Mechanisms of Ribozyme-Mediated RNA Cleavage

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Contents

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I. Introduction

Ribozymes are RNA molecules which adopt three-dimensional structures that allow them to catalyze chemical reactions, usually with the participation of one or more metal ions. As such, they are generally regarded as a distinct class of metalloenzymes.[1] A key feature of ribozymes, or catalytic RNA, and the observation that revolutionized our understanding of biological chemistry, is the fact that proteins are not involved in the key chemical process.[2,3] Formerly, biochemical dogma dictated that proteins performed all biological chemical reactions, and nucleic acids merely carried the genetic information that encoded these proteins. Now, however, we know that nucleic acids are capable of a broad repertoire of chemical transformations despite their relative lack of chemical functional groups. The most biologically relevant function of ribozymes is the processing of RNA, which includes activities of self-cleavage and splicing. The concept of RNA as a catalyst raises the intriguing possibility of an "RNA world", one where RNA enzymes predated protein enzymes and catalyzed all biochemical reactions, ultimately including peptide bond formation.[4] In fact, in vitro selection techniques have "evolved" new ribozymes with unique abilities.[5−7] Thus, it is seems plausible that ribozymes preceded protein enzymes in an evolutionary sense. Although ribozymes are capable of performing a variety of reactions, the focus of this paper will be the mechanism(s) of ribozyme-mediated RNA cleavage. Emphasis will be given to biologically derived ribozymes.

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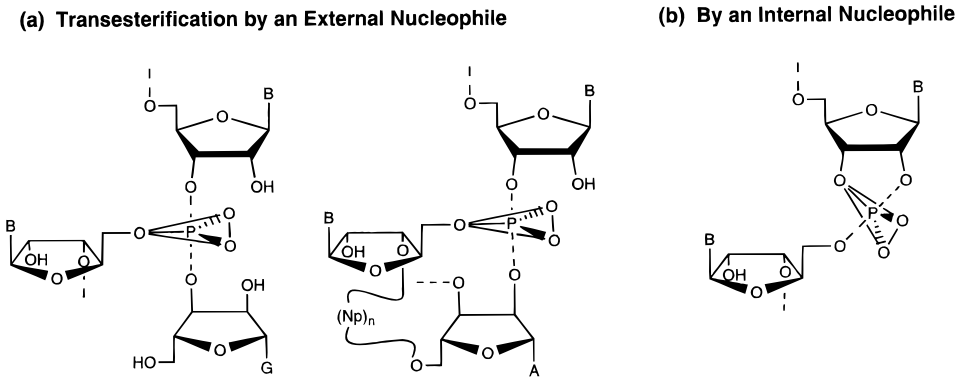


Figure 1. Possible transition state structures for (a) group I (left) and group II (right) introns, and for (b) hammerhead or hairpin ribozymes.

Since the initial landmark discovery of RNA with self-cleavage activity nearly 15 years ago,[8−10] several additional classes of ribozymes have been identified which are categorized on the basis of the reaction pathway or mechanism. Currently recognized groups are the group I intron, the group II intron, the RNA subunit of ribonuclease P, the hammerhead ribozyme, the hairpin ribozyme, and the hepatitis delta virus (HDV) ribozyme. The group I and II intron ribozymes and the ribonuclease P ribozyme are relatively large ribozymes (hundreds of nucleotides). In many cases shortened forms or other smaller constructs retain high activity. The hammerhead, hairpin, and HDV ribozymes are substantially smaller (less than one hundred nucleotides). The shortened form of the *Tetrahymena* group I intron is the mechanistically best characterized of the larger ribozymes. Among the small ribozymes, the hammerhead is the best characterized and has been subjected to the most intense scrutiny, which has resulted in several X-ray structures.[11−13,92]

The formal mechanisms by which RNA cleavage occurs remain elusive although they can be categorized into two general classes based upon the nature of the products obtained. In most cases the reactions proceed as transesterifications with the attacking nucleophile being either a 2′-hydroxyl or a 3′-hydroxyl (Figure 1a). With the hammerhead,[14] hairpin,[15] and hepatitis delta[16] ribozymes, the 2′-hydroxyl adjacent to the scissile phosphodiester is employed as an internal nucleophile (Figure 1b), while the group I introns employ an external guanosine cofactor (Figure 1a).[10] The group II introns employ an external-like mechanism (Figure 1a), although the 2′-hydroxyl recruited as a nucleophile is located outside of the catalytic pocket,[17,18] it is technically part of the intronic sequence. In these latter cases, both the group I[19] and group II introns[20,21] as with the RNase P ribozyme,[22] a water molecule can be employed as the attacking nucleophile such that cleavage occurs as a hydrolytic event rather than a transesterification. The simple illustrations of Figure 1 do not take into account the role of metal cofactors, or the involvement of general acid/base catalysis.

II. The Involvement of Metal Ions

All known catalytic RNAs have an absolute requirement for divalent metal ions, usually Mg^{2+}, but similar metals can often be substituted.[23,24] Interpretation of ribozyme metal ion requirements is more complicated than for proteinaceous enzymes because RNA, unlike most proteins, requires divalent cations for the formation of its three-dimensional structure that is so important for catalysis. It is well-established that metal cations bind to specific sites of an RNA sequence to stabilize specific structures. X-ray crystallographic analyses of tRNA reveal that Mg^{2+} can stabilize sharp turns of the polyanionic backbone, stabilize hairpin loops, and link two single-stranded regions. Yeast tRNA has at least four strong, site-specific Mg^{2+} binding sites and many more weak binding sites.[25−29] Since metal ions are so critical in determining structure, and since structure determines function, the mere requirement of a divalent metal for ribozyme activity does not prove its direct involvement in the chemical cleavage mechanism. However, since the discovery of catalytic RNA and the identification of the metal ion requirement, it has been proposed, through lessons learned from protein enzymes, that metal ions could serve to deprotonate a nucleophile, activate an electrophile, stabilize a transition state, or protonate a leaving group. In fact, and not surprisingly, catalytic RNAs utilize metals both for proper folding and for active-site chemistry. Some metal ions can perform both functions, while others can either promote proper folding or only participate in active-site chemistry.

Those that participate in the chemistry can do so in a number of roles. Perhaps the simplest role for the metal is one of a general base in which the conjugate base of the metal hydrate functions to deprotonate the active-site hydroxyl (illustrated for an adjacent 2′-OH, Figure 2a). The pK_a values of the magnesium hexahydrate and manganese hexahydrate have been estimated to be 11.4 and 10.6, respectively.[30] Alternatively, the metal could coordinate to the active-site nucleophile (e.g., the 2′-oxygen, Figure 2b) and thereby reduce the pK_a of the hydroxyl, permitting proton dissociation and attack by the metal-stabilized oxyanion. Transesterification reactions at phosphorus proceed through a pentacoordinate transition state or intermediate, and the metal could stabilize the developing negative charge in the pentacoordinate species (Figure 2c). As the transition state or intermediate collapses, with breakage of the P−O bond, negative charge will begin to

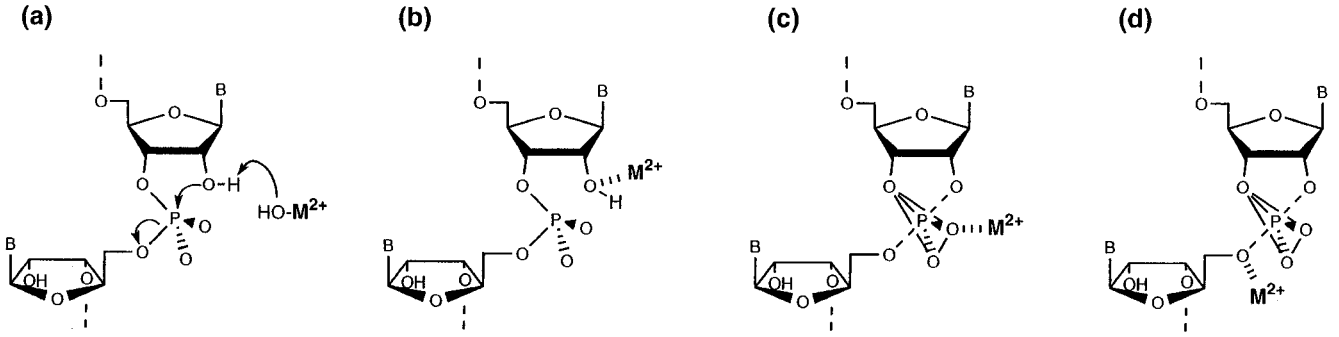


Figure 2. Possible roles for metal-catalyzed transesterifications or hydrolysis: (a) metal hydroxide as a general base, (b) metal stabilization of hydroxyl (or water), (c) metal stabilization of pentacoordinate transition state/intermediate, and/or (d) metal stabilization of leaving group.

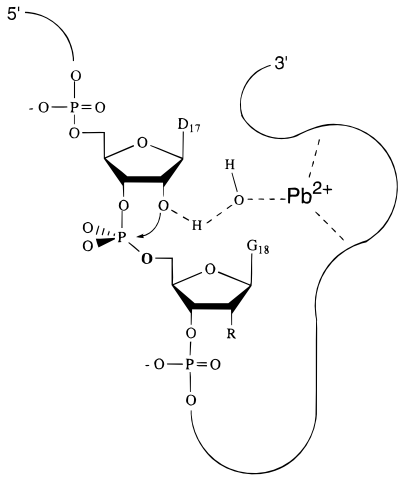


Figure 3. Possible transition state structure for the lead-based cleavage of $tRNA^{Phe}$ between dihydrouridine (D_{17}) and guanosine (G_{18}).[32,33]

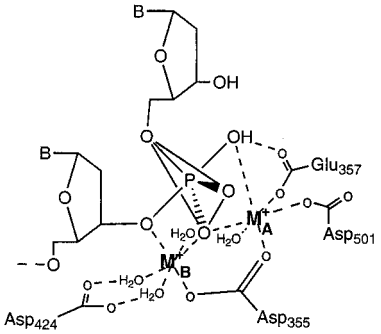
build on the leaving group oxygen and the metal can function to stabilize the leaving group anion (illustrated for breakage of the P−O5′ bond, Figure 2d). Additionally, a metal could participate in more than one of these roles, and likewise, more than one metal could be present, involved in multiple types of interactions.

It is worthwhile to consider the role of the metal ion in the nonphysiological, site-specific, Pb^{2+}-mediated cleavage of tRNA.[31−34] Although technically not a catalytic RNA, this reaction is structurally well-characterized through X-ray crystallography and biophysical studies and illustrates one way in which an RNA can utilize a metal ion to facilitate cleavage. Pb^{2+} ions catalyze the cleavage of $tRNA^{Phe}$ between residues D17 and G18 at pH 7.4 to give 2′,3′-cyclic phosphate and 5′-hydroxyl fragments. The Pb^{2+} ion is bound to O^4 of the base U59 and N^3 of the base C60, displacing a nearby Mg^{2+} in the native structure. The bound Pb^{2+} ion is located in the T loop, which is far from the cleavage site in the primary structure, but very close in the folded tertiary structure. It is proposed that a water molecule coordinated to the specifically bound Pb^{2+} ion (with a pK_a of 7.7) abstracts the proton from the 2′-OH group of D17 to generate a nucleophilic alkoxide (Figure 3).[32,33] The alkoxide then attacks the adjacent phosphodiester and cleaves the strand. Critical factors include flexibility near the cleavage site, positioning of the Pb^{2+} ion such that a coordinated water molecule is in a favorable orientation and distance to abstract the proton from the 2′-OH of D17, and the fact that the pK_a of the Pb^{2+}-bound water molecule is near neutrality.[32,33] The key point here is that the tertiary structure of the tRNA enables a metal ion to bind and to promote cleavage by functioning as a general base.

Biologically relevant protein enzymes also employ metal ions to cleave phosphodiester bonds. X-ray crystallography has revealed that the 3′,5′-exonuclease domain of DNA polymerase I uses two catalytic metals at the active site.[35,36] Protein residues bind and correctly orient the metal ions for catalysis; no protein side chain is implicated in the actual cleavage chemistry (see Figure 4a). In this case the first metal, metal A, facilitates formation of the attacking OH^- with its lone pair of electrons oriented toward the phosphorus atom and situated for in-line attack. Metal B is hypothesized to assist departure of the 3′-leaving group by acting as a Lewis acid and also by interacting with the nonbridging oxygen to stabilize the transition state. Other protein enzymes that are thought to use divalent ions to facilitate phosphoryl-transfer reactions include alkaline phosphatase (see Figure 4b),[37] the RNAse H domain of HIV reverse transcriptase,[38] P1 nuclease,[39] and phospholipase.[40] For many of these enzymes, the cleavage mechanism does not depend on the chemical properties of the protein side chains. Thus, with the notion that the total number of different mechanisms by which proteins achieve catalysis is quite small compared to the number of enzymes that catalyze a particular reaction type; it has been proposed that catalytic RNAs could use a similar "two-metal" mechanism.[41]

Yarus has outlined a number of distinguishable ways in which metal ions could participate in RNA catalysis: coordination, strain, electrophilic catalysis, general acid/base catalysis, and stabilization of an anionic leaving or attacking group.[42] Coordination can help to shield negative charges and allow folding that would otherwise be excluded by electrostatic repulsion, thereby specifying structure. Coordination can also provide a substantial ordering effect by bringing reactants into apposition and providing an entropic contribution to catalysis, which is potentially one of the largest contributors to protein catalysis.[43] Metal cations, being centers of positive charge, can

(a) *E. coli* DNA Pol. I



(b) *E. coli* Alkaline Phosphatase

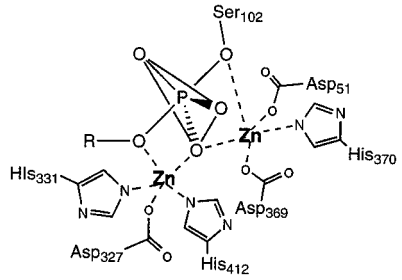


Figure 4. Proposed transition-state structures for protein-based phosphoryl transfer reactions as exemplified by (a) the exonuclease site of *E. coli* DNA polymerase I and (b) the active site of *E. coli* alkaline phosphatase.

induce strain at the active site, comprising a negative phosphate oxygen. The tetrahedral O−P−O bond angle is 109°, which must reduce to 90° in the pentavalent transition state, and this requires a translocation of negative charge. The metal ion could facilitate this movement and alter the bond angle. The 106-fold greater hydrolysis rate of methylethylene phosphate compared to trimethyl phosphate has been ascribed solely to a reduction in the O−P−O bond angle from 109° to 99°.[44,45] In an electrophilic mode of catalysis, the metal cation can coordinate to a nonbridging phosphate oxygen and polarize the phosphorus atom, rendering it more susceptible to nucleophilic attack, although this is expected to make a small contribution.[46] Except for terminal phosphate moieties, nucleic acids have no functionalities that are ionizable near neutrality, and so they cannot, by themselves, exploit acid/base catalysis.[47] However, water molecules bound to certain divalent cations do ionize at physiological pH,[30] and so these can be recruited as proton acceptors or donors. Metal cations could also function as Lewis acids to stabilize an anionic leaving or attacking group. In this case there is direct interaction between the metal and the electron-rich group. Since the negative charge is substantially formed in the transition state, coordination to a metal ion should stabilize the transition state and accelerate the reaction. Bashkin and Jenkins have also described some of the possible roles of metals in the nonbiological, hydrolytic cleavage of nucleic acids.[48]

III. Internal Transesterification

The reaction pathway of internal transesterification involves an adjacent 2′-OH group. The nucleophilic 2′-OH group attacks the phosphorus center to produce a pentacoordinate transition state or intermediate. The pentacoordinate transition state breaks down to yield two fragments: a 2′,3′-cyclic phosphate product and a 5′-hydroxyl product. This is the same general pathway that exists for the nonenzymatic, random cleavage of RNA linkages in alkali solutions and/or in the presence of metal ions. In this scheme several opportunities exist for acid/base catalysis. First, a base can deprotonate the 2′-OH to generate a more nucleophilic alkoxy species. Also, a metal ion could coordinate to the nonbridging phosphate oxygen to render the phosphorus atom more electrophilic and more susceptible to attack. A metal coordinated to the nonbridging phosphate oxygen could also help stabilize the pentacoordinate transition state. An acid catalyst could help to neutralize the developing charge on the 5′-leaving group, thus lowering the energy of the transition state. In all of these scenarios single or multiple divalent metal ions, or a water molecule coordinated to a metal ion (a hydrated metal ion), could fill these roles (Figure 5).

Any RNA linkage is susceptible to metal-catalyzed cleavage, especially by metal ions whose attached water molecules have a low pK_a, such as Pb^{2+} and Eu^{3+}.[31,49,50] In highly structured RNAs, such as transfer RNAs, certain sites are especially prone to cleavage.[51] The folded structures of these RNAs contain metal binding pockets that position the metal ions to facilitate cleavage of a specific phosphodiester bond. Although not necessarily biologically relevant, the Pb^{2+}-mediated site-specific cleavage of tRNA (described above) is a good example of metal-mediated cleavage of RNA by the above-described pathway.

A. Hammerhead Ribozyme

The hammerhead, so-called because of its secondary structure, is the best characterized ribozyme, thanks in part to its small size and interest in its potential therapeutic application. Several general reviews are available.[52−56] It is derived from a structural motif present in the RNA genomes of several plant pathogens, where it is believed that RNA-mediated cleavage events are an essential step in the viroid's replication pathway.[57−59] The hammerhead complex consists of 3 helical stems and

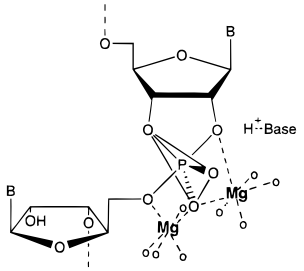


Figure 5. Possible transition-state structure for the hammerhead ribozyme showing two possible roles for the metal cofactors.

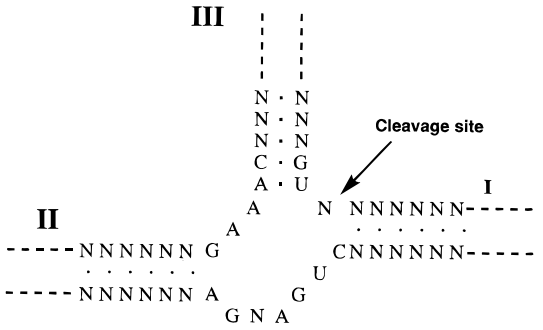


Figure 6. Consensus sequence for the hammerhead ribozyme illustrated the three helical stem regions (I, II, and III) and the conserved, nominally single-stranded core sequence.

includes 11 consensus nucleotides that form the catalytically active core (Figure 6).[14,57,60] Nine of the 11 conserved nucleotides are nominally single-stranded. The hammerhead ribozyme is capable of cleaving *in cis* or *in trans*.[61] The *in trans* construct allows for multiple turnover and enables virtually any sequence to be targeted for cleavage. Classical sequence mutagenesis was used to identify the impact of nucleotide substitutions in the catalytic core.[60] With the exception of U7, all substitutions led to a dramatic decrease in cleavage activity. Substitutions at G5 and A14 were especially detrimental and totally destroyed the cleavage activity.[62] Nucleotide analogue substitutions have identified a number of functional groups that are critical for catalysis, and these experiments are summarized in recent reviews.[62,63]

The hammerhead ribozyme has an absolute requirement for divalent metal ions.[14] Mg^{2+} is the preferred metal, but Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} also support cleavage activity.[64] Standard reaction conditions include 10 mM metal, pH 7.5, at temperatures between 27 and 50 °C. Under these conditions chemical cleavage rates of 1 min^{-1} are typical. In some cases additives that stabilize higher order nucleic acid structures, for example the polycations spermine or spermidine, or sodium ions, can minimize the divalent metal ion requirement, but in all cases the divalent metal is still needed for full activity.[64] These observations imply that the metal may serve a structural as well as a catalytic role. However, initial NMR experiments did not indicate a dramatic structural change upon addition of the divalent metal, at least not in the ground state.[65] A second study suggests that magnesium is required to form the active complex—even before the transition state is reached.[66] Fluorescent nucleotides have also been used to examine the structural changes upon addition of divalent metals.[67] Selected polycationic antibiotics inhibit the hammerhead cleavage reaction.[68,69] The mode of inhibition is thought to be ionic, possibly involving the displacement of the metal cofactor.[70]

As mentioned, hammerhead-mediated cleavage yields fragments that contain a 2′,3′-cyclic phosphate and a 5′-hydroxyl. Experiments employing phosphorothioate diesters at the cleavage site (Figure 7 a,b) have demonstrated that the cleavage reaction proceeds with inversion of configuration at the phosphorus center via an in-line, S_N2-type mechanism.[71] This was proved by incorporating an R_p phosphorothioate at the cleavage site, inducing the ribozyme reaction, and then directly analyzing the stereochemistry of the terminal 3′-nucleotide. The presence of the sulfur atom in the 2′,3′-cyclic phosphate makes two stereoisomers possible, *endo* and *exo*, which are chromatographically distinguishable. The observation that only the *endo* isomer is formed proves that the reaction proceeds with inversion of configuration and strongly suggests an in-line S_N2-type mechanism.[71]

Phosphorothioates have been used not only to define the stereochemical course of hammerhead-mediated cleavage, but also to probe for important phosphate interactions. Introduction of R_p phosphorothioates can be accomplished by transcription using T7 RNA polymerase and an appropriate S_p-α-thiotriphosphate.[72] Unfortunately, however, enzymatic incorporation of the corresponding S_p isomer is not

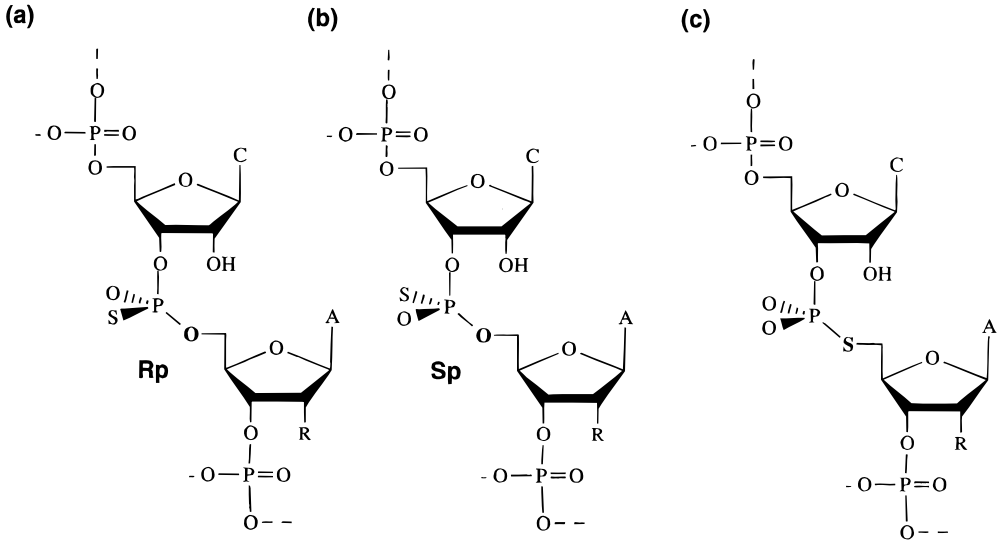


Figure 7. Internucleotide phosphodiester analogue linkages in which one of the nonbridging oxygens has been replaced by sulfur, generating either the R_p (a) or S_p (b) phosphorothioate diastereomer. Replacement of the 5′-bridging oxygen with sulfur generates the phosphorothiolate derivative (c).

possible because the R_p-α-thiotriphosphates are not substrates for the polymerase. Nonetheless, phosphorothioate interference experiments have located a number of critical phosphate groups (that is, *pro*-R_p oxygens) in the central core.[64,73−77] An especially important phosphate group that has been identified by these interference experiments is that of the cleavage site itself, as revealed by a dramatic reduction in the cleavage rate upon introduction of an R_p phosphorothioate.[64,73−75] Just as interesting, however, is the fact that cleavage activity can be substantially restored by replacing the Mg^{2+} with Mn^{2+}. These observations suggest that, in the transition state, the metal cofactor interacts with the *pro*-R_p oxygen at the cleavage site. The metal specificity is expected when one considers the relative affinity of oxygen for Mg^{2+} or Mn^{2+}, compared to the relative affinity of sulfur for the same cations. Oxygen binds Mg^{2+} much more strongly than Mn^{2+}, whereas sulfur binds the two metals more equally.[78,79] Therefore, "rescue" of the cleavage activity upon switching to Mn^{2+} suggests a direct interaction between the metal ion and the *pro*-*R* oxygen at the cleavage site. In contrast, an S_p phosphorothioate incorporated at the cleavage site does not significantly alter the hammerhead cleavage rate and little preference is seen for Mn^{2+} vs Mg^{2+}.[74] These results provided the first real indications of how the essential metal cofactor lowers the transition-state energy. A recent reevaluation of these results suggests that a metal ion does not interact with the *pro*-R_p oxygen.[73] Nevertheless, a subsequent X-ray structure of a freeze-trapped intermediate clearly shows that a metal ion does in fact bind to the *pro*-R_p oxygen and this intermediate may represent a step toward the transition state.[13]

The hammerhead ribozyme's response to changes in pH provides additional clues about the cleavage mechanism. The log of the cleavage rate increases linearly with pH between pH 5.7 and 8.9 to give a slope of unity.[14,80] This observation indicates that a single deprotonation is required for cleavage. Moreover, pH rate profiles with a series of divalent metals correlate with the metal's pK_a values, which suggests that a metal hydroxide bound to the hammerhead acts as the base in the cleavage reaction.[80] There is good precedent for this scenario in the nonphysiological and well-characterized cleavage of tRNA by Pb^{2+} ions. Thus, we would seem have experimental evidence of a second role for the essential metal cofactor, that of a general base. Solvent isotope effect experiments carried out on the hammerhead ribozyme reveal a substantial isotope effect ($k_{H_2O}/k_{D_2O} \approx 2-4$).[81,82] On the surface this would appear to demonstrate that a proton transfer does indeed occur in the transition state. However, it has been argued that the observed isotope effect can be attributed solely to a reduction in the concentration of the metal hydroxide species as a result of using D_2O as solvent rather than H_2O, which alters the pK_a of the metal hydroxide.[82] It was further argued that the observed isotope effect actually proves the nonexistence of a proton-transfer step, after a correction is applied to account for the pK_a difference. In this scenario, one that does not involve a proton transfer, a Lewis acid can be invoked instead.[82]

Some proteinaceous phosphotransfer enzymes utilize a "double-metal" strategy to stabilize the transition state of their reaction mechanism.[36] It has been proposed that various catalytic RNA could employ a similar strategy.[41] In this scenario, one metal would deprotonate the 2′-hydroxyl group at the cleavage site and/or bind to the *pro*-R_p oxygen of the scissile phosphodiester while a second metal would interact with the 5′-oxygen as a Lewis acid to help neutralize the developing negative charge. Gas-phase ab initio calculations on model compounds have shown that this idea is theoretically plausible in the case of the hammerhead ribozyme.[83−85] To experimentally test the notion of metal interaction at the 5′-leaving group, two different groups prepared substrates in which the 5′-oxygen at the cleavage site was replaced with a sulfur atom (Figure 7c), thus creating a bridging phosphorothioate.[81,86−88] Examination of the cleavage kinetics as a function of metal ion type should reveal possible metal ion interactions at the 5′-leaving group, in analogy with the nonbridging phosphorothioate experiments described above. In one set of experiments, a mostly DNA substrate was used under standard reaction conditions (pH 7.5, 10 mM metal). This system did not reveal a metal ion preference or a dramatic change in the cleavage rate compared to the rate of cleavage of the control phosphodiester sequence (reflecting the so-called thio effect). These findings imply that a metal ion does not interact with the 5′-leaving group, and moreover, that the departure of the 5′-leaving group is not the rate-limiting step of hammerhead ribozyme-mediated RNA cleavage.[81,87] In a second set of experiments performed by a different group, an all-RNA substrate was used under nonstandard reaction conditions (pH 6.0, 0.3 mM metal). The results at this pH value were largely the same as those obtained for the mostly DNA substrate (at pH 6), except a greater difference was observed between the phosphorothioate and control (phosphodiester) substrates.[88] This difference can be ascribed to the nonstandard reaction conditions that were employed in the second study, since pH rate profiles reveal that the difference is much greater at low pH, where the curve becomes nonlinear.[89] However, this rate differential has been taken by some as evidence for rate-limiting departure of the 5′-leaving group.[88] Pontius et al. provide a somewhat different interpretation of these data.[90]

These differences in mechanistic interpreations with this type of sulfur-containing analogue reflect, in part, the observation that the general trend in results was unanticipated. The hallmarks of metal involvement at the site in question are (i) a significant thio effect (defined as the ratio of cleavage rates for the oxo and thio substrates); Cech's study of the group I ribozyme (see below) reported a 1000-fold difference[91] in rates (O ≫ S) for the native and analogue substrates under standard conditions (Mg^{2+} affinity for sulfur is about 30000-fold less than for oxygen), and (ii) metal rescue when a more thiophillic metal is added (e.g., Mn^{2+}). Cech's data for the group I ribozyme shows almost complete thio rescue with

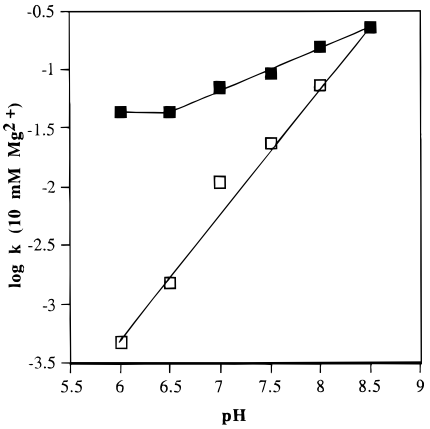


Figure 8. Sample pH vs rate profiles for the cleavage reaction of the hammerhead RNA (10 mM Mg^{2+}) in which the scissile phosphodiester contains either a native P−O bond (□) or an analogue P−S bond (■).

Mn^{2+}. The hammerhead ribozyme exhibits neither of these effects.[86,88] As pH decreases, the rates for cleavage of the thio and oxo substrate in the presence of Mg^{2+} diverge (Figure 8). While at higher pH there is little difference in the two rates (O vs S), as the pH decreases the cleavage rate for the thio substrate is actually faster (in the presence of Mg^{2+}) —the group I ribozyme study suggests cleavage of the thio substrate would be slower, since the Mg^{2+} should complex more poorly with the leaving group thiolate than with the corresponding oxylate. No metal specificity has been observed (in either study); the presence of the more thiophillic Mn^{2+} did not enhance the rate of cleavage, as might be expected. In principle, a mechanism involving coordination to the leaving group anion (as Cech reported for the group I intron) should exhibit an enhanced cleavage rate with the "softer" metal.

A significant milestone in our understanding of the three-dimensional ground-state structure of the hammerhead ribozyme was reached with the determination of two X-ray structures.[11,92] These structures represent the first detailed, three-dimensional pictures of a catalytic RNA molecule. In the first structure, a DNA "inhibitor" substrate was complexed with a *trans*-acting hammerhead.[11] A second structure soon followed that employed an all-RNA substrate with a noncleavable 2′-OMe nucleotide at the cleavage site (Figure 9).[92] Despite the substrate differences (DNA vs RNA) the two structures are remarkably similar and are virtually superimposable. The X-ray analyses reveal a Y-shaped tertiary structure with stems I and II forming the Y and stem III forming the base. Stems II and III are nearly collinear with almost continuous base stacking. Various solution-phase experiments have pointed to a similar overall structure.[65,93,94] The C3-U4-G5-A6 sequence forms a sharp "uridine turn" at the base of stem I, a common motif in tRNA structures. The catalytic core also contains a tandem GA mismatch as well as non-Watson−Crick base interactions. Thus the picture of the catalytic core is one of a tightly folded structure. The X-ray structures also reveal up to six bound metal ions, one of which is positioned near the active site, within striking distance of the scissile phosphodiester, but, unfortunately, not arranged in a manner which would allow for inline attack on the phosphorus center by the 2′-OH group.[92] On the basis of their crystal structure, Scott et al. have proposed a reaction mechanism that involves a single Mg^{2+} atom and a conformational change at the active-site (to allow for inline attack by the 2′-OH on the scissile phosphodiester).[92]

Figure 9. Crystallographically derived structure[92] of the hammerhead ribozyme with an RNA substrate containing an inhibitory 2′-OCH_3 group. The sites for a number of potential metal binding sites are also shown.

Scott et al. have produced a third hammerhead ribozyme crystal structure, one that contains no modified residues.[13] This native structure is identical in almost all respects to the previous two structures that contained either a deoxyribonucleotide substrate or a substrate with a 2′-OMe residue at the cleavage site, both of which had been introduced to prevent self-cleavage during crystallization. Agreement between the three structures shows that the modifications to the substrate (DNA or 2′-OMe) do not significantly alter the RNA fold of the ribozyme's ground state. Scott et al. also demonstrated that crystals grown in the absence of divalent metals could undergo cleavage when metal solutions were soaked into the crystals, showing that the crystalline form is indeed an active form. Moreover, it was possible to use time-resolved crystallography to capture the structure of the actual intermediate species. These experiments revealed a conformational intermediate with an additional Mg^{2+} bound to the scissile phosphate. Upon addition of the metal, major conformational changes were restricted to the active site of the ribozyme. The new metal ion in the intermediate is bound to the *pro-R* oxygen of the scissile phosphodiester, in perfect agreement with earlier phosphorothioate experiments. On the basis of these data, Scott et al. have proposed several variations of a reaction mechanism, favoring one whereby a Mg^{2+}

ion binds to the *pro-R* oxygen and subsequently induces a conformational change at the active site to allow inline attack.[13] The same metal that binds to the *pro-R* oxygen is envisioned to provide the hydroxide that initiates the base-catalyzed step of the cleavage reaction.

In a novel contribution, Westhof et al. have used molecular dynamics simulations of the freeze-trapped hammerhead ribozyme intermediate to suggest that the active site metal (site 6), and an adjacent metal ion (site 1) is actually a μ-hydroxo-bridged magnesium dimer.[95] This intriguing finding provides a new insight into the possible role of the metal ion. The molecular dynamics simulations show that at least one bridging OH^- is required to stabilize the two Mg^{2+} ions (sites 1 and 6 in the crystal structure) and moderate their electrostatic repulsion. Water molecules placed between the two Mg^{2+} ions caused expulsion of the site 1 ion during the simulation, which is the most tightly bound metal in the X-ray structure. Such a μ-hydroxo bridged dimer would be superior to a single Mg^{2+} in stabilizing a high local concentration of OH^- in the area of the active-site. It was proposed that after trapping an OH^- between the two Mg^{2+} ions, a simple conformational flip from C3′-*endo* to C2′-*endo* pucker at the cleavage site moves the attacking 2′-hydroxyl near the hydration sphere of the Mg^{2+} bound to the scissile *pro-R* phosphate oxygen. The μ-bridging OH^- between the two metal ions then abstracts a proton from a metal-bound water molecule, which activates the 2′-hydroxyl group for attack on phosphorus. Proton transfer from a metal-bound water molecule to the 5′-leaving group could also facilitate cleavage, and additional proton shuffling within the hydration sphere of the metal dimer would regenerate its initial state.[95]

B. Hairpin Ribozyme

As with the hammerhead ribozyme, the hairpin ribozyme can function both *in cis* and *in trans*.[96] The hairpin function is derived from the negative-polarity strand satellite RNA associated with tobacco ringspot virus.[15,97−99] It performs specific cleavage and ligation reactions required for minus strand replication. The corresponding plus strand replication utilizes the hammerhead motif. As such, these two ribozymes perform in concert to carry out a rolling circle replication strategy.[100] Although the hammerhead and hairpin ribozymes perform similar functions, they do not share a common structural motif. The shortened form of the hairpin ribozyme (Figure 10a) is larger than the minimum hammerhead sequence (50 nucleotides vs ∼30 nucleotides). Moreover, the secondary structure bears little resemblance to the hammerhead. The hairpin contains four helical regions and two single-stranded loops. This prediction is based on minimum energy folding,[101] but has experimental support through mutagenesis experiments,[15,98,101,102] phylogenetic comparisons,[103,104] in vitro selection libraries,[105−108] and analogue substitutions.[109,110] Most of the nucleotides in loops A and B are critical for activity.

The hairpin ribozyme, like the hammerhead, effectively uses Mg^{2+}, Sr^{2+}, and Ca^{2+} metal ions. However, unlike the hammerhead, the hairpin ribozyme is inhibited by Mn^{2+} and Co^{2+} unless spermine is also present.[111] This relationship implies that the metals perform both a structural and a catalytic role; apparently the Mn^{2+} and Co^{2+} can fulfill the catalytic role, but not the structural one. In the case of Mg^{2+}, Sr^{2+}, and Ca^{2+}, the metal can both induce proper folding and also facilitate catalysis. The hairpin shares many of the preferred reaction conditions with the hammerhead ribozyme, i.e., 10 mM metal, pH 7.5, 27−50 °C, and the chemical cleavage rate is comparable (∼1 min^{-1}). However, in contrast to the hammerhead, the ligation rate is an order of magnitude greater than the cleavage rate.[112]

In addition to the differences noted above, the hairpin ribozyme exhibits two important additional differences that suggest a very different reaction mechanism than that used by the hammerhead ribozyme. First, the cleavage rate of the hairpin is

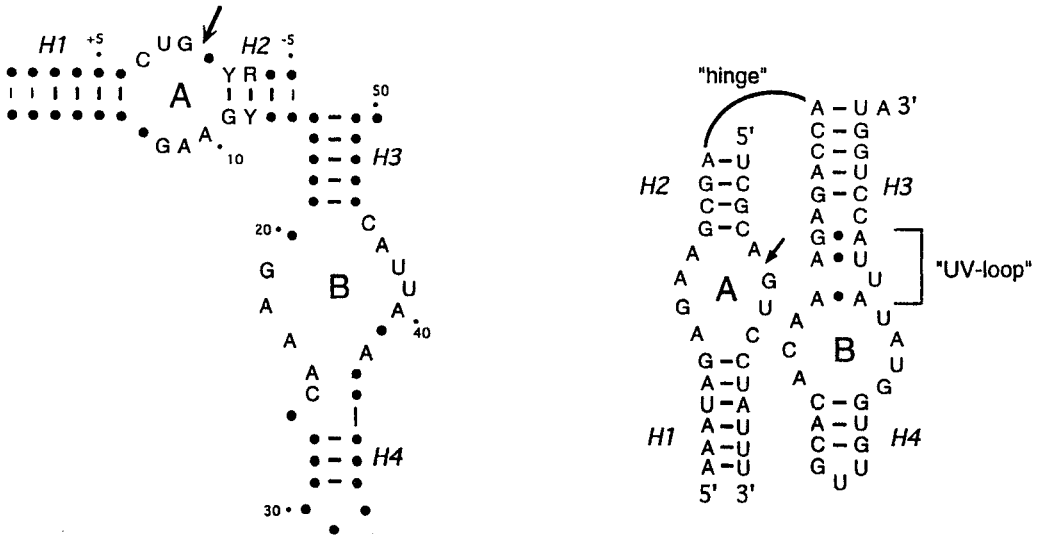


Figure 10. (a) Consensus sequence for the hairpin ribozyme and (b) suggested rate-determining conformational change preceding transesterification.[187]

unaffected by pH changes between pH 5.5 and 8.[15] This is in stark contrast to the hammerhead ribozyme, which has a linear response (slope of unity) in the same pH range.[80] This observation suggests a very different rate-limiting step for the hairpin ribozyme, at the very least excluding the abstraction of the 2′-OH as the rate-determining step. The second critical difference is the hairpin ribozyme's response to phosphorothioate substitution. The hairpin ribozyme effectively cleaves a phosphorothioate at the target site (both R_p and S_p isomers),[113−115] which is inconsistent with interaction between a metal ion and either nonbridging phosphate oxygen at the scissile phosphate.[71] Moreover, intriguing new data reveal that the hairpin ribozyme effectively catalyzes cleavage in the presence of EDTA and inert cobalt (III)complexes.[115] These results imply that metal ions do not directly participate in active-site chemistry of the hairpin and play only a passive role and are therefore required for structural purposes only.[114,115] In this respect, the hairpin ribozyme presents a mechanistically distinct class of catalytic RNA.

It has been suggested that a conformational change, one in which the two domains of the complex bend about a single stranded "hinge" region in order to generate the active complex (Figure 10b) in which the looped regions interact, is the rate-determining step.[116] Such a mechanism would be consistent with the reports that metal ions, although required for catalysis, play a more passive role[115,117] in the chemical events characterizing cleavage by the hairpin ribozyme. A guanosine amino group has previously been reported[118] to be implicated as a possible general base in the cleavage reactions, but a mechanistic pathway remains to be developed for this cleavage reaction.

C. HDV Ribozyme

The hepatitis delta virus (HDV) is a human pathogen possessing a circular RNA genome that contains a self-cleaving domain. The virus is thought to replicate via a double rolling−circle mechanism where both the positive and negative polarity strands promote self-cleavage.[119,120] Shortened forms of the HDV ribozyme are ∼85 nucleotides in length and, as with the other ribozymes described above, it is possible to design *trans*-acting constructs.[16,121−127] Several secondary-structure models have been proposed for the HDV ribozyme, and mutagenesis experiments have identified four helical regions.[16,121−123,125,128−133] Many of the conserved nucleotides are located in helix II and its hairpin. The structural motif resembles neither the hammerhead nor the hairpin ribozymes. Several secondary structure models have been proposed,[122,125,128,134] but the pseudoknot model is well-supported by mutagenesis experiments and chemical probing studies.[16,133,135−141]

As with the hairpin, the cleavage rate of the HDV ribozyme is unaffected by pH between pH 5 and 9.1.[142] The HDV ribozyme requires a divalent metal for activity and effectively uses both Ca^{2+} and Mg^{2+}. The metals Mn^{2+} and Sr^{2+} also support cleavage, and to a lesser extent Co^{2+}, Pb^{2+}, and Zn^{2+}.[142−145] Unlike the hairpin, *pro*-R_p phosphorothioate substitution at the cleavage site interferes with cleavage.[141] An apparently unique feature of the HDV ribozyme is its remarkable tolerance for denaturants and high temperatures. Full cleavage activity is maintained in the presence of 20 M formamide or 10 M urea, and at temperatures up to 80 °C, which speaks to the extremely stable secondary structure of this ribozyme.[129,146−148]

In the case of a *trans*-acting HDV ribozyme targeted against a 13-mer substrate,[149] the log of the cleavage rate increased linearly (with slope of ∼1) between pH 4.0 and 6.0 and showed a maximum cleavage rate at pH 7. Also, an R_p phosphorothioate was strongly inhibitory to this construct, but the S_p phosphorothioate had very little effect. Moreover, cleavage of the R_p phosphorothioate could not be rescued by replacing Mg^{2+} with the more thiophilic Mn^{2+} ion, indicating that the metal cofactor does not interact with the *pro*-*R* phosphate oxygen, but that the metal is important for the formation of the active complex consisting of the ribozyme and its substrate. Indeed, the order of addition of the reaction components was observed to have a significant influence on both the cleavage rate and the final amount of product that was produced, which essentially reflects the amount of active complex.[149]

IV. External Transesterification

By definition, external transesterification involves the recruitment of nucleophiles from outside the cleavage site. This is the strategy employed by all of the known "large" ribozymes, such as the group I and II introns and the RNA subunit of RNAse P. The relatively large size of these catalytic RNAs, in contrast to the much smaller internal transesterification ribozymes, is likely a function of the structural necessity of adopting a conformation that can bind and properly position the external nucleophile. In addition, these RNAs catalyze splicing function as well, so they are inherently more complex. In vivo, proteins or other RNAs generally help facilitate the cleavage, but these are not necessarily required. All of these ribozymes activate an exogenous nucleophile to facilitate cleavage, yielding a 3′-OH and a 5′-phosphate. Nucleophiles can include water or nucleotide hydroxy functionalities. The reaction pathway involves nucleophilic attack on phosphorus to generate a pentacoordinate transition state or intermediate. Displacement and protonation of the 3′-oxy species gives rise to a 3′-hydroxy fragment and a 5′-phosphate mono or diester. The mechanism of external transesterification is necessarily very different from that of internal transesterification. However, the opportunities for catalysis or transition state stabilization are similar. For instance, metal ions coordinating to a nonbridging oxygen of the scissile phosphate moiety can activate the phosphorus atom toward attack by increasing the electrophilicity of the phosphorus center. In addition, bases can help to deprotonate the nucleophile. In the transition state a coordinating metal ion can help to delocalize developing negative charge on the 3′-leaving group and Brønsted acids can protonate the

3′-oxyanion. Again, metal ions can help to stabilize the pentacoordinate transition state, and with all of these ribozymes, metal ions are essential.

A. Group I Intron Ribozyme

It is not uncommon for eukaryotic genes to be interrupted by stretches of noncoding DNA (introns). These noncoding regions are transcribed by RNA polymerase along with the coding DNA (exons) to give large precursor RNAs, which are later processed, or spliced, to give mature RNAs consisting of appropriately ligated exons. In many cases, the RNA is not only the substrate, but also the catalyst. It is these self-catalytic RNA molecules that we wish to examine. Ribozymes derived from group I introns generally consist of hundreds of nucleotides and adopt catalytically competent structures that are not yet well-defined at the molecular level. An example of one of the smallest group I ribozymes, the *Anabaena* ribozyme, is illustrated in Figure 11. At 241 nucleotides it is significantly smaller that the better understood *Tetrahymena* ribozyme, although the catalytic cores are thought to adopt similar structures.

The well-studied *Tetrahymena* ribozyme is derived from a self-splicing group I intron and is presented here as a prototypical example of this class. In vitro, these ribozymes self-splice by carrying out two consecutive transesterifications that result in excision of the intron and subsequent ligation of the flanking exon sequences.[23,150] We will be concerned here with the first step of the sequence of events, the cleavage reaction. The *Tetrahymena* ribozyme employs an external guanosine nucleoside or nucleotide cofactor

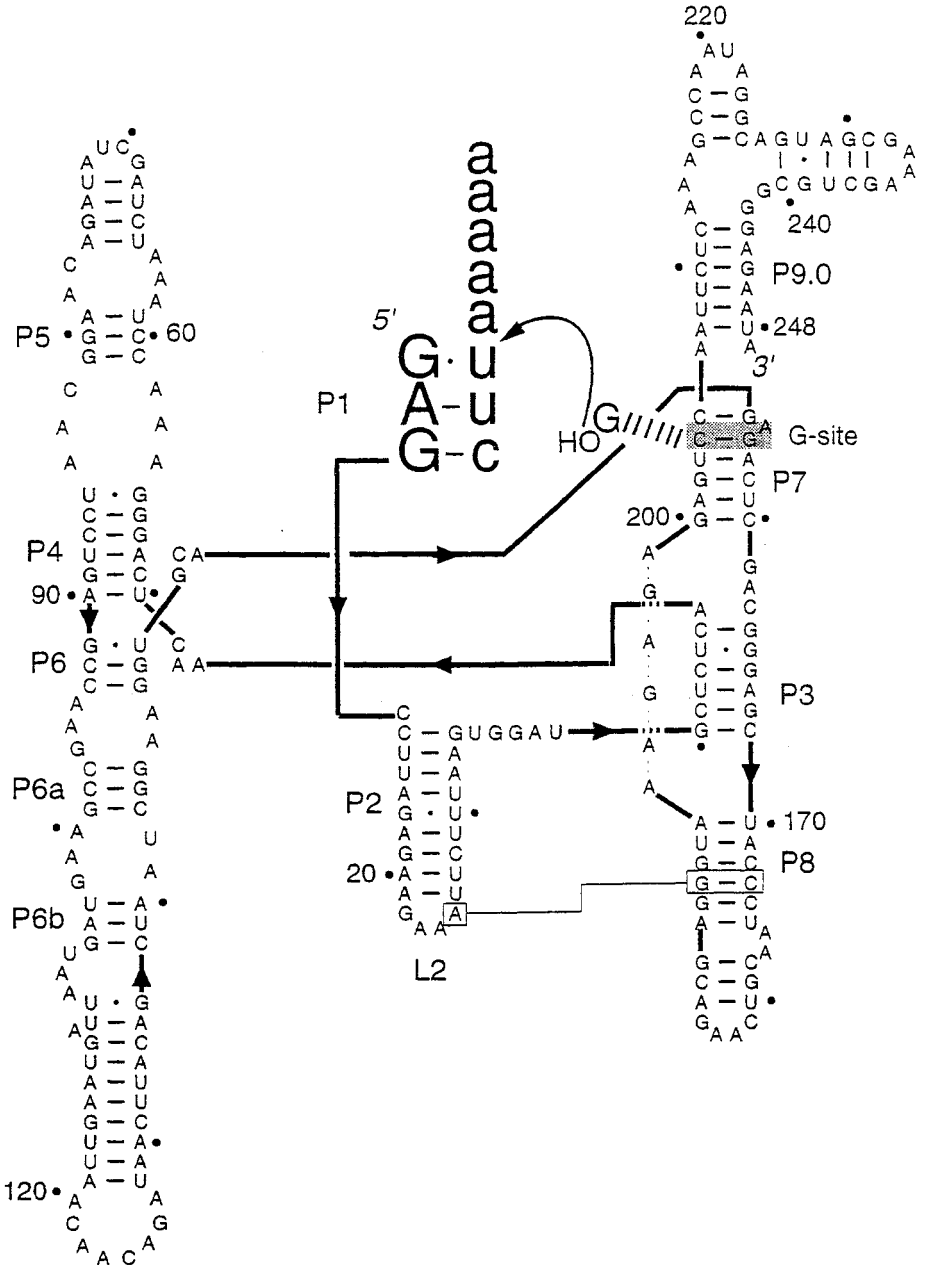


Figure 11. Secondary structure of the *Anabaena* ribozyme illustrating identified tertiary interactions, including the site for guanosine cofactor binding.[188]

as a nucleophile. The guanosine binds at a saturable binding site in the core of the ribozyme through at least three different interactions.[19,151−153] The 3′-hydroxyl of the guanosine acts as the nucleophile, which results in the extension of the intron sequence by one nucleotide after cleavage.[154,155] Water can replace guanosine to give a sequence-specific hydrolysis reaction.[19] The *Tetrahymena* ribozyme requires either Mg^{2+} or Mn^{2+} ions for activity.[156−158] Shortened forms of the ribozyme bind to and catalyze sequence-specific cleavage of both DNA and RNA oligonucleotides, and these processes have been well-characterized both kinetically and thermodynamically.[19,159−162] The substrate binds by base pairing to an internal guide sequence on the ribozyme and, in the case of RNA, also by interactions with specific 2′-hydroxyl groups.[162−164] Reaction conditions for shortened forms of the *Tetrahymena* ribozyme are comparable to those used for the smaller ribozymes, namely, pH 7, 10 mM metal, 2 mM guanosine, 30−50 °C. Typical rate constants for cleavage under these conditions are 0.01 min^{-1}.

As with the hammerhead ribozyme, phosphorothioates have proven to be valuable probes in revealing details about the cleavage mechanism of the *Tetrahymena* ribozyme. Nucleophilic attack of the target phosphodiester by the 3′-hydroxyl group of the external guanosine cofactor proceeds with inversion of configuration at phosphorus, which infers an in-line, S_N2-type reaction (see Figure 1a).[154,155] Substitution of the *pro-*R_p oxygen at the cleavage site with sulfur produces only a small thio effect that can be attributed solely to the intrinsic chemistry differences between sulfur and oxygen.[165,166] This observation argues against a direct metal interaction at this site in the transition state, in contrast to the hammerhead ribozyme. Interestingly, however, replacement of the *pro-S* oxygen at the cleavage site with a sulfur atom generates a very large thio effect, but one that cannot be "rescued" by replacing Mg^{2+} with the more thiophilic Mn^{2+} ion.[91] Therefore, some functionality is most certainly interacting with the *pro-S* oxygen at the cleavage site, but not necessarily a metal ion. The 3′-leaving group of a substrate for the *Tetrahymena* ribozyme has also been replaced with a sulfur atom, thus producing a 3′-bridging phosphorothioate.[91] Compared to the native phosphodiester sequence, the bridging phosphorothioate was cleaved 1000 times more slowly in the presence of Mg^{2+} ions. However, the addition of Mn^{2+} or Zn^{2+} ions almost completely restored, or rescued, the cleavage rate. The fact that these thiophilic metals fully rescued the cleavage provides compelling evidence that a metal ion interacts directly with the 3′-oxyanion in the transition state (or sulfur in the analogue complex, Figure 12). Presumably this interaction stabilizes the developing negative charge on the leaving group, thereby lowering the transition-state energy. There is good precedent for this mode of catalysis in the nonenzymatic reactions of phosphoesters.[48]

Recently the ligation reaction of the *Tetrahymena* ribozyme has been explored with a bridging 3′-phosphorothioate.[167] As with the cleavage reaction,

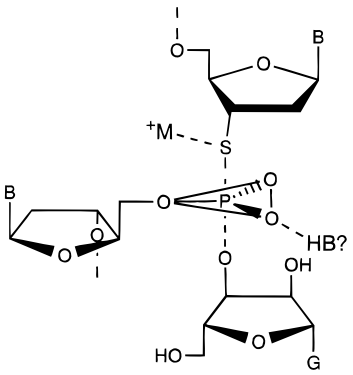


Figure 12. Proposed transition state for the *Tetrahymena* group I intron, illustrated with an analogue 3′-phosphorothiolate.

activity requires the presence of a thiophilic metal ion and a similar metal specificity switch is observed. On the basis of the principle of microscopic reversibility, the metal ion is proposed to activate the nucleophilic 3′-hydroxyl of the guanosine in the first step of splicing.[167] Taken together, the data from the cleavage and ligation reactions support the model of a two metal ion active site for the group I ribozyme.[41]

B. Group II Intron Ribozyme

Group II introns are commonly found in mitrochondrial genes in plants, fungi, yeast, and other lower eukaryotes. Like the group I introns, they are generally large fragments of RNA consisting of a series of helical domains and nominally single-stranded regions of RNA (Figure 13). Catalytic competency requires the correct folding and the binding of requisite metal cofactors.[168]

As with the group I intron, the group II introns catalyze two sequential reactions that result in release of the intron and ligation of the flanking exon sequences. However, the group II introns do not require a free guanosine cofactor. Instead, the group II introns exploit the 2′-hydroxyl of a specific internal adenosine nucleotide that is part of the intron sequence (see Figure 1a).[169−171] Transesterification results in a branch point adenosine (Figure 14), and upon cleavage a lariat structure is formed which contains a 2′-5′-phosphodiester bond at the branch site. The second of the two reactions, ligation of the flanking exonic sequences, will not be discussed here, but is thought to occur in an analogous fashion to the group I intron. Group II introns bear no apparent structural similarity to group I introns (see Figure 13). Efficient in vitro cleavage requires high Mg^{2+} concentrations (up to 100 mM) and monovalent cations (up to 1.5 M K^+ or NH_4^+),[172] and under physiological salt conditions a hydrolytic mechanism, one in which a water molecule is positioned for attack (rather than the 2′-OH of the branch point adenosine), tends to dominate in in vitro analyses.[173] In addition to transesterifcations (with a 2′-OH nucleophile) or hydrolysis (H_2O or metal-coordinated H_2O as the nucleophile), both resulting in a 3′-OH leaving group, group II introns can also catalyze the reverse reaction (debranching) in which the nucleophile is the

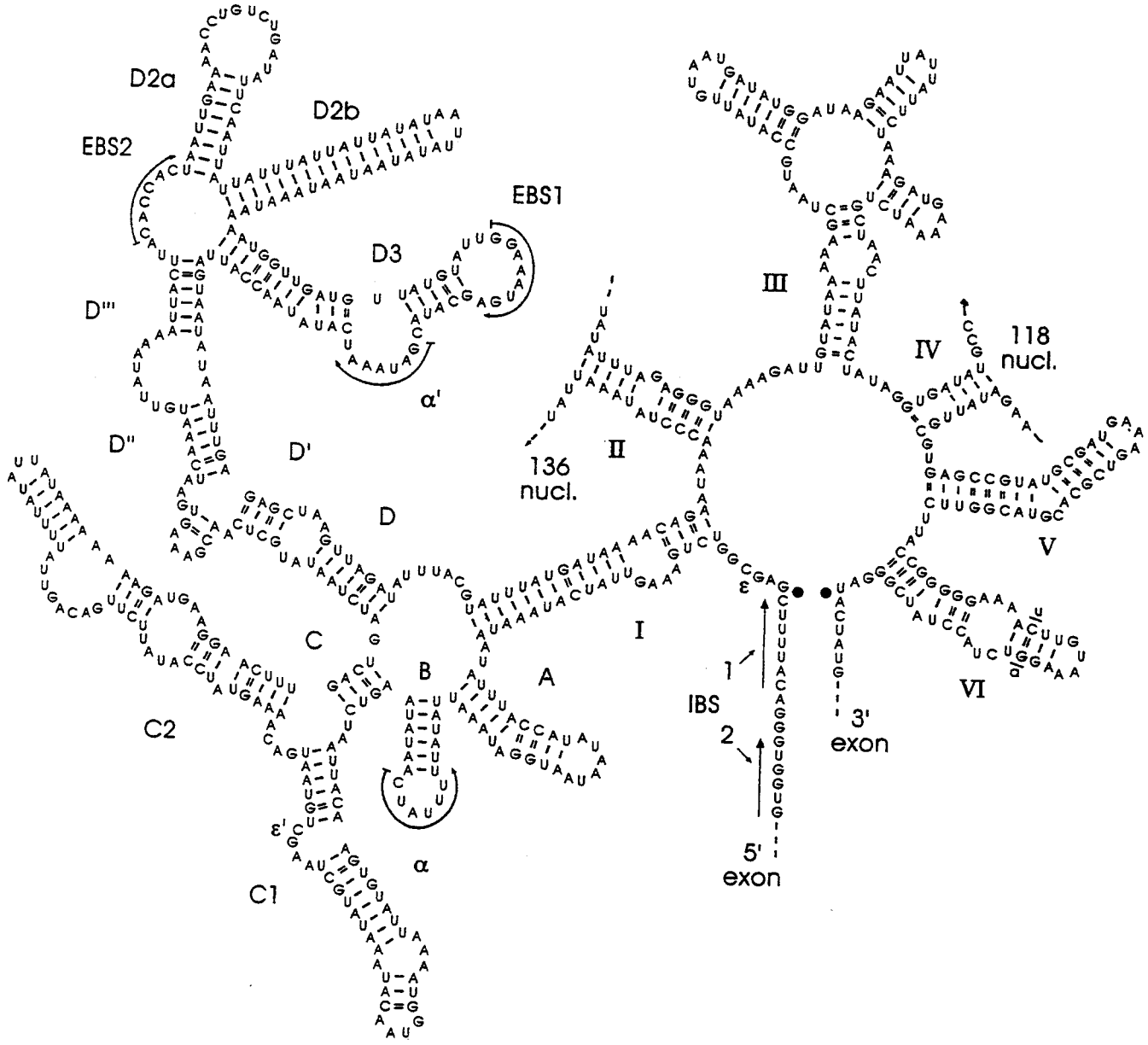


Figure 13. Secondary structure of the ai5g group II intron of yeast mitochondrial cytochrome oxidase.[168]

3′-OH and the leaving group is the 2′-OH.[174] The intron can recognize both the 5′-3′ linkage as well as the 2′-5′-3′ linkage, and in some cases even a triphosphate.[175] These activities suggest a certain degree of flexibility in the active-site, and the nature of the active-site residues.

In the transesterification reaction the 2′-hydroxyl from a specific adenosine, located within the intron region, functions as the active-site nucleophile. Transesterification results in a branch point with the adenosine esterified at all three (2′, 3′, and 5′) hydroxyls. The substitution of this adenosine by guanosine severely reduces the extent of transesterifcation.[176] Similarly, the use of nucleotide analogues, those containing subtle alterations in the functional group character, can either reduce the extent of the reaction, or result in cryptic branch point formation—a new site is selected at the adjacent 5′-position.[176,177]

The structure responsible for the catalytic activity is not well-defined. In a two-dimensional representation, group II introns can be organized into a set of six helical domains about a central wheel (Figure 13).[168] The only interdomain contact that has been suggested based upon phylogenetic and mutational studies is the interaction of the GAAA tetraloop of domain five (V) with the stem loop region of domains one (I).[178] The catalytic activity of the group II intron is centered about domain V. Despite its small size (34 nucleotides) domain V mediates the activity of the much larger intron sequence,[179,180] and many of the phylogenetically conserved nucleotides are located within the D5 stem/loop sequence. Mutations in this portion of the sequence can block the splicing reaction.[181]

C. RNAse P Ribozyme

The RNA subunit of bacterial ribonuclease P (RNAse P) specifically cleaves precursor sequences from the 5′-ends of pre-tRNA molecules to produce mature tRNAs. RNAse P is unique in that it consists of both RNA and protein subunits, but all of the

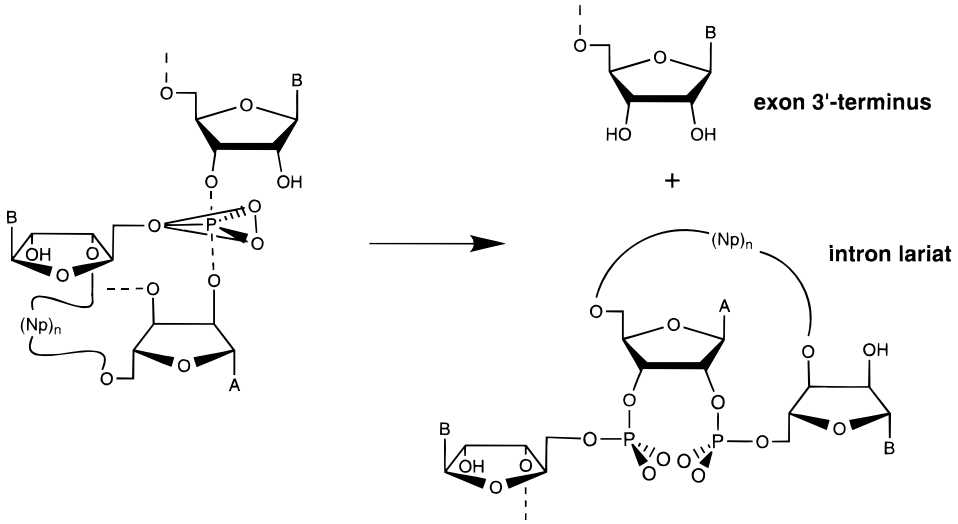


Figure 14. Proposed (transesterification) transition state for a group II intron generating the exon's 3′-terminus and the intron lariat structure.

catalytic activity resides in the RNA subunit. The protein subunit is necessary for in vivo activity, but under high Mg^{2+} and monovalent salt concentrations the RNA portion itself is sufficient for catalysis. The high salt requirement in the absence of protein is thought to be necessary to allow the proper folding of the RNA by screening unfavorable electrostatic interactions of the folded, polyanionic RNA. A detailed secondary structure model of the bacterial RNAase P (Figure 15), which consists of ~400 nucleotides, has been derived through phylogenetic comparative analysis,[189] and cross-linking studies[190] have revealed some of the higher order structural features. Interestingly, divalent cations are not required for proper folding and substrate binding, as demonstrated by intermolecular cross-linking.[190] The homologous core structure of RNAse P is only about 60% the size of the native molecule,[191] and a minimized structure containing this core sequence retains activity.

Unlike other ribozymes, the primary mechanism of phosphodiester cleavage is one of hydrolysis rather than transesterification.[182,183] Multiple Mg^{2+} ions appear to be required for cleavage activity, both to labilize a water molecule for attack at the scissile phosphodiester, as well as to stabilize the resulting transition-state structure. The most plausible mechanism, based upon phosphorothioate substitution,[184] is a simple in-line S_N2 displacement at the phosphorus center, with a divalent metal or metals functioning as critical cofactors. Hydrolysis results in the cleavage by loss of the O3′-oxygen from the upstream ribose. It is necessary for the hydroxide to be oriented for attack from the apical positions,[185] and the divalent metal may be a key to the orientation of the nucleophile and its target. One of the possible transition-state structures is illustrated in Figure 16a and based upon the models suggested by Guerrier-Takada et al.[182] and Smith and Pace.[183] In this model, one of the required hydrated magnesium ions is coordinated to the *pro-S* oxygen of the scissile phosphodiester, two of the coordinated water molecules from this catalytic metal are involved in hydrogen bonding interactions—one to the attacking

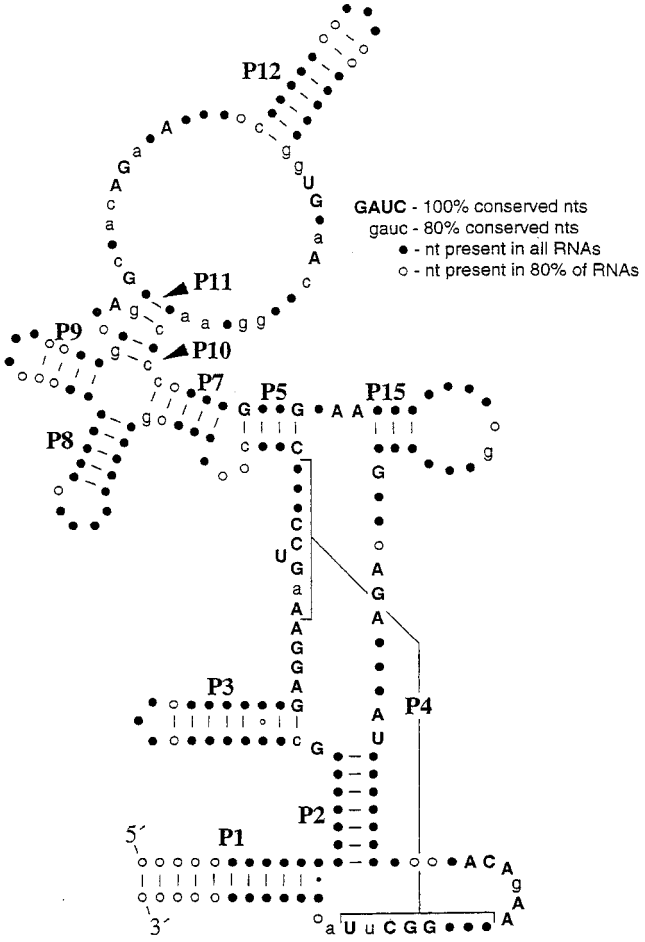


Figure 15. Phylogenetic minimum consensus sequence for bacterial RNase P RNA.[191]

hydroxide, and the second to the adjacent 2′-OH. A second metal appears to be coordinated to the *pro-R* oxygen.[184] A more recent publication suggests that the two metals might be oriented such that they are interacting with both the attacking hydroxide nucleophile as well as the leaving 3′-alkoxide (Figure 16b).[186]

Substrate recognition appears to result from the fully folded tRNA-like pre-RNA since mature tRNAs

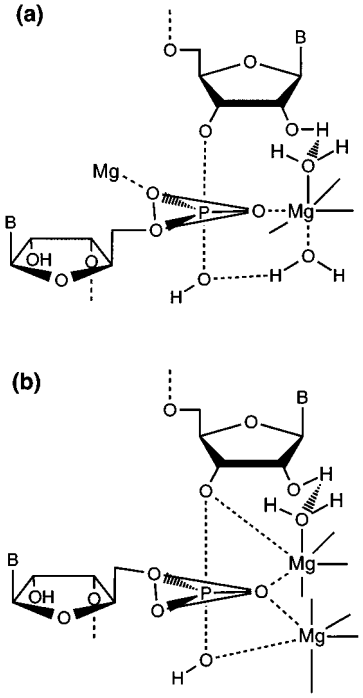


Figure 16. Proposed transition states showing two different formats for metal-stabilized pentacoordinate transition state/intermediate with apical attack of a metal hydrate stabilized hydroxide.

bind to the ribozyme with similar affinities ($K_m \approx 10$ nM). These experiments also suggest that sequences outside of the mature RNA are not employed, so precursor-specific recognition sites to align the ribozyme are largely absent. The recognition elements of the mature or precursor tRNA seem to reside on the helix formed from the coaxial stacking of the acceptor stem and the TΨC stem (and may include the CCA terminus) based upon modification interference and cross-linking experiments.

Although the intact RNase P ribozymes contain an RNA of about 400 nucleotides in addition to a protein of about 120 amino acid residues, the core RNA sequence as illustrated in Figure 15 is the phylogenetic minimum structure for catalytic activity.[191] A small RNase P consisting of this core structure maintains high catalytic activity. But even within this core structure relatively few nucleotides are conserved. Provided that proper folding of the core structure can be achieved, relatively few specific sequences are necessary to promote activity. The orientation of the various secondary structural elements, primarily helices, has been described in detail, and permits the generation of a three-dimensional structure for the catalyst which can be further tested by cross-linking experiments. For example, a number of such interactions appear to involve GNRA tetraloops interacting with Watson−Crick base pairs to form a series of base triplets.[192] Such three-base interactions have been suggested to be responsible for the interaction of P8 (See Figure 15) with two noncore tetraloops found on P14 and P18.[192]

Although the details by which the RNA, protein, and metal cofactors facilitate the cleavage of the precursor tRNA substrates are not yet fully elucidated, the continued development of structural-based analyses will provide further insights into the mechanism employed by this RNA catalyst.

V. Conclusion

RNA structures capable of catalyzing phosphodiester cleavage reactions, either by mechanisms of transesterification or hydrolysis, are many and varied. Although a ubiquitous reaction in cellular processes, a simple S_N2 mechanism can be used in most instances to characterize each process. However, other than mechanistic type, the formal explanation of any specific, detailed mechanism for the ribozymes described here, remains elusive. In all cases metal cofactors are required, and in some cases a nucleoside cofactor is also necessary, but their precise role is less clear. Metal cofactors, in the simplest analysis simply provide a small but mechanistically significant concentration of $^-$OH in order to facilitate base catalyzed transesterification or hydrolysis. However, the electrostatic properties of the metal ions may be important in stabilizing negative charge as it develops either on the nonbridging or bridging oxygens of the internucleotide phosphorus. Understanding the role of the metal atoms is complicated by the observation that the concentrations of metal cofactor in the low millimolar range are typically necessary for effective cleavage reactions, yet at such concentrations the RNA is likely decorated with many weakly and/or nonspecifically bound divalent metals. Separating out the effects of the nonspecifically bound metal atoms, from the effects of what are likely to be only a few specifically bound metal centers critical to the catalytic nature of the reaction, will continue to attract researchers to the study of ribozyme-mediated cleavage events.

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