ions such as Pb^{2+} and Eu^{3+} promote rapid hydrolysis of RNA at neutral pH (*8, 31–33*). These metals coordinate hydroxide ion, which presumably deprotonates the adjacent 2′-OH at the cleavage site (Fig. 2A). Metal-catalyzed RNA hydrolysis can occur at any phosphodiester linkage, although cleavage at specific sites often occurs in highly structured RNAs, such as transfer RNA (tRNA) (*8, 32*) and virusoid RNAs (*5*). Within their folded structures, these RNAs contain metal binding "pockets" that direct attack toward particular phosphodiester linkages. Although these intramolecular reactions are not strict examples of RNA catalysis, highly specific interactions between nucleotide functional groups and aquated metal ions stimulate phosphodiester cleavage, just as they do in intermolecular systems.

The specificity of certain metal-cata-

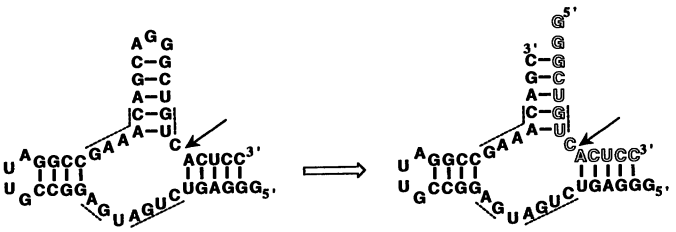

Fig. 3. Site-specific RNA cleavage transformed into multiple-turnover ribozyme activity. Shown is the conversion of (**left**) a metal-dependent, self-cleaving virusoid RNA into (**right**) the hammerhead ribozyme through synthesis of the virusoid RNA as two pieces. An arrow indicates the cleavage site in each figure. The hammerhead ribozyme mediates metal-dependent cleavage at a single site along the bound oligonucleotide substrate (open letters, right). Dotted lines indicate nucleotides of a phylogenetically conserved sequence in virusoid RNAs. On the same principle, specific Pb^{2+} cleavage of tRNA forms the basis of a two-piece ribozyme that is dependent on Pb^{2+} (*35, 36*).

lyzed RNA hydrolysis reactions has been exploited in the design of multiple-turnover ribozymes. Several RNAs that undergo intramolecular self-cleavage have been redesigned to perform intermolecular RNA cleavage as true enzymes. Virusoid RNA was synthesized in two pieces to produce the hammerhead ribozyme, which contains the uncleaved part of the molecule, and its "substrate," containing the natural cleavage site (Fig. 3) (*5, 34*). Similarly, the single lead cleavage site in tRNA became the reaction site of a two-piece tRNA ribozyme (*35–37*). These examples illustrate that there is often little more than a semantic distinction between ribozyme-catalyzed reactions that proceed through M1 and specific metal-catalyzed hydrolysis of certain structured RNA molecules.

RNA catalysis by a trinucleotide. The smallest true ribozyme described to date is the trinucleotide UUU, which with Mn^{2+} catalyzes specific cleavage between G and A of the GAAA tetranucleotide through m_A (*38*). The reaction proceeds best in the presence of Mn^{2+}, although some activity is also observed with Cd^{2+}. This ribozyme is a pared-down version of a larger Mn^{2+}-dependent ribozyme (*39*). The discovery of this tiny ribozyme is dramatic evidence that simple RNA structures can direct specific hydrolysis by metal ions. It is intriguing to speculate why this triplet, and not others, can promote such a highly specific reaction. Kazakov and Altman (*38*) proposed that two Mn^{2+} ions bind through association with adenine N7 residues on the GAAA substrate, a hypothesis consistent with the tendency of Mn^{2+} to bind both nitrogen and oxygen ligands. Although this is plausible, it does not help to explain why other purine tetramers are not cleaved by a complementary pyrimidine trinucleotide. It may be significant that the UUU-AAA pairing is the weakest, most deformable of the 64 possible triplet pairs (UUA-AAU is energetically similar) (*40*). The conformational flexibility of this particular duplex may enable backbone and base functional groups to form a specific Mn^{2+} binding site that is not energetically permissible in other triplet pairs. Although the reaction conditions would not normally be expected to stabilize such a short duplex (*40*), Mn^{2+} appears to impart a substantial amount of energetic stabilization. The UUU-AAA pair may be an exceptional extended ligand for Mn^{2+} ions poised to attack adjacent phosphodiester linkages.

The hammerhead ribozyme. Because there are only two invariant nucleotides in the substrate sequence recognized by the hammerhead ribozyme, it can be designed to cleave many different target sequences (Fig. 3) (*12, 34, 41*). A variety of cations stimulate cleavage by the hammerhead ribozyme, which can use divalent metal ions that are less reactive toward random RNA hydrolysis. The observed cleavage of RNA is efficient in the presence of Mg^{2+}, Mn^{2+}, Co^{2+}, and Ca^{2+}, slower with Sr^{2+} and Ba^{2+}, and very slow with spermidine or monovalents in place of divalent cations (*42*). Significant progress has been made in defining the role of metal ions in catalysis by the hammerhead ribozyme.

Direct Mg^{2+} interaction with a single nonbridging phosphate oxygen at the cleavage site was implicated by the results from a metal specificity switch (M2) (Fig. 2A). In this experiment, a single phosphorothioate linkage was inserted at the substrate cleavage site, resulting in the replacement of the nonbridging *pro*-R_P phosphate oxygen atom with a sulfur atom (*42*). Ribozyme cleavage of this substrate was strongly inhibited in the presence of Mg^{2+}, which prefers oxygen ligands. Dramatic restoration of cleavage efficiency was observed in the presence of Mn^{2+}, which coordinates oxygen and sulfur. Similar specificity switches may implicate a role for metal ions that are directly involved at other points in the mechanism (open circles in Fig. 2A). Although divalent metal ions are important for catalysis by the hammerhead ribozyme, nuclear magnetic resonance studies performed in the presence of a noncleavable DNA substrate suggest that the ribozyme structures in the presence and absence of Mg^{2+} are similar (*43*). In the presence of monovalent cations or spermine, it appears that catalytically important metal ions bind to existing sites on the hammerhead ribozyme, exerting effects on chemistry rather than RNA folding. If other cations are present for structural stabilization, divalent metal ions may act only in the transition state of this ribozyme.

The hairpin and hepatitis delta ribozymes. These ribozymes are somewhat larger than the hammerhead ribozyme, and although they use a form of m_A to catalyze sequence-specific RNA cleavage, their molecular structures are distinct from those of other known ribozymes (*44, 45*). Efficient RNA cleavage by the hairpin ribozyme is only observed in the presence of the alkaline earth metals Mg^{2+}, Ca^{2+}, and Sr^{2+} (*46*). Unlike the reaction by the hammerhead ribozyme, that of the hairpin ribozyme is actually inhibited by transition metals such as Mn^{2+} and Co^{2+}, which appear to prevent complex formation between the ribozyme and its substrate. Another important difference from the hammerhead ribozyme lies in the fact that the hairpin ribozyme efficiently cleaves a phosphorothioate linkage (*47*), a result inconsistent with direct interaction between a metal ion and the *pro*-R_P phosphate oxygen. These results indicate that, despite overt similarities in their mechanisms, the hairpin and hammerhead ribozymes may use metal ions in different ways. It will be interesting to compare the metal ion specificities of these ribozymes with those of the hepatitis delta ribozyme, which is known to catalyze efficient RNA cleavage in the presence of Mg^{2+} and Ca^{2+} (*4*), and of the large *Neurospora* ribozyme (*48*).

Attack on Phosphorus by an External Nucleophile

Ribozymes that activate an external nucleophile, including those derived from the self-splicing group I and group II introns and the RNA subunit of ribonuclease P, react through m_B (Fig. 2B). All ribozymes in this family catalyze the attack of an exogenous nucleophile, yielding 3′-OH and 5′-phosphates in a reaction that is markedly different from m_A. Phosphodiester cleavage can proceed in the presence of water as a nucleophile (*3, 49, 50*), although in specific instances other nucleophiles are preferred (*51, 52*). These ribozymes tend to be relatively large (>100 nucleotides), with requirements for both catalytic and structurally essential metal ions. Because these ribozymes do not use the adjacent 2′-OH as a nucleophile, they must adopt complicated structures that help bind exogenous nucleophiles. The larger size of group I and group II intron ribozymes may also be due to the fact they are derived from RNAs that carry out multistep tasks (such as RNA splicing),

rather than single cleavage events. Their added mechanistic and structural complexity allows these ribozymes to catalyze reactions as well as cleave RNA phosphodiesters. These ribozymes can cleave DNA (*15, 16*), and it has been shown that a ribozyme based on the group I intron can catalyze reactions at carbon centers (*22*). Several of these ribozymes have been shown to catalyze ligation (*1*), polymerization (*17*), and insertion into the middle of nucleic acid strand (reverse splicing) (*53, 54*). It should be noted that the hairpin ribozyme, which acts by m_A, can also catalyze a ligation reaction. It will be interesting to see if there is a relation between the structural complexity of a ribozyme and either its mechanism of catalysis or the diversity of reactions that it can catalyze. Ribozymes that act through m_B have different metal ion requirements, and the role of metals in their individual mechanisms may not be the same. There are five ways that metal ions may be involved in this reaction (Fig. 2B).

The Tetrahymena *ribozyme.* This catalytic RNA is derived from a self-splicing group I intron (*14*). Shortened forms of the intron catalyze sequence-specific cleavage of oligonucleotides in the presence of Mg^{2+} and a bound guanosine cofactor. The results of early work on metal ion requirements for the *Tetrahymena* ribozyme showed that Mg^{2+} or Mn^{2+} is required for reactions, although other divalent metal ions such as Ca^{2+} do not support catalytic activity (*55*).

Catalysis by structurally complex RNAs reflects the sum of metal-ion requirements for folding, substrate binding, and chemistry. Therefore, it was of interest to differentiate the effect of metal ions on individual steps in catalysis by the *Tetrahymena* ribozyme. Ribozyme folding, independent of catalytic activity, was first monitored by the technique of hydroxyl-radical footprinting, which distinguishes solvent-accessible nucleotides from those that are protected inside a folded RNA structure (*56*). Folding as a function of different metal ions was monitored, leading to the observation that Ca^{2+} and Sr^{2+} promote formation of a folded RNA tertiary structure that is almost identical to the Mg^{2+}-dependent structure (*57*). With a gel-mobility shift assay, it was shown that although a Ca^{2+} form of the ribozyme cannot cleave the substrate, it can fold and bind the substrate (*58*). Taken together, these results showed that there is a distinct role for Mg^{2+} in the chemical step, although RNA folding and substrate binding can proceed in the presence of other divalent cations.

As in the hammerhead ribozyme, the strong preference of Mg^{2+} for oxygen ligands and the tendency for Mn^{2+} to bind sulfur and oxygen have helped to identify the precise location of a metal ion in the *Tetrahymena* ribozyme active site. In a synthetically challenging experiment, the bridging 3′-oxygen at the cleavage site was replaced with a sulfur atom. This substrate was highly resistant to cleavage by the ribozyme in the presence of Mg^{2+}. However, the rate of cleavage was restored in the presence of Mn^{2+} and other thiophilic metal ions such as Zn^{2+}, provided that stabilizing concentrations of Mg^{2+} were also added (*59*). This specificity switch is consistent with direct metal coordination to the bridging 3′-oxygen (M3) (Fig. 2B) in the transition state of the chemical step, in which the metal ion stabilizes the developing negative charge on the leaving group. The result provides support for a unified model in which two specific metal ions (M3 and M4) (Fig. 2B) promote catalysis by m_B ribozymes (*60*). The cleavage of RNA by the *Tetrahymena* ribozyme is significantly faster than DNA cleavage, which suggests that the binding of M3 may be facilitated by additional coordination to the 2′-OH group at the cleavage site. This would be consistent with the results reported by Sugimoto and co-workers, who observed that an apparent Mg^{2+} binding constant depends on the presence of a 2′-OH at the cleavage site (*61*). Metal ions that are involved in other functions such as 2′-OH coordination probably will also be probed through metal-switch experiments. Despite the essential role of metal ions, they do not provide the only interactions that contribute to catalysis by this and probably other ribozymes. Ribonucleotide functional groups that participate in catalysis have also been identified (*62, 63*).

A switch in metal ion specificity has also been used to identify an array of metal binding sites that affect progress toward the transition state in the *Tetrahymena* ribozyme. This group of sites includes both structurally and chemically important divalent ions potentially involved in substrate alignment and catalysis in the phylogenetically conserved ribozyme core (Fig. 1B). A pool of self-splicing ribozymes containing randomly distributed phosphorothioate linkages was allowed to self-cleave in the presence of Mg^{2+} and Mn^{2+} (*64, 65*). The molecules that reacted were isolated, and their phosphorothioate linkages cleaved with iodine. The positions of iodine cleavage were mapped on a sequencing gel for both the Mg^{2+}- and Mn^{2+}-dependent reactions. The positions that predominated only in the presence of Mn^{2+} are likely metal binding sites of structural or chemical importance (Fig. 1B).

The group II intron. No discussion of group II intron chemistry would be complete without a more thorough definition of the term "intron." After RNA is transcribed from DNA, it often contains extra sequences that must be removed before the RNA can be translated or used in other functions. The splicing of RNA is the process by which adventitious RNA (the intron) is excised from the middle of functional RNA sequences (the exons). In two of the several categories of introns, the group I and group II intron families, sequences within the intron catalyze the cleavage and ligation reactions involved in RNA splicing (*1, 51*). Additional protein or RNA cofactors are not necessarily required, although they may facilitate the process in vivo. Like the *Tetrahymena* ribozyme derived from a group I intron, there are ribozyme derivatives of the group II intron, which catalyze cleavage and ligation reactions in the presence of divalent cations (*50, 54, 66, 67*). For group II introns, the preferred nucleophile is a specific adenosine 2′-OH within the intron. Because the group II intron uses a 2′-OH nucleophile, it might be described as an m_A ribozyme, like the hammerhead. However, the 2′-OH nucleophile of group II introns is located far in sequence from the cleavage site, and the organizational demands of properly orienting this group within the active site necessarily require a high degree of structural complexity. Mechanistic parallels to eukaryotic pre–messenger RNA splicing have led to the hypothesis that all RNA splicing, including forms that require protein cofactors, is catalyzed by RNA molecules (*6, 68–70*). Therefore, the metal ion requirements of group II intron splicing are important because they may reflect the function of divalent cations in catalysis of all eukaryotic RNA splicing. The group II intron bears no apparent structural similarity to group I introns or to ribonuclease P, and unfortunately, little is known beyond its absolute metal ion requirements. Efficient self-splicing is not observed without high concentrations of Mg^{2+} (60 to 100 mM) and a monovalent cation (0.5 to 1.5 M K^+ or $NH4^+$) (*66, 71*), and even under these optimal conditions many group II introns have no self-splicing activity.

Ribonuclease P RNA. The RNA subunit of ribonuclease P (RNase P) was one of the first catalytic RNA molecules discovered (*2*). At high concentrations of divalent and monovalent cations, this RNA cleaves 5′-terminal nucleotides from precursor tRNA molecules, producing functional tRNAs (*3*). The Ca^{2+} form of this RNA is reported to be catalytically active, although reactions are most efficient in Mg^{2+} or Mn^{2+} (*72–74*). Structurally and chemically essential metals were differentiated by the application of a photocross-linking assay, which covalently links RNase P–tRNA complexes under a variety of reaction conditions (*73*). Results showed that monovalent cations can promote the formation of a ribozyme-tRNA complex, although divalent cations

are still required for chemistry. A protein associated with RNase P provides a charge-screening function in vivo and in vitro. This protein can take the place of many structurally important metal ions, lowering the salt requirements for RNase P activity (*75*), but does not alter the requirement for divalent cations in the chemical step.

Catalysis of Nucleophilic Displacement at Carbon

Biochemical dogma suffered another blow in 1992 with two reports that RNA was involved in the catalysis of nucleophilic displacements at carbon. Before this time, it was widely accepted that the chemistry of RNA was limited to reactions at phosphate and that the only ribozyme substrates were nucleic acid molecules. However, workers in the Cech and Noller laboratories reported that the *Tetrahymena* ribozyme could catalyze nucleophilic attack on an activated carbon ester that was selectively inserted into the normal phosphodiester cleavage site of the ribozyme substrate (*22*). This result was accompanied by a report from the Noller lab that showed that ribosomes (particles composed of both protein and RNA) almost completely stripped of proteins could still catalyze a reaction analogous to the peptidyl transfer reaction, whereby a single amino acid is transferred from its cognate tRNA to the growing protein chain (*21*). These studies indicate that ribozymes can catalyze nucleophilic displacement at carbon centers and that this reactivity may be used to perform a ubiquitous transformation important to protein synthesis.

Both of these reactions require divalent cations, and given that the chemistry of RNA can be extended to new molecular substrates, metals may be shown to adopt significant new catalytic functions. Like the function of protein enzymes, the reactivity of an RNA molecule may be modified by the identity, coordination environment, and oxidation state of the bound metal ion. Therefore, as novel reactivities of RNA are discovered, new enzymatic functions for unusual metals may also be identified.

Ribozymes and Redox Chemistry

The field of bioinorganic chemistry is replete with studies on the redox chemistry of metalloenzymes. Protein enzymes commonly use transition metals to catalyze electron transfer and oxidation and reduction reactions. There may be examples of this chemistry among RNA enzymes, which might seem unlikely given the sensitivity of the sugar-phosphate backbone to attack by free radicals (*76*). However, the peptide backbone of proteins is also susceptible to attack by highly reactive metal complexes (*77–79*). Only by the careful placement of transition metals, regulation of their oxidation states, and steering of their chemistry can protein enzymes direct the reactivity of their metal centers. An RNA scaffolding could possibly be constructed that accomplishes these objectives. Although normal RNA may be an acceptable scaffold, an additional strategy exists to protect RNA in vulnerable positions.

Natural RNA molecules, especially tRNA (*80*), ribosomal RNA (*81*), and the small nuclear RNAs (*82*), are commonly functionalized after transcription by the strategic placement of base and backbone modifications. The extent of modification depends on the organism and the function of the RNA molecule. Naturally occurring 2′-*O*-methyl groups protect the ribose sugar from hydrolytic degradation, whereas modified bases such as 3-(3-amino-3-carboxypropyl)uridine provide side chains capable of metal chelation. Modified RNA active sites that are protected from nonspecific attack and that extend their metal binding sites away from the RNA itself may be capable of catalyzing extremely vigorous chemical transformations. This protection may help to explain the large clusters of base modifications within the phylogenetically conserved interior of many functional RNAs (*81*), particularly in thermophilic organisms (*83*).

Ribozyme redox reactivity may explain the results of a 1967 report by Sakai, who observed that a ribonucleoprotein from sea-urchin egg catalyzed a disulfide exchange reaction (*84*). With great foresight, he conducted careful controls to show that activity was dependent on both RNA and protein components of the reaction. The citation of this finding as an example of ribozyme redox chemistry may seem far-fetched, and at best the RNA subunit may serve only a structural role in the reaction. However, this example underscores the possibility of isolating redox-active metalloribozymes or creating them through in vitro evolution.

Directed Molecular Evolution of New Metalloribozyme Activities

Although the isolation of RNA molecules with unusual catalytic properties has been productive, we can now speed up the process of discovery by creating new ribozymes through in vitro selection (*23, 85*). Because RNA molecules possess both function and information content, we can select and amplify molecules with particular properties of interest. In particular, we can create new ribozymes with the ability to direct the activity of unusual metal ions. The careful choice of in vitro selection conditions and of metal ions may lead to the generation of RNA molecules with completely novel forms of chemical reactivity. It may be possible to generate ribozymes with enzymatic capabilities unknown even for protein enzymes.

Two examples now exist of ribozymes that were selected for novel catalytic metal ion specificities. Although catalysis by the wild-type *Tetrahymena* ribozyme is almost undetectable in Ca^{2+} (and any activity observed may be attributable to contaminating Mg^{2+}), variants have been selected that function in the presence of Ca^{2+} (*86*). After eight rounds of selection, amplification, and mutation of a sequence-randomized pool, Lehman and Joyce selected ribozyme variants with a high level of Ca^{2+}-mediated activity. This result shows that ribozymes can be created that can stimulate catalysis using metals not normally favored for a particular reaction.

Conversely, can new ribozymes be selected that specifically direct the chemistry of highly reactive metal ions without self-destructing? This will be essential for the evolution of ribozymes with new types of chemical reactivity. For example, Pan and Uhlenbeck have used in vitro selection to produce a series of lead-dependent ribozymes that efficiently cleave RNA substrates with multiple turnover (*19, 37*). Although the cleavage of RNA by Pb^{2+} is rapid at neutral pH, lead-dependent ribozymes bind Pb^{2+} and direct its reactivity to specific spots on other RNA molecules (*36*). A particular variant of the "leadzyme" promotes RNA cleavage through m_A but then catalyzes an additional step (*19*). Unlike other ribozymes that promote the formation of 2′-3′-cyclic phosphate termini, this tiny ribozyme goes further, catalyzing hydrolysis of the cyclic phosphate to a 3′-terminal phosphate. By harnessing the chemistry of a metal center highly reactive toward phosphodiester hydrolysis, the leadzyme is both efficient at catalyzing a specific target reaction and capable of performing a second, unanticipated reaction. Perhaps in vitro selection and RNA modification can be combined to generate RNA enzymes that can bind and direct redox chemistry with metals such as iron, manganese, or copper. The expansion of RNA chemistry into new fields and new reaction centers will depend on the creative application of the principles of bioinorganic chemistry.

Conclusion

The title of this article defines ribozymes as a distinct class of metalloenzymes. However, we must consider whether this is an artificial distinction. Although the compositions of RNA and of proteins are different, there are many similarities in their methods of promoting catalysis. Both types of en-

zymes commonly recruit specific metal ions for structural stabilization and both use metals to catalyze similar reactions. With the discovery of new ribozyme reactions, the in vitro selection of novel ribozymes, and the extension of RNA catalysis by the use of unusual metals and modified nucleotides, we may see direct parallels between the chemistry of RNA and protein enzymes in all classes of reaction mechanism.

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