

How to Kinetically Dissect an RNA Machine

Rhiju Das* and Rick Russell*



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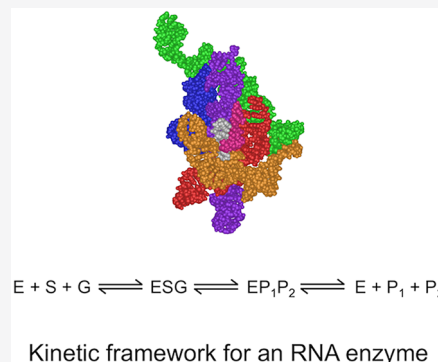
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ABSTRACT: RNA-based machines are ubiquitous in Nature and increasingly important for medicines. They fold into complex, dynamic structures that process information and catalyze reactions, including reactions that generate new RNAs and proteins across biology. What are the experimental strategies and steps that are necessary to understand how these complex machines work? Two 1990 papers from Herschlag and Cech on “Catalysis of RNA Cleavage by the *Tetrahymena thermophila* Ribozyme” provide a master class in dissecting an RNA machine through kinetics approaches. By showing how to propose a kinetic framework, fill in the numbers, do cross-checks, and make comparisons across mutants and different RNA systems, the papers illustrate how to take a mechanistic approach and distill the results into general insights that are difficult to attain through other means.



Breakthrough discoveries in the early 1980s led to the startling realization that chemical catalysis in biology was not the exclusive domain of proteins. Indeed, it was demonstrated that some RNAs also catalyze chemical reactions. These discoveries had profound implications for biology, providing strong support for an early form of life in which RNA provided both information storage and the catalytic machinery to maintain and propagate it. They also propelled an explosion of interest in RNA biology and biotechnology that revealed RNA catalysis in the similarly intricate machines that underlie the fundamental processes of splicing and protein translation and ultimately uncovered the existence of CRISPR-Cas RNA-protein machines that are now being repurposed for medicine.¹ In addition, the discoveries introduced the possibility of a new field of enzymology, with comparisons between the catalytic strategies of RNA and protein catalysts, and they introduced key model systems for this novel field. The *Tetrahymena* group I self-splicing intron was one of the first two RNA molecules demonstrated to have catalytic activity, along with the RNA component of the bacterial ribonucleoprotein RNase P.^{2,3} The intron appears to be a selfish element, excising itself from a rRNA of the ciliate *Tetrahymena thermophila*, likely without any functional impact on the host organism. Despite the lack of obvious biological significance, the intron’s thermostability and its biochemical properties, most notably its ability to carry out a fascinating multistep splicing reaction without any protein cofactors, helped it become a widespread model system for RNA structure, folding, conformational changes, and catalysis, with much of the pioneering work carried out in the Cech lab.

The first few years of the Cech lab’s work on the *Tetrahymena* intron RNA contributed important advances by defining the

reaction steps and nucleotide positions of cleavage and ligation,^{4,5} defining key structural elements within the intron,^{6–8} and generating a “ribozyme” version of the intron that lacks the exons and functions as a true multiple-turnover enzyme by binding and cleaving an oligonucleotide mimic of the 5′ splice site.^{9,10} However, there remained relatively little known about the reaction kinetics and mechanism, and early forays in this area produced puzzling results, such as the finding that mismatches between the oligonucleotide substrate and the ribozyme could actually increase the observed reaction rate of the substrate.¹¹ In 1990, two papers by Herschlag and Cech that were published back to back in *Biochemistry* had an enormous impact on the understanding of the *Tetrahymena* group I intron ribozyme as a catalyst.^{12,13} Beyond the immediate impacts of the specific results for understanding and future studies of the *Tetrahymena* ribozyme, these papers transformed the field of RNA enzymology, enabled breakthrough studies of RNA folding, and had lasting impacts on biochemical studies of ribonucleoprotein enzymes that act on RNA or DNA and use base pairs for substrate recognition. On a personal note, the papers also impacted our own research in fundamental ways during our time as trainees in the Herschlag lab and in our own subsequent independent careers, points to which we return below.

The *Tetrahymena* intron catalyzes its own removal from the precursor RNA (self-splicing) using a chemical mechanism that

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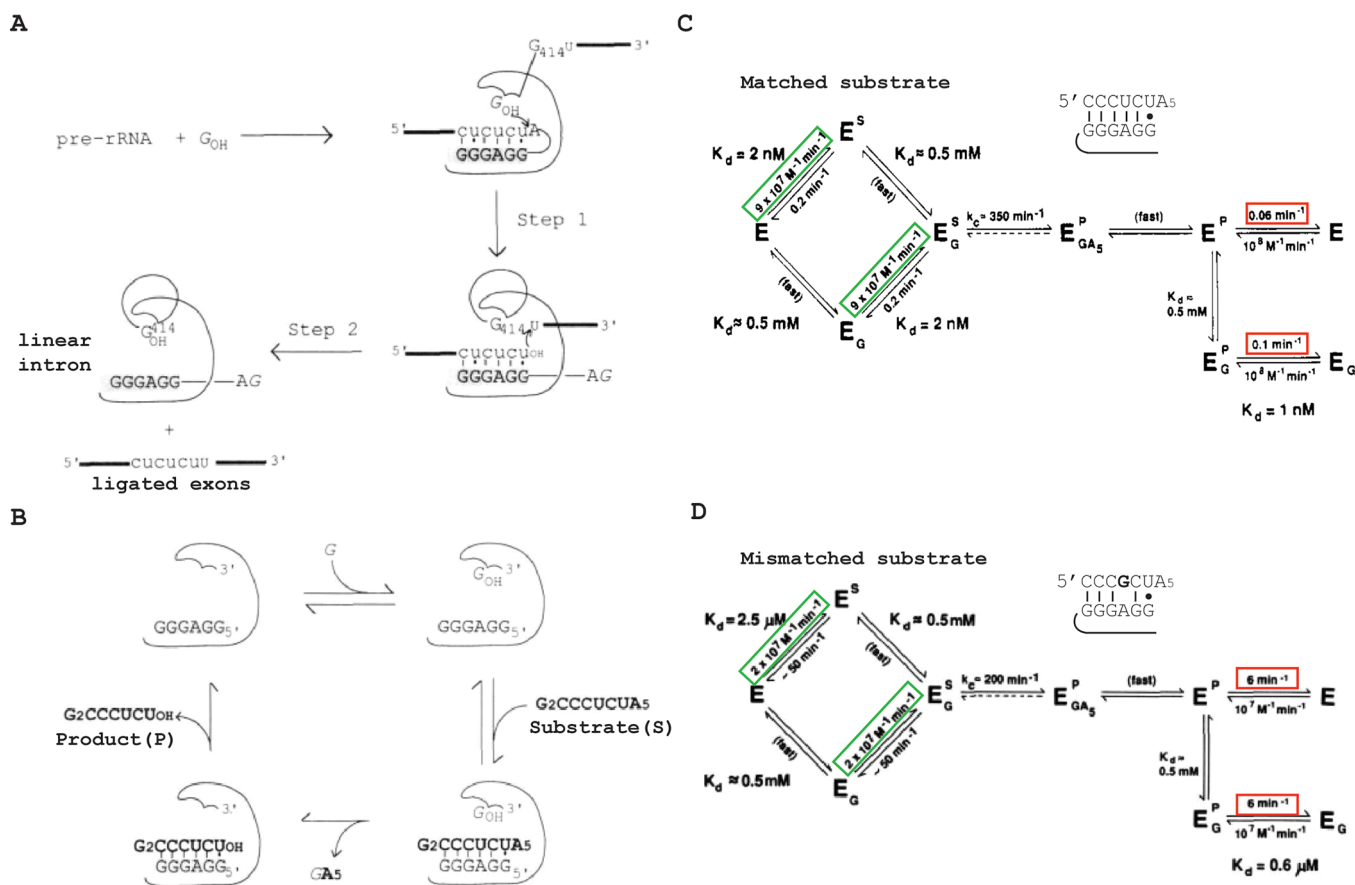


Figure 1. Reactions of the *Tetrahymena* group I intron and ribozyme. (A) Self-splicing reaction of the intron. Starting from the pre-rRNA containing the intron, the two-step splicing reaction produces the ligated exon product and free linear intron. (B) Reaction of the ribozyme. The substrate (S) binds the ribozyme by base pairing, mimicking interactions formed by the 5' splice site region in panel A. Substrate cleavage is mediated by a guanosine nucleotide from solution (G) in a reaction that is analogous to the first step of splicing. (C and D) Kinetic frameworks for ribozyme-catalyzed reactions with a matched substrate and a mismatched substrate at position -3 , respectively. Schematics of the substrates and base pairs with the ribozyme are shown above each framework. Product release rate constants are highlighted with red boxes, and substrate binding rate constants are highlighted with green boxes. All panels are adapted from refs 12 and 13.

is shared by all group I introns and consists of two steps. In the first step, the 5' splice site is cleaved by an exogenous guanosine nucleotide, and in the second step, the liberated 5' exon attacks the 3' splice site to generate the products: the ligated exons and linear intron (Figure 1A). The ribozyme is a shortened version of the intron that lacks the exons and can bind an oligonucleotide substrate that mimics the 5' splice site (Figure 1B). It can also bind a guanosine nucleotide from solution to cleave the substrate in a reaction that mimics the first step of splicing. In the first of the two papers, Herschlag and Cech used single-turnover kinetics methods to measure rate and equilibrium constants for nearly all of the individual steps of this minimal reaction. The result is a "kinetic framework" for the oligonucleotide cleavage reaction of this RNA (Figure 1C). In the second paper, they used the same methods to analyze the reaction of a substrate that included a mismatch with the intron RNA (Figure 1D), providing an immediate application of the framework to resolve a paradox in RNA enzyme specificity, described below.

It is clear from a casual inspection of panels C and D of Figure 1 that a completed kinetic framework is rich with information. The most immediately groundbreaking aspect of this work was that individual steps of the reaction were measured, achieving a level of mechanistic dissection that was unprecedented for an

RNA system. In addition, a distinctive feature was that some of the measurement strategies were unconventional and highly creative. For example, a key parameter that had previously been mysterious, and turned out to be quite important for understanding earlier results, was the rate constant for release of one of the oligonucleotide products from the cleavage reaction (Figure 1B). In this paper, the rate constant for product release was determined for the first time with a novel strategy, by measuring the competition between the release of the product and an artifactual reaction of the product with a second molecule of the substrate.¹² [Later, the field developed a more direct method to measure this rate using an electrophoretic mobility shift assay (EMSA) and obtained consistent values.] An additional feature of the work was that the kinetic framework was highly rigorous and definitive, particularly because the measured rate and equilibrium constants were cross-checked where possible with independent experiments. For example, values of a key parameter that defines an enzyme's efficiency, k_{cat}/K_M were found to be equivalent in multiple-turnover and single-turnover reactions, as expected.

Overall, the *Tetrahymena* ribozyme, and catalytic RNA in general, turned out to be exceptionally well-suited for this type of dissection because key reaction intermediates are sufficiently long-lived that they can be populated by nearly the entire

population and/or give distinct kinetic phases, allowing individual reaction steps to be isolated and measured. Indeed, the time scales are long enough (seconds or minutes) that most of the rate constants could be measured by hand; remarkably, the entire reaction scheme could be characterized without specialized chemical syntheses, mixing equipment, or advanced spectroscopy. The entire study was carried out with radiolabeled RNA, hand-mixing, and gel electrophoresis of reaction products, establishing a paradigm for mechanistic enzymology of RNA that was then applied widely in the field (discussed below). The avoidance of specialized equipment also makes achieving a similar framework for new RNA-based systems feasible for any current student with access to standard gel equipment and an appetite for understanding molecular mechanisms. Aside from the difference that the modern practitioner might consider replacing the radiolabel with a fluorescent tag, the experimental strategies and methods used in these papers are just as relevant now as they were in 1990.

What did the kinetic framework reveal about RNA function? An immediate consequence was that the ribozyme reaction kinetics could be analyzed in terms of the individual reaction steps. Notably, previous studies revealed that the overall ribozyme reaction was quite slow, but these studies used multiple-turnover conditions, under which the ribozyme has to capture the substrate, catalyze phosphoryl transfer, release the products, and complete this cycle multiple times.^{10,11} As noted above, this new kinetic framework revealed that release of the product oligonucleotide is very slow, but it also showed that, under single-turnover conditions, the intrinsic ribozyme chemical reaction is actually quite fast. Indeed, for this first round of capturing the substrate and performing chemical catalysis, the RNA attains a level of rate acceleration that matches that of many protein enzymes. It even achieves a limited definition of a “perfect enzyme” by reaching a level at which substrate binding is rate limiting for the reaction under subsaturating conditions and occurs approximately as fast as possible for this binding reaction [$\sim 10^8 \text{ M}^{-1} \text{ min}^{-1}$, similar to the rate constant for RNA helix formation in solution (Figure 1C)]. In a nutshell, the first of these papers put RNA on the map as a “legitimate” catalyst.

The comparison of ribozyme substrate binding to solution RNA helix association rates is one of several examples of a new understanding that was derived from insightful comparisons of detailed kinetic parameters with measurements of other systems. As another example, comparisons of the substrate binding equilibrium provided evidence for tertiary interactions, as the substrate bound much more tightly than expected from nearest neighbor rules for pure helix formation, by ~ 6 kcal/mol, corresponding to 10^4 -fold stabilization. This insight about tertiary interactions at the ribozyme active site was suggested from previous studies by Sugimoto and Turner that examined interactions of a circular form of the intron with product oligonucleotides.^{14,15} Nevertheless, the insight was significantly strengthened through the isolation of the substrate binding step enabled by this study.

Paper 2 provided a further concrete example of the usefulness of the framework (Figure 1D).¹³ Prior to this work, there was a mysterious finding that a substrate that included a mismatch three nucleotides upstream of the cleavage site (changing a C to a G, abbreviated -3G) was cleaved by the ribozyme 100-fold faster than the fully matched substrate.¹¹ Paper 2 investigated the reaction steps of this mismatched substrate further and showed that the increased reactivity was in fact a straightforward

consequence of rate limiting product release, which limits the reaction rate of repeated turnovers (i.e., under steady state conditions). The mismatch in the substrate weakens the ribozyme's hold on the product of the reaction; that faster release, in turn, enables the next substrate to be bound by the ribozyme faster, giving an overall acceleration when multiple enzyme turnovers are measured. In contrast, for a single enzyme turnover, the mismatched substrate was no more reactive than the matched substrate because there is no longer a need for the product to be released.^{12,13} Under these conditions, the mismatch was shown to actually decrease the reaction rate slightly. The relatively small magnitude of this effect (<5 -fold) turned out to be because substrate binding is rate limiting for the matched target, and the mismatch has just a small effect on the binding rate constant of the substrate. Notably, the mismatch was shown to substantially decrease the binding affinity of the substrate (by ~ 1000 -fold), but this weakened binding arises almost entirely from a large increase in the rate constant for dissociation, not the substrate binding rate.

An enduring impact from this set of results is on thinking about specificity from base pairing interactions. The result that the -3G substitution had a very large effect on substrate affinity but a much smaller effect on the binding rate constant (4.5-fold) resulted in the ribozyme displaying little discrimination against the mismatch, as substrate binding was shown to be fully rate limiting under subsaturating concentrations for the matched substrate and partially rate limiting for the mismatched substrate. The ideas that (1) base pairs are stable enough that binding is often rate limiting and (2) only a few base pairs are formed in the transition state for helix binding and therefore contribute to specificity were developed further in a *Proceedings of the National Academy Sciences of the United States of America* paper that used the data from paper 2 to make the point that additional base pairs in a substrate recognition sequence do not increase specificity indefinitely and can even decrease specificity.¹⁶ This effect arises because when the overall binding becomes sufficiently strong to be rate limiting for the downstream steps, only a fraction of the base pairs—the ones that form early in binding—contribute to specificity. The ideas had enduring value for thinking about broad groups of enzymes that select their targets by base pairing, notably the Ago complexes of RNAi and miRNAs and CRISPR-Cas endonucleases.¹⁷ For readers who are familiar with the notion of a special “seed” subregion of a target that is particularly important for the rates or specificity of RNA silencing or gene editing, consider that the underlying mechanisms can be understood from the kinetic frameworks first developed in these *Tetrahymena* ribozyme papers. The seed sequences are the sites of highest specificity, and they are also the base pairs that form first.

Arguably the most enduring value of the framework itself was that it allowed the design of future experiments, even ones using radically different experimental approaches or dissecting distinct RNA and RNA-protein systems, in a way that allowed measurement of the specific reaction step or steps of interest. The two papers hinted at the presence of tertiary contacts holding the substrate-containing helix to the ribozyme active site. A series of subsequent papers from Herschlag and Cech and then from the Herschlag group relied on the framework to thoroughly dissect the network of tertiary contacts that permit “docking” of the substrate-containing helix into the active site of the ribozyme (Figure 2A),^{18–20} resulting in atomic level insights that were confirmed and extended by structural studies on other

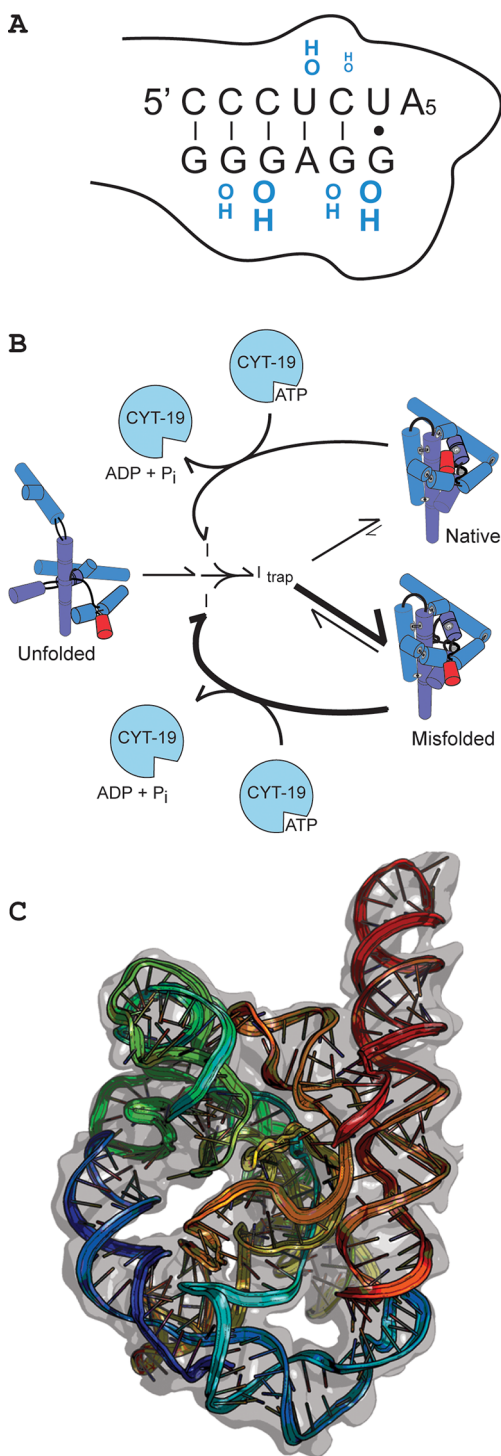


Figure 2. Further discoveries that were propelled by the kinetic framework. (A) Tertiary contacts of the *Tetrahymena* ribozyme's substrate-containing helix mediated by 2'-OH groups. The presence of a label (OH) indicates a tertiary contact contributing at least 0.5 kcal/mol to docking of the helix into tertiary contacts with the ribozyme core, and the size of the label increases with the strength of the tertiary contact to 3.0 kcal/mol.²⁰ (B) Kinetic folding pathway of the *Tetrahymena* ribozyme (shown as cylinders) and the impact of CYT-19, a DEAD-box RNA helicase protein with RNA chaperone activity. The arrow thickness corresponds to relative rates of individual steps. Assays for the time dependence of native ribozyme formation used catalytic activity and relied on the substrate binding, dissociation, and cleavage rate constants from the kinetic framework established by Herschlag and Cech. Panel B is adapted from ref 36 with permission.

Figure 2. continued

(C) Three-dimensional structure of the *Tetrahymena* ribozyme. The functional dissection of this ribozyme made it a first choice for piloting the application of cryo-EM to RNA-only systems (coordinates from Protein Data Bank entry 6LWS; cryo-EM density map in gray from EMD 21840).

group I introns.^{21,22} The expanded framework then enabled the first study of catalysis and folding of any RNA at the single-molecule level,²³ as well as studies that probed local folding steps during the catalytic cycle.^{24,25} In addition, a series of papers from the Herschlag group and colleagues used the framework and atomic mutagenesis and rescue experiments to illuminate the roles of specific Mg²⁺ ions in the active site,^{26,27} attaining a deep atomic level understanding of how metal-dependent RNA catalysis works and setting a paradigm for understanding mRNA splicing within human cells.²⁸

Beyond the catalytic reaction that occurs on the folded ribozyme, the framework of Herschlag and Cech turned out to be critical for dissecting the kinetic folding pathway of the RNA. While the structure of the intact *Tetrahymena* ribozyme remained unsolved for many years, the kinetic framework was used by one of us (Russell) to design an enzymatic assay through which catalytic activity could be used as a probe of native folding of the ribozyme, revealing a multibranch pathway involving native, partially folded, and misfolded states (Figure 2B). This complete framework encompassing both folding and catalysis was further leveraged to learn about helicase proteins as chaperones of RNA folding,^{33–35} including the surprising result that chaperones can “misfold” RNAs under conditions in which the native conformation is only marginally more stable than a misfolded conformation.³⁶ Finally, having a clearly interpretable functional assay motivated making the *Tetrahymena* ribozyme a first choice for piloting the application of cryogenic electron microscopy (cryo-EM) to RNA-only systems in recent work including one of us (R.D.) (Figure 2C).^{37–39}

Overall, the two papers comprising Herschlag and Cech's “Catalysis of RNA Cleavage by the *Tetrahymena thermophila* Ribozyme” greatly advanced the fields of RNA enzymology and biophysics, with impacts over three decades. The papers provided a foundation for interpreting past studies and guiding future studies on a paradigmatic model system for RNA structure and function. They also illuminated new principles, including the concept of a “perfect enzyme” for RNA and how RNA systems can have minimal specificity against mismatched base pairs when binding is rate limiting for the overall reaction. Finally, the papers more broadly transformed the field of RNA catalysis both by demonstrating the power of a kinetic framework for the catalytic reaction and by providing a generalizable blueprint for how to obtain such a framework, inspiring analogous work on systems ranging from small nucleolytic ribozymes and group II introns (from which eukaryotic spliceosomes likely descended) to completely non-natural RNA, and even DNA, enzymes that were evolved *in vitro*.^{40–45} Although each system presents its own challenges, which must be confronted on a case-by-case basis, the general strategies outlined in these papers are broadly applicable, as demonstrated by the very large range of RNAs, RNA-protein complexes, and even protein enzymes to which they have been successfully applied by investigators who honed their craft within the Herschlag group.^{46–50} Indeed, current and future scientists who wish to understand and re-engineer RNA-guided

and RNA-based machines will want kinetic frameworks for their systems, too, and there's no better place to see and learn how it is done than Herschlag and Cech's two papers.

AUTHOR INFORMATION

Corresponding Authors

Rhiju Das – Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305, United States; Department of Physics, Stanford University, Stanford, California 94305, United States; orcid.org/0000-0001-7497-0972; Email: rhiju@stanford.edu

Rick Russell – Department of Molecular Biosciences, The University of Texas at Austin, Austin, Texas 78712, United States; orcid.org/0000-0002-4879-8563; Email: rick_russell@cm.utexas.edu

Complete contact information is available at:

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