University of Dundee

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Lilley, David M. J.

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REVIEW

How RNA acts as a nuclease - some mechanistic comparisons in the nucleolytic ribozymes

David M. J. Lilley

Cancer Research UK Nucleic Acid Structure Research Group,
MSI/WTB Complex,
The University of Dundee,
Dow Street,
Dundee DD1 5EH,
U.K.

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Editorial enquiries to Professor Lilley:
Tel: (+44)-1382-384243
FAX: (+44)-1382-385893
Email: d.m.j.lilley@dundee.ac.uk

Ribozymes are catalytic RNA molecules, some of which catalyse extremely important cellular reactions, notably processing tRNA and mRNA (*1*) and condense amino acids to form polypeptides (*2-5*). Ribozymes are very widespread; for example ribonuclease P (*6, 7*) processes precursor tRNA in all domains of life. However, the mechanisms of their chemical reactions are incompletely understood, and sometimes controversial.

The catalytic resources of RNA

RNA would appear to be very poorly equipped to perform chemical catalysis, particularly when compared with proteins. The twenty different amino acid side chains offer a broad chemical space with a range of different aqueous chemistry, displayed on an electrically-neutral polypeptide backbone. The protein enzymes catalyse a wide variety of reactions, and can generate huge catalytic rate enhancements. In marked contrast, the potential catalytic resources of RNA are limited to four similar nucleobases, ribose with a secondary alcohol (2'-hydroxyl) and an anionic phosphodiester with associated hydrated metal ions that can bind site-specifically (inner-sphere complex) or more dynamically in atmospheric mode. In addition, RNA is an excellent ligand for many small molecules (exemplified by the riboswitches (*8, 9*)) and ribozymes can recruit additional molecules as coenzymes (*10*) or reactants (*11*).

The nucleolytic ribozymes

The nucleolytic ribozymes are a diverse group of smaller RNA species that generate site-specific cleavage and ligation of RNA. The rate of discovery of new ribozymes has recently increased significantly, as the Breaker laboratory bioinformatic pipeline has generated a flurry of new ribozymes (*12, 13*). There are now nine distinct members of this group. Crystal structures have now been determined for the majority of these ribozymes (*14-25*). The reaction is initiated when an O2' atom attacks the adjacent phosphorus in an S_N2 transesterification reaction leading to the formation of a 2'3'-cyclic phosphate and a 5' hydroxyl group (Figure 1). Ligation is the reverse of this reaction (*26-30*), and occurs if the cleavage product RNA is held in place by the structure of the ribozyme. The hammerhead (*31, 32*), hepatitis delta virus (HDV) (*33, 34*) and twister (*12*) ribozymes are widely dispersed in prokaryotic and eukaryotic genomes. While their biological roles are not yet fully understood, some are expressed and their positions suggest possible functions in genetic control.

Mechanisms of RNA-mediated catalysis

Examination of the standard mechanism of the nucleolytic ribozymes (Figure 1) suggests four possible catalytic strategies. These are :

1. Facilitation of in-line attack. The SN2 transesterification reaction requires a colinear alignment of the nucleophile (O2' in the cleavage reaction), the phosphorus atom and the leaving group (O5' in the cleavage reaction), in a trigonal bipyramidal phosphorane transition state (or this should at least be a high-energy intermediate that closely resembles the transition state). This requires that the nucleotides that flank the scissile P are splayed apart, which is quite different from how they are present in duplex RNA. The best example of this arrangement is observed in the crystal structure of a vanadate transition-state analog of the hairpin ribozyme (*35*), which is constrained to be in line by the coordination of the vanadium. However, such a clear picture is not always found in crystal structures of ribozymes (e.g. see the discussion of twister below), and normally some chemical insight and extrapolation needs to be applied. Facilitation of the in-line trajectory is unlikely to give more than a factor of 100 (and quite possibly less) to the rate enhancement.

2. Stabilization of the phosphorane. All enzymes stabilize their transition states in one or more ways. The phosphorane-related transition state is formally dianionic and juxtaposition of positive charge could lower its free energy. The obvious candidate would be a metal ion. While for the majority of the nucleolytic ribozymes there is no evidence for a role for site-specifically inner-sphere-bound ions, the majority function in high concentrations of monovalent ions (*36*) and so it is likely that the transition state is stabilized electrostatically by diffuse ion interactions. The phosphorane structure could also be stabilized structurally by specific formation of hydrogen bonds.

3. *Deprotonation of the nucleophile*. An alkoxide is a much stronger nucleophile than an alcohol, so a general base that can deprotonate the 2'-OH should accelerate the cleavage reaction.

4. *Protonation of the oxyanion leaving group*. A neutral hydroxyl group should be a better leaving group than its corresponding oxyanion, so a general acid that protonates the oxygen should contribute significantly to the rate enhancement.

Strategies 3 and 4 together constitute general acid-base catalysis. Catalytic strategies

1 through 4 have been termed α through δ (*37*). The protein enzyme ribonuclease A uses the imidazole side chains of two histidine residues to achieve this strategy (*38*). It should be noted that because the acid and base must be in their correct states of protonation, and so the reaction rate will be pH dependent. Current evidence indicates that all the nucleolytic ribozymes employ general acid-base catalysis, although evolution has found different ways to achieve this over the group as a whole.

The role of nucleobases in ribozyme catalysis

All the nucleolytic ribozymes employ nucleobases in their catalytic mechanisms, mostly involved in proton transfers in general acid-base catalysis although they can also participate in transition state stabilization. Purine bases are most prevalent, and the majority of ribozymes use a deprotonated guanine nucleobase as a general base in the cleavage reaction (*16, 19, 39-45*).

Several ribozymes use a combination of adenine and guanine nucleobases in coordinated general acid-base catalysis. In spite of very different overall architecture, the topological arrangement of the components of the hairpin and VS ribozyme active centers is essentially identical (*43*), probably resulting from convergent evolution. The general acid and base must each be in its correct state of protonation (i.e. protonated and unprotonated respectively) and thus the rate of reaction is strongly pH dependent. This leads to a classic bell-shaped dependence of reaction rate on pH (*46*), and the adenine and guanine nucleotides were shown to be the general acid and base respectively in the cleavage reaction by phosphorothiolate substitution (*45, 47*). The twister ribozyme also uses an adenine plus guanine combination, but with two significant differences as discussed below.

While all the nucleolytic ribozymes probably use at least one nucleobase, some employ other elements. The hammerhead (and probably pistol) uses a 2'-hydroxyl group as a general acid, while HDV and the TS ribozymes use a water molecule in the inner coordination sphere of a bound divalent metal ion as general base (*24*). These cases are discussed below. Lastly, the GlmS ribozyme (*10*), which functions as a riboswitch, uses the amine of its glucosamine-6-phosphate as a general acid (*17, 18, 42, 48, 49*).

The twister ribozyme - four catalytic strategies

The twister ribozyme (*12*) was the first of the new group that has emerged in the last few years from the Breaker lab bioinformatic pipeline (*13*). The secondary structure is that of a long stem-loop interrupted by internal loops that can form two tertiary interactions. We solved the 3D structure using X-ray crystallography (*19*) (Figure 2). The overall fold is a unique reversed-double-pseudoknot that brings together a number of highly-conserved nucleotides to form the active site in the center of the ribozyme. Each molecule is paired with a symmetry-related one, that has the result of pulling the uridine 5' to the scissile phosphate (U-1) out from the active center, and places the nucleophile (were it present) in a position that is far removed from the required in-line geometry. However, we showed that a simple rotation of U-1 would bring the O2' in-line with the phosphate, unhindered in its path (*19*). This positions U-1 in the major groove, stacked under G33. Thus although the geometry is not in-line in the crystal, its easy to see how to adjust the structure to achieve the required trajectory for S_N2 attack.

The rate of cleavage exhibits a bell-shaped pH dependence corresponding to two ionizations with apparent pK_a values of 6.9 and 9.5, consistent with a concerted general acid-base catalytic mechanism involving bases of neutral and high pK_a. Mutation and study of the pH dependence strongly supported a role for G33 in catalysis (*19*), and in the remodelled structure of the active site N1 of G33 is juxtaposed with the O2' nucleophile.

If G33 is the general base, what might serve as the general acid ? Its apparent pK_a is 6.9, suggesting either adenine or cytosine in a very electronegative environment. This was narrowed down to a single nucleobase, and the result was rather unexpected. It turned out to be A1, i.e. the adenine immediately 3' to the scissile phosphate, which is completely unprecedented (*50*). Perhaps even more surprising, we showed that the proton is transferred not from the usual N1, but rather the more acidic N3 atom. A1 is located in a rather special environment at the base of the P4 helix. The exocyclic N6 of A1 is hydrogen bonded to successive phosphates in the T1 backbone, the negative charges of which raise the pK_a of this adenine to near-neutrality. This compensates for the low fractional protonation of N3. The position in the pocket holds the nucleobase in a *syn* conformation, directing its N3 towards the O5' as required for the

general acid. It furthermore provides the splayed out conformation required for an in-line trajectory.

Lastly, we have identified a fourth component of the catalytic mechanism. We (*50*) and others (*12, 51*) have noted that there is marked stereospecific effect of phosphorothioate substitution at the scissile phosphate. Replacement of the *pro*R oxygen by sulfur resulted in a cleavage rate that was one hundred-fold slower, whereas *pro*S substitution left the rate effectively unaltered. This effect was not alleviated by addition of thiophilic metal ions, so was not a result of direct coordination by a metal ion. However, we found that it was markedly affected by substitution at G33. Nucleotides that retained the N2 exocyclic amine (guanine, 2-aminopurine) retained the stereoselective effect, whereas those lacking the N2 amine (inosine, adenine) resulted in a loss of stereoselectivity. We conclude that the key interaction in the transition state is between the *pro*R oxygen and the N2 exocyclic amine of G33.

Thus all four catalytic strategies combine to generate the overall rate enhancement of the twister ribozyme (Figure 2). The entire fold of the RNA molecule has clearly evolved to generate the environment in which G33 and A1 catalyse the cleavage of the phosphodiester backbone. G33 is positioned to act as general base (N1) and stabilize the phosphorane transition state (N2). In addition, A1 is pulled out of the helix to generate an in-line trajectory, and juxtaposed with the O5' to act as general acid (N3) with its pK_a substantially raised by its interaction with two backbone phosphate groups (N6).

The TS ribozyme - a probable metalloenzyme

One of the newest group of nucleolytic ribozymes was called twister sister (*13*), because it was thought likely to resemble twister. I shall refer to it here as the TS ribozyme. Weinberg *et al*. (*13*) found that reaction rate increased with pH and exhibited a steep dependence on Mg^{2+} ion concentration. No reaction was observed in 1M monovalent ions apart from very weak activity in Li^+, and no cleavage occurred in $Co(NH_3)_6$ (III) ions. These properties suggest a possible direct role for a hydrated metal ion in the cleavage reaction. We explored this further, finding that while the ribozyme was active in divalent metal ions (e.g. Mg^{2+} and Mn^{2+}), activity in 1 M Li^+

or 1 mM $Co(NH_3)_6$ (III) ions was extremely low (k_{obs} = 5.7x10^{-5} and <1x10^{-5} s^{-1} respectively) (*24*). The activity in these ions is significantly lower than for other ribozymes such as the hairpin or twister ribozymes, and suggested a direct role for a water molecule bound to a divalent metal ion in the cleavage reaction. This expectation was supported by our observation that the rate of cleavage exhibited a log-linear dependence on pK_a in a series of divalent metal ions (*24*).

We crystallized a form of the TS ribozyme and solved the structure at a resolution of 2.0 Å (*24*). Two co-axial helical stacks of helices are organized by a three-way junction and two tertiary contacts (Figure 3). The scissile phosphate lies in a loop region that is quasi-helical and stabilized by a network of hydrogen bonding. Like the twister ribozyme the geometry at the cleavage site is not in-line; this must reflect a pre-reactive conformation, and will certainly rearrange as it advances towards the active state. Interestingly however, we see a divalent metal ion bound directly to the nucleobase 5' to the scissile phosphate, positioning an inner-sphere water molecule juxtaposed with the O2' nucleophile. This water molecule would be well positioned to act as a general base in the cleavage reaction. Thus, current evidence points to the TS ribozyme acting as a metalloenzyme, at least in part.

The hammerhead ribozyme - the role of a 2'-hydroxyl group

The hammerhead ribozyme was the first nucleolytic ribozyme to be discovered (*52-54*), and yet its catalytic mechanism is still incompletely understood. Early forms of the ribozyme lacked a key tertiary contact element (*55*) required to facilitate folding (*56*) and permit the active site to adopt the correct conformation (*16, 57*). Cleavage rate rises with pH across the range, indicating that there are no reaction participants of low pK_a. Two guanine nucleotides were implicated in the mechanism, G8 and G12 (*41*). The crystal structure of the active site of the ribozyme with the additional tertiary contact (*16*) showed that G12 is oriented with its N1 poised to accept a proton from the O2' nucleophile, consistent with a role as general base (Figure 4). However, there was no nucleobase in a position consistent with general acid catalysis. Instead, the O2' of G8 was positioned adjacent to the O5' of the scissile phosphate, consistent with phosphorothiolate substitution data of Thomas and Perrin (*58*). At first sight the normally-high pK_a of a 2'-hydroxyl makes this a rather unlikely candidate as a general acid. However, on the basis of hybrid QM/MM simulation Lee and York (*59*) propose

that a Mg^{2+} ion observed in the crystal moves to bind in a bridging position in the active conformation, where it could stabilize the general acid. This could lower the pK_a of the O2' significantly, bringing it into play in the catalysis.

A proposed mechanistic classification of the nucleolytic ribozymes

Table 1 presents the majority of the known nucleolytic ribozymes, with a proposed grouping according to their probable general acid-base mechanisms. The most common feature that emerges from this summary is the use of guanine N1 as a general base for the cleavage reaction. The hairpin and VS ribozymes are essentially catalytically identical, employing the N1 atoms of adenine and guanine as general acid and base respectively. The twister ribozyme is mechanistically similar, except that the N3 of adenine is used, and unlike the other ribozymes this nucleotide is immediately 3' to the scissile phosphate.

The hammerhead ribozyme also uses guanine N1 as general base, but there is now strong evidence that the general acid is a 2'-hydroxyl group, probably activated by a metal ion. I shall also tentatively place the new pistol ribozyme (*13, 60*) into the same group. The pH dependence of this ribozyme is similar to that of the hammerhead. The overall structure is reminiscent of that of the hammerhead (*23, 25*), and the structure of the active center is consistent with a role for a guanine N1 and an O2' as general acid. This requires more detailed mechanistic investigation.

In the next group I am placing the hepatitis D virus (HDV) ribozyme (*61-63*) and the TS ribozyme. Structural and mechanistic evidence indicates that the HDV ribozyme uses metal ion-bound water as the general base, and a cytosine nucleobase as a general acid (*15, 64-67*). The TS ribozyme also appears to employ a hydrated metal ion as the general base, but at the present time we have not identified the general acid with confidence. However, modification of a conserved cytosine results in a very large reduction in ribozyme activity, and this may yet prove to be a catalytic element.

Lastly the GlmS ribozyme is placed in a group on its own. Like all the ribozymes except for those of the HDV group it uses a guanine N1 as general base in the cleavage reaction. But uniquely it appears to use a bound small molecule as the general acid (*17, 18, 42, 48, 49*). This creates an interesting precedent that

immediately suggests how ribozymes might greatly expand their chemical repertoire by recruitment of coenzymes, either in an ancient RNA world, or even in contemporary biology (*68*).

In conclusion

Great progress has been made in understanding the chemical origins of catalysis in the nucleolytic ribozymes. This has been helped greatly by the recent discovery of new ribozymes, so that comparisons can be made and common themes discerned. This has enabled us to begin a classification of the ribozymes by mechanism, although this is still very tentative in places, and much work remains to establish some mechanisms with greater certainty. And relatively little is known about the hatchet ribozyme (*13*), so we cannot attempt to classify this at the present time. Given recent progress in finding new ribozymes we hope that more general principles will emerge, and perhaps in time we may discover new RNA molecules exhibiting a wider range of chemistry.

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Acknowledgements

Table 1.

	base	acid
hairpin	G N1	A N1
VS	G N1	A N1
twister	G N1	A N3
hammerhead	G N1	O2'
pistol (1)	G N1	O2'
HDV	Mg^{2+} OH_2	C N3
TS	Mg^{2+} OH_2	(2)
GlmS	G N1	glnP

Table 1. Tabulation of the nucleolytic ribozymes grouped by the catalytic moities. At the present time the hatchet ribozyme (*13*) has not been included.

(1) The mechanistic data for the pistol ribozyme are still incomplete, so this classification is somewhat tentative.

(2) The general acid in the TS ribozyme is not known with certainty at this time.

FIGURE LEGENDS

Figure 1. The mechanism of the nucleolytic ribozymes, and potential catalytic strategies. In the cleavage reaction (left to right), the O2' makes a nucleophilic attack on the adjacent phosphate, generating the central phosphorane that should resemble the transition state. Departure of the O5' leaves a cyclic 2'3' phosphate. Four potential catalytic strategies are indicated. AH^+ is a general acid, and B^- a general base.

Figure 2. The twister ribozyme. The structure was determined by X-ray crystallography (*19*); these images were prepared from PDB 4OJI. (**A**) : The overall fold, with the different helical sections (*P*) and tertiary contacts (*T*) labeled. The structure is a reversed double-pseudoknot. The scissile phosphate is shown by the magenta sphere. (**B**) : Closer view of the active center for the remodeled structure in which U-1 has been rotated under G33 to position the O2' for in-line attack. The key interactions for the catalytic mechanism are indicated by the broken lines.

Figure 3. The TS ribozyme structure. The structure was determined by X-ray crystallography (*24*); this image was prepared from PDB 5T5A. The structure is based on two coaxial stacks of helices connected by a three-way helical junction. Two tertiary contacts (T1 and T2) lock the helices together. The scissile phosphate is shown by the magenta sphere. Note the hydrated metal ion (*M*) coordinated to the uridine nucleotide 3' to the scissile phosphate.

Figure 4. The active site of the hammerhead ribozyme. The structure was determined for the ribozyme from *Schistosoma mansoni* which contains a key tertiary contact that allows the active center to adopt the conformation required for full activity (*16*). This image was prepared from PDB 2OEU. The O2' nucleophile is approximately in-line, and the N1 of G12 is positioned to act as general base in the cleavage reaction. The only group that is well placed to act as the potential general acid is the O2' of G8.