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Mechanistic Debris Generated by Twister Ribozymes

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Abstract

Twister RNAs represent a recently-discovered class of natural ribozymes that promote rapid cleaving of RNA backbones. Although an abundance of theoretical, biochemical, and structural data exists for several members of the twister class, disagreements about the architecture and mechanism of its active site have emerged. Historically, such storms regarding mechanistic details typically occur soon after each new self-cleaving ribozyme class is reported, but paths forward exist to quickly reach calmer conditions.

The strategies and tools that researchers bring to bear on the mechanistic analyses of self-cleaving RNAs¹ have been progressively advancing over the last 30 years, ever since the first reported discovery of members of the hammerhead² and hairpin³ ribozyme classes. The main goal for each of these studies is to determine *precisely* how small structured noncoding RNAs can rally their limited chemical make-up to function as high-speed ribozymes. From such insights, we can better appreciate how some ribozymes persist in modern cells despite seemingly overwhelming competition from protein-based enzymes. A deep mechanistic understanding of ribozyme catalysis also can empower molecular engineers who seek to create new biocatalysts by sculpting them from random-sequence RNAs, or perhaps to improve existing examples. Moreover, we can better speculate on how ancient ribozymes might have guided primitive metabolic processes in the RNA World.^{4,5}

The chemical pathway catalyzed by the nine known¹ classes of natural small self-cleaving ribozymes involves a straightforward phosphoester transfer reaction (Figure 1) that splits the RNA backbone. Despite the small number of chemical groups available to RNA for promoting a limited number of catalytic strategies,^{6,7} definitive conclusions regarding precisely how each self-cleaving ribozyme class functions usually are reached only after following very stormy paths. Recently, such disputes have centered on twister RNAs (Figure 2), which represent a distinct natural self-cleaving ribozyme class first reported in 2014.⁸ Improvements in biochemical and computational strategies, coupled with advances in RNA x-ray analysis methods, now permit detailed mechanistic data to be generated on new

ribozyme classes within a few months of their reported discovery. Indeed, in the two years since the initial description of twister ribozymes,⁸ there have been four atomic-resolution structural models reported,^{9–12} along with considerable additional data derived from biochemical^{8,9,11,13} and molecular simulation studies.^{14,15} Unfortunately, the speed of data generation has not eliminated the seemingly traditional disagreements among researchers and their models for catalytic function. Discussed below are some key points of contention, along with some concepts and facts that can help sort through the complex characteristics of the active site formed by twister ribozymes.

Typically, there are three problems that commonly lead to difficulty in reaching mutual understanding for each self-cleaving ribozyme mechanism. The first problem is that researchers frequently use different terms to discuss the same catalytic effects. Based on the words used by our community, a new scholar to this field might infer that self-cleaving ribozymes can draw from as few as two or as many as six or more possible catalytic strategies to promote RNA strand scission by internal phosphoester transfer. To help overcome this confusion, we developed a simplified framework⁶ for understanding the strategies that could be used by any catalyst, regardless of whether it is made from protein, RNA, or another medium entirely. In this framework (Figure 1), there are only four predominant catalytic strategies that contribute to the catalytic rate enhancement of RNA cleavage by internal phosphoester transfer. Specifically, these catalytic strategies include (**α**) in-line geometry adopted by the reactive atoms, (**β**) neutralization of the negative charge on a non-bridging phosphate oxygen, (**γ**) deprotonation of the 2'-oxygen nucleophile, and (**δ**) neutralization of the developing negative charge on the 5'-oxygen leaving group.

Confusion ensues when researchers begin to overlay various different frameworks for rationalizing the mechanisms of catalysts. Particularly problematic is conflation of *concepts* like 'catalysis by approximation' or 'transition state stabilization' with various individual *strategies* that can be used to hold reactive groups in close proximity or stabilize a transition state. General concepts are very effective for analyzing and comparing the function of diverse enzymes. However, these concepts do not constitute additional catalytic strategies to be included with the list of four strategies described above. Indeed, how can a ribozyme stabilize the pentacoordinate intermediate (Figure 1, middle structure) during RNA cleavage? It must use one or more of the α , β , γ , and δ catalytic strategies. Importantly, one cannot count these four catalytic strategies more than once simply by renaming them in the context of the transition state stabilization framework. Furthermore, individual catalytic strategies employed to stabilize the transition state can be achieved in multiple different ways, but their outcomes can be the same. For example, either protons (via the action of specific acid or general acid), or the close approach (e.g. inner-sphere coordination) of a metal cation could be used to promote β or δ catalysis, but the maximal catalytic rate enhancements are effectively the same regardless of the source of the positive charge.⁶

The second problem involves diverse biophysical datasets and the divergent structural models that can result. For twister ribozymes, the x-ray datasets reveal either that a Mg^{2+} ion is precisely positioned in the active site to promote catalysis,^{11,12} or that the active site is entirely devoid of metal ions!^{9,10} Since subsequent molecular dynamics simulations were built upon these disparate structural models, computer modeling therefore yields very

different predictions of certain aspects of the RNA cleavage mechanism. For example, simulations indicate either that the adenosine nucleotide located immediate downstream of the labile phosphodiester linkage (named A1, Figure 2) acts as a general acid to help stabilize the developing negative charge on the 5'-oxygen leaving group (Figure 1, δ catalysis),¹⁴ or that it cannot act as a general acid.¹⁵ Thus, the disparity among the x-ray structure models confounds subsequent efforts to unify the conclusions regarding what catalytic strategies are used by twister ribozymes. Perhaps as starting points, each computer simulation campaign could consider all biophysical data sets available for the ribozyme of interest to avoid embracing only a single potentially flawed model.

To be clear, securing high-resolution structural models of self-cleaving ribozymes is not easy. We most desire a snapshot of the precise structure at the active site that reveals all the catalytic strategies in action. Of course, under such conditions the RNAs would rapidly self-cleave, which would likely only yield data on the cleaved state of the ribozyme and a distorted view of the active site. Therefore, all structural models of twister have used modified substrate molecules that are missing the nucleophile (2'-deoxy),^{9,10,11} or that carry a methylated nucleophile (2'-O-methyl).¹² As a result, we are trying to model the active site from non-ideal data, and using related biochemical data and our intuition (guesses or simulations) to predict the catalytic state. Substrates modified in this way can distort the active site to give structure models that do not have the reactive atoms in the right geometry. We know that the labile linkage needs to conform to an in-line configuration, but there is plenty of room for error when making such adjustments.

The third problem is that several research groups can generate essentially the same biochemical data, but then draw very different conclusions from these near-identical findings. To illustrate this problem, in the following paragraphs I will describe in detail the challenges of conducting and interpreting experiments with sulfur-modified ribozyme substrates. Three groups^{8,12,13} have evaluated the effects of replacing the two non-bridging oxygen atoms (called *pro-R* and *pro-S*) (Figure 3A) with sulfur atoms at the site of RNA strand scission. Initially, my laboratory made two claims regarding the effects of sulfur at these two positions. Since our phosphorothioate substrate was not separated into the two distinct *pro-R* and *pro-S* isomers, we could only conclude (i) that at least one of the two positions was a contact point for β catalysis by the active site and (ii) that neither position was likely to be involved in forming an inner-sphere interaction with a divalent metal ion during catalysis.⁸

Subsequently, the laboratories of Ronald Micura¹² and David Lilley¹³ generated very similar data by using stereochemically pure phosphorothioate substrates. Both laboratories determined that there is a strong (nearly two orders of magnitude) disruption of ribozyme activity when a sulfur atom replaces the *pro-R* non-bridging oxygen at the cleavage site. Moreover, the sulfur-mediated reduction in rate constant cannot be rescued by supplementing the reaction mixture with a thiophilic metal ion such as Mn^{2+} or Cd^{2+} . These findings suggest that the *pro-R* atom is not serving as a site for inner-sphere coordination of a metal ion to promote β catalysis,^{12,13} just as was more generally concluded previously.⁸ In other words, we all seem to agree that the ribozyme is achieving β catalysis by forming a

contact with the *pro*-R oxygen, but without exploiting this atom as a direct ligand to a divalent metal ion.

All models of the active site proposed so far have suggested that the *pro*-R atom is involved in forming a hydrogen bond either with the strictly conserved guanosine nucleotide located at the base of the right shoulder of stem P2 (Figure 2, G33),^{12,13,16} or possibly with the adenosine nucleotide located immediately 3' of the guanosine.¹⁰ Both model possibilities correspond well with the phosphorothioate biochemistry data. A sulfur atom at the *pro*-R position disrupts this contact with a ribozyme functional group, and this disruption cannot be rescued by using a thiophilic metal ion. By using functional group mutagenesis, the Lilley group¹³ provided compelling evidence that *pro*-R oxygen hydrogen bonding with the 2-amino group of G33 is the most likely source of β catalysis (Figure 3B).

With β catalysis achieved via ribozyme action at the *pro*-R oxygen, is there any useful role for the *pro*-S oxygen? Both the Micura and Lilley laboratories also demonstrate^{12,13} that replacing the *pro*-S oxygen atom with sulfur has essentially no effect on the maximum rate constant for the ribozyme reaction when conducted with Mg^{2+} as the sole divalent metal ion, which again is consistent with our previous claim.⁸ However, an odd characteristic of the reaction results complicates the subsequent interpretations. The supplementation of the reaction with a thiophilic metal ion appears to reduce somewhat the rate constants observed with the normal substrate, whereas the *pro*-S phosphorothioate substrate is processed normally. This gives the appearance of a very modest thiophilic metal “rescue” of the reaction rate constant, which (along with x-ray data)¹¹ is used as support by the Micura lab to create an active site model wherein a metal ion is a critical catalytic component.^{12,16} In contrast, the Lilley lab states that this very modest thio effect at the *pro*-S position “leaves open the possibility of a specific interaction between a Mg^{2+} ion and the scissile phosphate”.¹³ However, they use these data (again supported by their own x-ray results)^{9,10} to propose a model for the active site that is devoid of divalent metal ions.¹³ Alas, even with near identical biochemical results, the two models are in complete opposition.

Even without generating additional data, there is a path forward to address these discrepancies. Specifically, more detailed considerations of the possible role of the *pro*-S oxygen in catalysis reveal that it is very unlikely that a metal ion is present at the active site to promote RNA strand scission. Are the characteristics of the *pro*-S phosphorothioate substrate consistent with metal-dependent β catalysis? If the *pro*-S oxygen were an inner-sphere ligand for a divalent metal ion, then the presence of a sulfur atom at this position should have dramatically eroded the affinity of Mg^{2+} at this binding site. Any loss of Mg^{2+} affinity at this binding due to insertion of a *pro*-S sulfur atom should have directly and adversely affected β catalysis, unless of course the concentration of divalent metal ion was well above the dissociation constant (K_D) for this altered binding site (which seems unlikely). Moreover, when a more thiophilic Mn^{2+} or Cd^{2+} is combined with Mg^{2+} in the reaction mixture,¹² the ribozyme reaction is only negligibly increased (up to ~2-fold above the rate constant measured with Mg^{2+} alone). Again, this result suggests that there was no loss of divalent metal binding affinity at the *pro*-S site, and therefore no loss of rate enhancement via β catalysis to restore by saturating this phosphorothioate substrate using a thiophilic metal ion. Finally, twister ribozymes retain most activity when Mg^{2+} is replaced

with cobalt hexammine,⁸ which is a mimic of fully hydrated Mg^{2+} ions. Since cobalt hexammine cannot form inner-sphere interactions, any role for such an interaction at the *pro*-S oxygen must be dispensable without severely affecting the rate constant for twister ribozymes.

Can an enzyme promoting RNA scission by internal phosphoester transfer gain anything by interacting with both non-bridging phosphate oxygen atoms simultaneously? This appears to not be the case for RNA phosphodiester linkage degradation via specific acid catalysis occurring free in solution. The kinetic characteristics of the acid-promoted reaction are consistent with only two protonation events,^{6,17} and these have been interpreted as (i) neutralization of the charge on one of the two non-bridging phosphate oxygen atoms ($\text{p}K_{\text{a}} \sim 1$) and (ii) protonation of the 5'-oxygen leaving group ($\text{p}K_{\text{a}} \sim 3.5$). These two events involving substrate protonation constitute β and δ catalysis, which become more optimized as the pH falls. Compared to specific acid catalysis in solution, the effect of β catalysis might already be achieved by twister ribozymes via the interaction of a general base (G33) with the *pro*-R oxygen. Therefore, a metal ligand simultaneously located at the *pro*-S atom seems superfluous.

However, there is some evidence that RNase A, the well-studied protein enzyme that catalyzes the same chemical transformation, might interact with both non-bridging phosphate oxygen atoms of its RNA substrate.^{18,19} This leaves open the possibility that twister RNAs are forming an active site that, like its sophisticated protein counterpart, might exploit interactions with both the *pro*-R and *pro*-S atoms. Moreover, it does seem possible that interactions between the ribozyme active site and both non-bridging oxygen atoms might be a way to maximize β catalysis if each independent interaction were imperfect. In other words, such interaction redundancy might serve as a sort of β catalysis buffer to overcome a deficiency in either single interaction. A survey of the predominant active site models for the hammerhead,²⁰ hairpin,²¹ HDV,²² VS,²³ and *glmS*²⁴ self-cleaving RNA classes reveals that none of these five natural ribozyme classes exploit simultaneous ligands to both non-bridging phosphate oxygen atoms, although there is some evidence that hairpin ribozymes might make both contacts.²⁵ Thus, most other self-cleaving ribozyme classes appear to attain their high speeds without using this structural feature, and so twister ribozymes would be very unusual if they did.

Since the phosphorothioate substrate data actually disfavors a model involving direct and robust β catalysis by inner-sphere coordination with a metal ion, what other data supports this model? Two x-ray crystallography studies^{11,12} generated data supporting an active site model wherein a Mg^{2+} ion forms an inner-sphere interaction with the *pro*-S oxygen. Unfortunately, these structures only exist when a highly-conserved substructure (stem P1, Figure 2) is not formed. Indeed, formation of this base-paired P1 stem would preclude the formation of active site structure containing the metal ligand. Although twister ribozymes with truncated 5' ends that cannot form P1 still promote RNA strand scission, RNA constructs with base-paired P1 stems promote catalysis much more efficiently.⁸ Also, the presence of a P1 hairpin in the type P3 and type P5 ribozyme configurations (Figure 2) strongly suggests that this region indeed base-pairs. Therefore, active site models built with

incomplete or misfolded substructures might not be reflective of the ribozyme's true active state.

There are also other features of the active site of twister ribozymes that were not discussed above (Figure 3B). For example, there is general agreement that the labile RNA linkage must adopt an in-line geometry to undergo productive nucleophilic attack by the 2'-oxygen atom, and that the ribozyme therefore promotes α catalysis. Also, there is evidence suggesting that the 5'-oxygen leaving group is protonated by the N3 group of the adenosine nucleotide (A1, Figure 2) located immediately 3' of the cleavage site.^{10,13} The adenosine N3 seems like a very poor functional group for a general base to promote δ catalysis, but it is important to note here that enzymes or ribozymes do not have to employ each catalytic strategy to its maximal efficiency to operate with a speed so great that it approaches other chemical limitations.^{6,7} Finally, there is some ambiguity in the models regarding the source of γ catalysis (deprotonation of the 2'-hydroxyl group), which now seems certain to be the N1 of nucleotide G33.¹³ Regardless, the high speed of twister ribozymes could be attained even when discounting any effect of γ catalysis, we have noted that the pH profiles for members of this ribozyme class are consistent with a shift in pK_a of the 2'-hydroxyl group (normally ~ 13.7) to near neutral. Thus, it is possible that twister ribozymes employ all four catalytic strategies, each at least with partial efficiency, to promote rapid RNA strand scission.

As has happened for all five of the classes of self-cleaving ribozymes whose discovery preceded twister, additional biochemical and structural data will be needed to bring complete clarity to the mechanism of this ribozyme class. Coming to agreement on the catalytic strategies used by twister cannot come too soon, as additional data on the structures and mechanisms of the newest self-cleaving ribozyme classes called twister sister,²⁶ hatchet²⁷ and pistol²⁸ are already beginning to be published.^{29,30} Moreover, it is almost certain that other natural self-cleaving ribozyme classes await discovery. To assess the mechanisms of these new ribozyme classes, we would do well to use a common mechanistic language, accurately interpret all the various biochemical results, and cautiously interpret structural models based on x-ray data. Careful attention to the possible catalytic strategies and their fundamental limitations will help make the path to mechanistic clarity for each of these ribozymes much less stormy than those of the past.

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Catalytic Strategy

- α = Arrange the 2' oxygen, phosphorus, and 5' oxygen atoms to adopt an in-line attack geometry
- β = Neutralize the negative charge on the non-bridging phosphate oxygen
- γ = Deprotonate the 2' oxygen
- δ = Neutralize the developing negative charge on the 5' oxygen

Figure 1.

The predominant catalytic strategies for cleavage of the RNA backbone by an internal phosphoester transfer mechanism. The theoretical basis for each of these catalytic strategies is described in detail elsewhere.⁶ This figure has been adapted with edits from a previous publication.⁸

Figure 2.

Consensus sequence and secondary structure model for twister ribozymes. Red, black and gray nucleotides represent >97%, >90%, and >75% conservation among natural representatives. Red circles indicate that a nucleotide of any sequence identity is present in >97% of the representatives. Green shading indicates strong evidence for sequence covariation to maintain base-pairing. Base-paired stems P1, P2 and P4 are depicted, whereas P3 and P5 stems are optional. Catalysis at the cleavage site (arrowhead) is promoted in part by highly-conserved nucleotides A1 and G33 (Figure 3B). Type P1, Type P3 and Type P5 configurations are present naturally, and are named for the stem that lacks a hairpin loop. Figure adapted from a previous publication.⁸

Figure 3. Phosphorothioate substrates and the twister ribozyme mechanism. (A) Replacing the *pro*-R oxygen atom with sulfur causes a substantial (~ 100 -fold) loss of twister ribozyme activity, whereas replacing the *pro*-S oxygen with sulfur has a marginal (~ 2 -fold) effect. (B) A mechanism for twister ribozymes that is consistent with the biochemical data. Ambiguity in x-ray crystal forms does not rule out an unlikely role for a Mg^{2+} ion coordinated to the *pro*-S oxygen.