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RNA structure, metal ions, and catalysis

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Several new and unexpected insights into the metalloenzymology of ribozymes have been achieved in the past year. From a mechanistic point of view, the NMR and crystal structures of a small Pb^{2+}-dependent ribozyme have been particularly revealing.

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Abbreviation
HDV hepatitis delta virus

Introduction: the requirement for metal ions in ribozyme catalysis

Because of the volume of research devoted to understanding the mechanistic roles of divalent metal ions in ribozyme catalysis and because a fundamental tenet of ribozyme enzymology has been that all ribozymes are metalloenzymes [1–3], it was surprising to find that at least three of the four small, naturally-occurring ribozymes can function reasonably efficiently in the absence of divalent metal ions, providing that very high concentrations of monovalent cations are present (i.e. 4 M Li^+ or 4 M NH_4^+) [4•]. The hammerhead and hairpin ribozymes are derived from two small, self-cleaving RNAs that mediate rolling-circle replication in small single-stranded circular virus and virus-like RNAs that infect plants. The *Neurospora* VS ribozyme similarly is derived from a self-cleaving RNA motif that mediates rolling-circle replication of the Varkud plasmid of *Neurospora*. Finally, the HDV ribozyme is derived from hepatitis delta virus (HDV), a satellite RNA virus that is occasionally found associated with hepatitis B. This self-cleaving RNA mediates rolling-circle replication of the delta virus. In the cases of the hammerhead, hairpin and *Neurospora* VS ribozymes, monovalent cations such as those mentioned above apparently enable the RNA to fold in much the same way that divalent metal ions allow it to. (The crystal structures of the hammerhead ribozyme in the presence of 1.8 M Li_2SO_4 and in the presence of 10 mM $MgCl_2$ at low ionic strength are identical within experimental error [5].) It therefore appears that RNA folding accounts for much, if not all, of the catalytic enhancement over background rates found with these ribozymes. For example, $HH_{16.1}$, which is considered to be an optimized hammerhead ribozyme sequence [6], cleaves only three times more quickly in the presence of 10 mM $MgCl_2$ and 2 M Li_2SO_4 than it does in the presence of 2 M Li_2SO_4 alone [4•]. The activities of hairpin and VS ribozymes in 2 M Li_2SO_4 actually exceed those measured under 'standard' low ionic strength conditions. One can actually titrate out Mg^{2+} with EDTA and thus completely eliminate the catalytic activity of each of these ribozymes. Upon further addition of EDTA, however, and therefore Na^+, the activity of the hammerhead, hairpin and VS ribozymes can be recovered, as shown in Figure 1.

It has been objected that 2 M Li_2SO_4 is hardly physiological, and therefore that the lack of a requirement for divalent metal ions is an artificial one, probably an *in vitro* artifact. Although this may be the case, a similar line of reasoning then must lead us to the conclusion that catalysis by Group I introns and bacterial RNase P must be *in vitro* artifacts. Group I introns and bacterial RNase P function as RNA–protein complexes *in vivo* [7,8]. The discovery that RNA can be catalytic [9–11] involved isolating the RNA components of these complexes *in vitro* and providing an environment of suitable ionic strength to compensate for the lack of the protein components. Under these *in vitro* conditions, which are also nonphysiological, the Group I intron RNA and the bacterial RNase P RNA are catalysts. But they appear to require their protein components in order to fold correctly and therefore in order for them to be catalytic *in vivo*. Is RNA catalysis in general therefore an *in vitro* artifact?

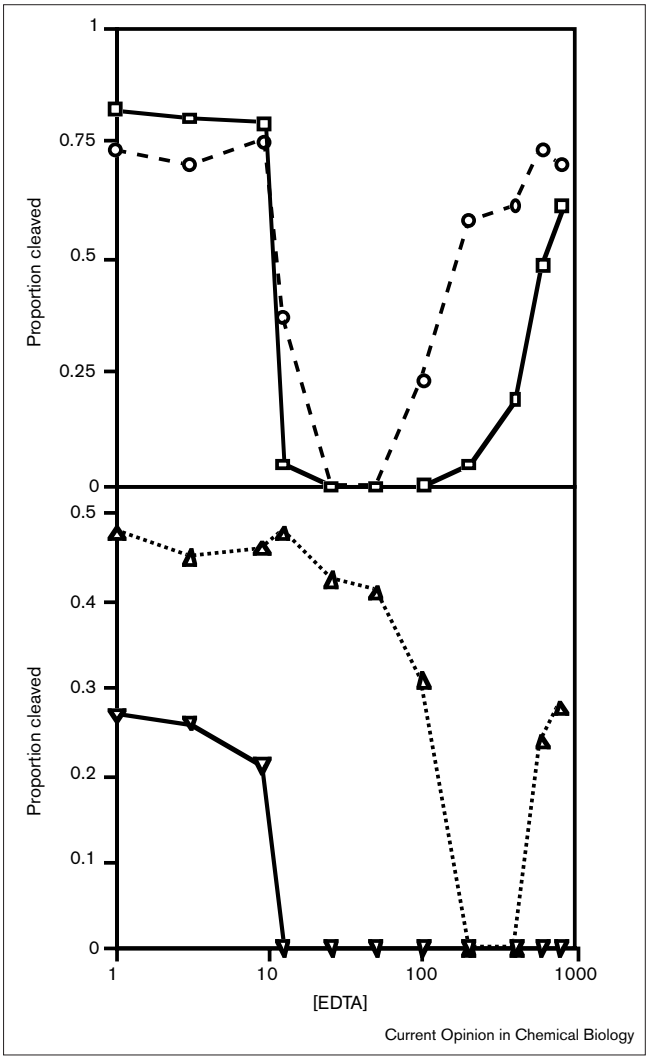
The importance of the discovery of catalytic RNA is that the protein components of these complexes are not fundamentally required for catalytic (or enzymatic) activity. They appear to play an ancillary structural role rather than a direct chemical role in ribozyme catalysis, and their apparent *in vivo* requirement can be substituted for *in vitro* by inclusion of nonphysiological concentrations of various salts. Similarly, metal ions are not fundamentally required for hammerhead, hairpin or *Neurospora* VS ribozyme catalysis, even if these catalytic RNAs rely on the presence of physiological concentrations of Mg^{2+} *in vivo* because, like the protein components of the larger ribozymes, one can find *in vitro* conditions in which the Mg^{2+} is not required for catalysis.

So far, the other naturally occurring ribozymes (i.e. the HDV ribozyme, as well as the larger Group I and Group II introns and RNase P) appear to be dependent upon divalent metal ions for catalysis, as does a small *in vitro* selected ribozyme that cleaves itself in the presence of Pb^{2+}. At the very least, conditions under which these ribozymes can function in the absence of divalent metal ions have not yet been discovered. This review focuses upon insights gained into the involvement of divalent metal ions in ribozyme catalysis in the period of October 1998 through October 1999 as viewed primarily from the perspective of macromolecular structure.

Catalytic metal ions and the HDV ribozyme structure

The 2.7 Å resolution crystal structure of the cleavage product of HDV was elucidated recently [12••]. In addition to

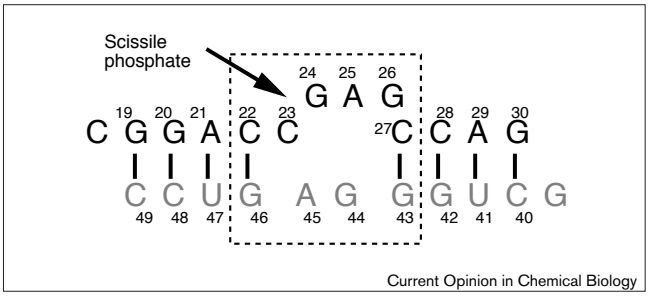
Figure 1



Na_3EDTA titrations demonstrate that Mg^{2+}-dependent ribozyme-catalyzed RNA cleavage reactions of the $HH_{16.1}$ (❑), hairpin (O) and VS (Δ) ribozymes, but not the HDV ribozyme (∇), are quenched by EDTA and stimulated by monovalent cations [4•].

revealing a novel double-pseudoknot fold, the absence of any catalytic divalent metal ion was particularly striking given that, among the small ribozymes, it alone appears to require divalent metal ions for catalysis and that the apparent K_{Mg} for the HDV ribozyme is significantly lower than those for the other small ribozymes, indicating much tighter metal binding. It may be that the catalytic metal ion simply disassociates from the ribozyme subsequent to cleavage, as Pb^{2+} has been observed to do so in tRNA crystals [13,14]; however, the authors instead present an argument in favor of a conserved cytosine functioning as the chemically active moiety responsible for inducing self-cleavage. If so, HDV might then join the other small naturally occurring catalytic RNAs in terms of containing all of the chemical determinants fundamentally required for catalytic self-cleavage (see also Note added in proof).

Figure 2



Leadzyme sequence used for crystallization, which, apart from the overhanging 5′ nucleotides, is the sequence LZ4 [16,17]. The numbering scheme corresponds to the tRNA sequence from which LZ4 was derived. The enzyme strand is shown in gray and the substrate strand is shown in black, with the cleavage site indicated with an arrow.

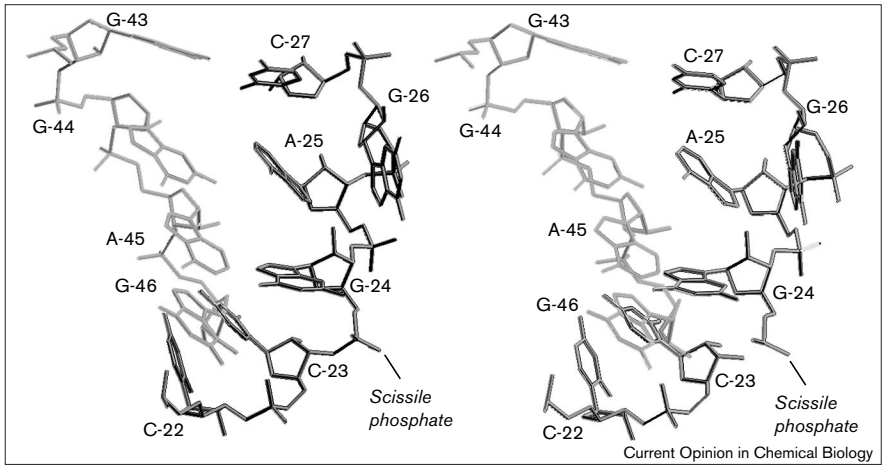
The leadzyme: a 'prototype' ribozyme that has a surprisingly rich chemistry

Before any structures of a catalytic RNA were available, $tRNA^{Phe}$, which cleaves itself in the presence of Pb^{2+} in a highly specific manner, served as the best model for understanding the relationship between RNA three-dimensional structure and catalysis. The Pb^{2+}/tRNA system is particularly well-suited as such a 'prototype' for three reasons: first, because crystal structures of both the initial state and the final state are available [13,14] (this is still unique in terms of self-cleaving RNA structures); second, it had been demonstrated that the system could be engineered to function as a true ribozyme/substrate system in the same manner as the small self-cleaving RNAs have been engineered into ribozymes [15]; and third, the cleavage chemistry — apart from involving Pb^{2+} rather than Mg^{2+} — is the same as that found in the four small catalytic RNAs. The utility of this system for understanding ribozyme catalysis thus was, and continues to be, of significant importance.

In the course of studying the Pb^{2+}/tRNA system using *in vitro* selection techniques, Pan and Uhlenbeck [16,17] discovered a small RNA motif having at most 10 essential nucleotides that form an asymmetric bubble in a duplex of RNA, which catalyzes self-cleavage in the presence of Pb^{2+}. The fold is different from that found in the Pb^{2+}/tRNA system, even though it was derived from the cleavage-active region. In addition, it catalyzes a slightly more complicated reaction. Like Pb^{2+}/tRNA and the small ribozymes, it initiates cleavage via attack of a 2′-oxygen nucleophile upon the adjacent phosphate, forming a 2′,3′-cyclic phosphate product. However, the cyclic phosphate is a true reaction intermediate in that the ribozyme then goes on to hydrolyze the cyclic phosphate in a manner similar to RNase A. Because of its small size as well as its surprisingly rich cleavage chemistry, the leadzyme (as it has been dubbed) itself has attracted considerable attention for biochemical and biophysical studies. How does such a simple RNA bind to and deploy a divalent cation to catalyze a reaction similar in complexity to that of

Figure 3

A stereoview of the conserved core region of the leadzyme NMR structure. The numbering scheme is that used in Figure 2, showing only residues contained within the dashed line box in that figure. The enzyme strand is shown in gray, and the substrate strand is shown in black, with the cleavage site indicated by the scissile phosphate.



RNase A (and more complex than that catalyzed by the four small naturally occurring ribozymes)? What are the structural principles that make this possible? It is in the context of these questions that the importance of the NMR and crystal structures becomes evident.

The NMR structure of the lead-dependent ribozyme

The NMR-derived structure of the leadzyme represents a major milestone in RNA structural biology for at least two reasons: it is the first ribozyme structure to be determined in its entirety by NMR, thus providing proof that NMR is indeed effective for elucidating ribozyme structures [18••]; and it provides the most direct physical evidence yet for the importance of conformational dynamics in RNA structures, and how these are affected by the presence or absence of metal ions [19••]. In addition, the leadzyme is only the second active ribozyme–substrate complex of which the structure has been determined in its entirety.

The leadzyme NMR structure was determined from RNA in solution with 100 mM NaCl and 10 mM phosphate buffer at pH 5.5. The effects of additional NaCl, $Pb(OAc)_2$ or $MgCl_2$ to this solution were assayed by comparing ^{1}H-^{13}C two-dimensional correlation spectra under the various conditions. The aromatic regions of the spectra remained surprisingly constant, indicating that the leadzyme exists in a stable folded conformation under all of these conditions and therefore, by inference, any metal binding sites for Mg^{2+} or Pb^{2+} must either be preformed (if the metals bind) or are not accessible under any of these conditions.

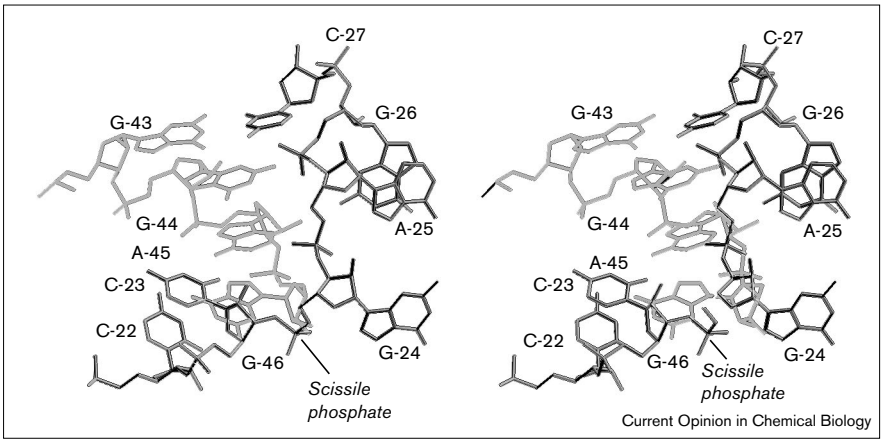
NMR ^{13}C relaxation experiments, however, revealed a richness in the molecular dynamics of the active-site region of the ribozyme taking place on several timescales. Using the tRNA numbering scheme depicted in Figure 2, residue A-25 showed dynamic fluctuations in the subnanosecond timescale, whereas the canonical helical regions appeared to be quite rigid. (The GAAA tetraloop linking the 3′ end of the substrate strand to the 5′ end of the enzyme strand in the construct used for the NMR studies, by contrast, revealed chemical exchanges taking place on the microsecond to millisecond timescale.) These experiments provide the first direct and unambiguous physical evidence of RNA conformational dynamics in the active site.

The NMR structure of the leadzyme (Figure 3) reveals that the canonical base-pairs stack to form standard A-form helices, whereas C-23 forms a base-pair with a protonated A-45 at pH 5.5. A-25 is also observed to form a sheared base-pair with G-44, whereas G-24 and G-26 each bulge out of the otherwise continuous helix. The cleavage-site phosphate, between C-23 and G-24, does not appear to be in a conformation amenable to an in-line attack mechanism. This situation is analogous to that observed in tRNA at the Pb^{2+} cleavage site, and also in the hammerhead ribozyme initial conformation. (An in-line [or $S_N2(P)$] attack mechanism has been demonstrated unambiguously for the hammerhead ribozyme and is assumed to pertain to the cases of tRNA and the leadzyme as well, although both in-line and adjacent mechanisms have been proposed for the Pb^{2+}/tRNA system.) It is therefore proposed that the leadzyme must undergo at least a local conformational change to a precatalytic conformation capable of initiating self-cleavage, in a manner similar to that envisaged for the hammerhead ribozyme.

The crystal structure of the lead-dependent ribozyme

Subsequent to publication of the NMR structure, we were treated to a crystal structure of the leadzyme consisting of two crystallographically independent and significantly different molecules in the asymmetric unit (Figure 4; [20••]). The crystals were obtained at a relatively low ionic strength with 20 mM $Mg(OAc)_2$, 1 mM spermine and 50 mM Na cacodylate at pH 6 using 20–25% 2-methyl-2,4-pentane-diol (MPD) as a precipitating agent. The MPD was found to inhibit the

Figure 4



A stereoview of the conserved core region of the leadzyme crystal structure. The numbering scheme is that used in Figure 2, showing only residues contained within the dashed-line box in that figure. The enzyme strand is gray and the substrate strand is black, with the cleavage site indicated by the scissile phosphate.

cleavage reaction in the presence of Pb^{2+} ~10-fold, perhaps due to its low dielectric constant, and the crystal contacts were found to have an additional 10-fold attenuation of the rate of cleavage. Nevertheless, the ribozyme does appear to retain catalytic activity in the crystal, and such reaction rate attenuation is quite common in crystals of protein enzymes, as in crystals of tRNA in the presence of Pb^{2+}. (The crystal contacts in the case of the hammerhead ribozyme, by contrast, appear to increase the rate of self-cleavage of the particular sequence crystallized [21], although the rate of cleavage is attenuated relative to optimized hammerhead sequences.)

The core region of the crystallized leadzyme differs somewhat from that found in the NMR experiments. The only potential direct contact between the enzyme and substrate strands in this region is a single hydrogen bond between the O2 of C-23 and the N6 of A-45. Unlike the NMR structure, there is no evidence for a protonated C–A^+ base-pair; and the G-44 to A-25 sheared base-pair does not form. Instead, the G-24, A-25 and G-26 triplets bulge out into two different conformations in the two molecules, stabilized by crystal packing contacts. The two molecules are also observed to bind metals in two distinct ways. Molecule 1 alone is observed to bind to two Mg^{2+} ions, and molecule 2 alone is observed to bind to a single Pb^{2+} ion soaked into the crystals. Each molecule binds Ba^{2+} when soaked into the crystal but does so differently from the other. Molecule 1 binds one Ba^{2+} ion, and molecule two binds two Ba^{2+} ions strongly and one more weakly (this being similar to the binding site in molecule 1). Similar to the NMR structure of the leadzyme, neither molecule in the crystal has a scissile phosphate geometry compatible with in-line attack, although molecule 2 is described as potentially having the conformational flexibility to allow such a configuration to form.

Molecule 2 can bind a Ba^{2+} ion within striking distance of the 2′-OH of the cleavage-site ribose at C-23; for that reason (as well as the conformational flexibility about the scissile phosphate) the authors propose that molecule 2 is in a 'pre-catalytic' conformation, and molecule 1 is in a less active conformation. Pb^{2+}, by contrast, binds to G-42 in the nonconserved helical region of molecule 2, in a manner approximating one of the Mg^{2+} sites in molecule 1, as well as the other major Ba^{2+} site in molecule 2. It is thus positioned approximately 10 Å from the cleavage site, presumably too far to be involved directly in the cleavage site chemistry. Additional Pb^{2+} sites potentially exist in the leadzyme, but crystals soaked with more than 1 mM Pb^{2+} quickly degraded, thus complicating identification of binding sites. Hence the authors hypothesize that the Ba^{2+} site near the scissile phosphate in molecule two might also be a second Pb^{2+} site that would be revealed if the crystals were tolerant to soaking in a higher concentration of Pb^{2+}.

Insights into metal-mediated ribozyme catalysis

Assuming that the Ba^{2+} site near the scissile phosphate is also a Pb^{2+}-binding site, the Pb^{2+} most probably initiates catalysis by aiding abstraction of the 2′-proton from the ribose at the cleavage site, thus generating the attacking nucleophile. A concomitant change in the ribose sugar pucker from 2′-endo to 3′-endo has also been postulated as a mechanism for bringing the attacking nucleophile in line with the departing 5′-oxygen of the scissile phosphate. Recalling that the leadzyme reaction is chemically identical to the two-step reaction catalyzed by RNase A, it is tempting to speculate that a second Pb^{2+}, or an RNA functional group capable of functioning as a general acid catalyst, might then assist protonation of the 5′-oxygen leaving group. The complementary roles played by a basic histidine and an acidic histidine in the RNase A active site allow catalysis of the first step of the reaction. In the process, the initially basic histidine gains a proton, and the initially acidic histidine loses one. The second step, hydrolytic ring opening of the 2′,3′-cyclic phosphate, is then catalyzed by the newly basic and newly acidic histidines, and the resulting proton transfers restore the histidines to their initial state, thus regenerating the catalyst. RNase A efficiently catalyzes this two-step process thanks to the pK_a of histidine being close to 7. Likewise, a hydrated lead ion can

function as both an efficient acid and base catalyst due to the fact that the pK_a of the lead hydroxide is also approximately 7. The similarity of the chemistry of the leadzyme to that of RNase A might then be more than coincidental; the two-step cleavage process near neutral pH probably depends upon the unique properties of Pb^{2+}.

Various physical and chemical trapping techniques have previously been employed to capture conformational changes that occur prior to cleavage in the hammerhead ribozyme, including the use of flash freezing crystals after initiation of the reaction, as well as employing a modified leaving group that creates a kinetic bottleneck in the reaction pathway [5,21]. In the case of the leadzyme, two conformations have apparently been selected from the many that are likely to exist in solution by the forces of crystal packing. One of these fortuitously appears to be a pre-catalytic conformation trapped by the constraints of the crystal lattice. As in the case of the hammerhead RNA, as well as of tRNA, additional conformational changes are required to form the presumed in-line transition state of the leadzyme. In the case of the leadzyme, this might be simply a sugar pucker conformational change in the active site, perhaps induced by Pb^{2+} binding or, as some have suggested is the case with the hammerhead ribozyme, might be a more dramatic rearrangement. For now, the simplest explanation seems the most plausible, unless of course Pb^{2+} turn out not to participate directly in the cleavage chemistry!

Note added in proof

Further evidence that the cytosine identified in the HDV ribozyme crystal structure is directly involved in catalysis has now appeared [22••]. Imidazole buffer is capable of rescuing cleavage activity in an HDV ribozyme in which this cytosine has been changed to a uracil, suggesting that imidazole, and therefore the original cytosine, serve as a general base catalyst in the ribozyme self-cleavage reaction. This again demonstrates that it is likely the RNA itself, rather than metal ions per se, that is directly involved in initiating catalysis.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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- •• of outstanding interest

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HDV is a satellite RNA of hepatitis B the presence of which invariably results in a much more lethal hepatitis infection. Like the plant satellite RNAs, HDV replicates via a rolling circle mechanism aided by a self-cleaving RNA. The crystal structure of the self-cleaved domain of HDV was obtained with the aid of a bound protein of known structure, and reveals a novel double-pseudoknot RNA fold.

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The first NMR-determined structure of a ribozyme is presented in the context of a Pb-dependent *in vitro* selected ribozyme. Evidence for a protonated C·A pair is presented, and its possible mechanistic relevance is examined.

19. •• Legault P, Hoogstraten CG, Pardi A: **Order, dynamics and metal binding in the lead-dependent ribozyme.** *J Mol Biol* 1998, **284**:325-335.

This accompanying article to [18••] discusses the resonance assignment process as well as experiments designed to probe the conformational dynamics of the ribozyme.

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The crystal structure of the Pb-dependent ribozyme is compared with the NMR structure, and several metal-bound states of the ribozyme – produced as heavy atom derivatives to aid in solving the phase problem – are also examined. Two conformations appear in the crystal, one of which is believed to be pre-catalytic. On the basis of the metal binding data, the authors propose a mechanism for Pb-mediated catalysis.

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The authors provide strong evidence that the cleavage-site cytosine is directly involved in initiating the self-cleavage reaction of the HDV ribozyme, serving the function of a general base catalyst. It is still possible that the apparently required divalent metal ion plays some other active role in the chemistry of catalysis, but it is clear that this cannot be regarded as a forgone conclusion.