

An Ontology for Facilitating Discussion of Catalytic Strategies of RNA-Cleaving Enzymes

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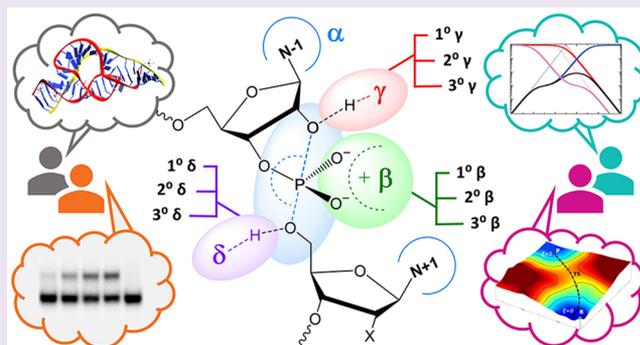
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ABSTRACT: A predictive understanding of the mechanisms of RNA cleavage is important for the design of emerging technology built from biological and synthetic molecules that have promise for new biochemical and medicinal applications. Over the past 15 years, RNA cleavage reactions involving 2'-O-transphosphorylation have been discussed using a simplified framework introduced by Breaker that consists of four fundamental catalytic strategies (designated α , β , γ , and δ) that contribute to rate enhancement. As more detailed mechanistic data emerge, there is need for the framework to evolve and keep pace. We develop an ontology for discussion of strategies of enzymes that catalyze RNA cleavage via 2'-O-transphosphorylation that stratifies Breaker's framework into primary (1°), secondary (2°), and tertiary (3°) contributions to enable more precise interpretation of mechanism in the context of structure and bonding. Further, we point out instances where atomic-level changes give rise to changes in more than one catalytic contribution, a phenomenon we refer to as "functional blurring". We hope that this ontology will help clarify our conversations and pave the path forward toward a consensus view of these fundamental and fascinating mechanisms. The insight gained will deepen our understanding of RNA cleavage reactions catalyzed by natural protein and RNA enzymes, as well as aid in the design of new engineered DNA and synthetic enzymes.



1. RNA CLEAVAGE REACTIONS

The subject of the present Perspective is RNA cleavage reactions^{1–4} catalyzed by nucleolytic RNA enzymes (ribozymes),^{5,6} as well as protein enzymes (ribonucleases)⁷ and several artificially engineered DNA enzymes (DNAzymes).^{8,9} While illustrative examples and discussion focus on ribozyme mechanisms, the concepts and terminology that we develop are equally applicable to protein and DNA enzymes.

RNA strand cleavage by 2'-O-transphosphorylation is universal in biology and has far-reaching implications for medicine.^{10,11} The goal of gaining a predictive understanding of the catalytic mechanisms of RNA cleavage reactions in the context of well-studied biological systems is important from a fundamental scientific perspective, as well as from a technology engineering viewpoint.^{12,13} Elucidating the diverse array of mechanistic strategies exhibited by well-studied biological systems will enable general principles to emerge. These general principles may be transferable outside the biological context and applied to guide the design of synthetic systems, such as

xeno nucleic acids¹⁴ and Hachimoji DNA and RNA,¹⁵ which have great promise for new biotechnological applications.^{12,13}

The mechanism and kinetics of RNA cleavage by Bronsted acids and bases, and metal ions has been extensively studied,^{2,3} providing a foundation for understanding biological catalysis. In this reaction, the RNA O2' is activated by deprotonation and makes an in-line nucleophilic attack on the phosphorus atom of the adjacent scissile phosphate to form a pentavalent dianionic transition state (or metastable intermediate), followed by departure of the O5' leaving group as an oxyanion that ultimately becomes protonated to form 2',3'-cyclic phosphate and 5'-OH cleavage products (Figure 1, left).

Although concerted and stepwise mechanisms are both possible, an idealized transition state (or metastable intermediate) is a dianionic pentavalent phosphorane in a

Received: March 13, 2019

Accepted: May 16, 2019

Published: May 16, 2019

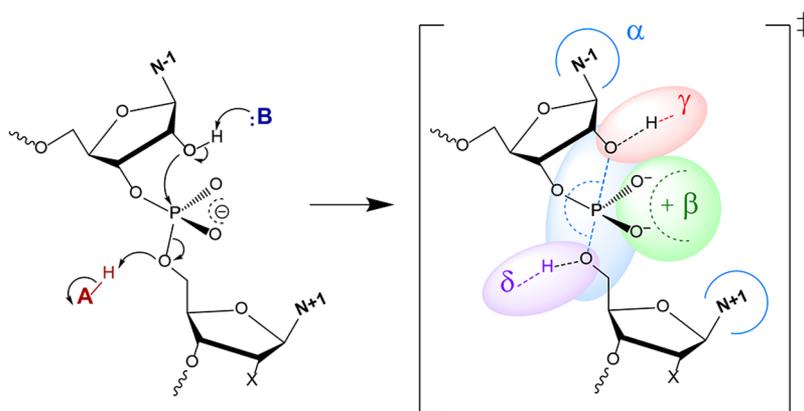


Figure 1. 2'-O-Transphosphorylation leading to cleavage of the RNA backbone (left of central arrow) and idealized transition state highlighting the general catalytic strategies¹⁶ (right of central arrow). α , Arrangement of the O2' nucleophile, P (of scissile phosphodiester bond), and O5' leaving group in an in-line attack geometry (facilitated by contacts, indicated by blue arcs, that splay the N-1 and N+1 nucleobases). β , Stabilization (neutralization/protonation) of the negative charge accumulation on the nonbridging phosphoryl oxygens (NPOs). γ , Activation (deprotonation) of the O2' nucleophile. δ , Stabilization (neutralization/protonation) of the accumulating negative charge on the O5' leaving group. Although this schematic uses a transition state model to illustrate the fundamental catalytic strategies, these strategies can impact any state along the reaction coordinate. Colored ovals highlight each strategy and encompass the primary atomic positions (defined in section 4 below) associated with the chemical space of bonds for each strategy.

trigonal bipyramidal geometry with nucleophile O2' and leaving group OS' ligands occupying apical positions (O2'–P–OS' angle near 180°)¹⁷ and the formal (2-) charge delocalized between the O2', OS', and nonbridge phosphoryl oxygens (NPOs) (Figure 1, right). Naturally occurring protein and RNA enzymes, as well as engineered enzymes such as DNAzymes, use multiple catalytic strategies in concert to enhance the rate of RNA cleavage by factors of typically 10⁵ to 10¹¹.^{1–4,16} Breaker has suggested a simplified framework for discussion of four basic catalytic strategies for RNA cleavage,¹⁶ designated α , β , γ , and δ , as illustrated in Figure 1. Concepts and terminology discussed in this Perspective apply to all enzymes catalyzing RNA cleavage via 2'-O-transphosphorylation; however we have focused our discussion and examples on small nucleolytic ribozymes.

2. NUCLEOLYTIC RIBOZYMES (SMALL SELF-CLEAVING RNAs)

Of key interest to the present discussion is how RNA cleavage is catalyzed by small catalytic RNA molecules known as nucleolytic ribozymes.^{5,6} Nucleolytic ribozymes serve as platforms for the design of new biomedical technology¹³ and therapeutics^{10,11} and as models for our understanding of RNA catalysis and its implications for theories of evolution.¹⁸ The central challenge is to gain a predictive understanding of precisely how these small RNA molecules, with their limited repertoire of chemical functional groups, are able to function as “high-speed” ribozymes.¹⁶ Such an understanding would enable molecular engineering and guide the design of new catalytic RNA-based technology¹³ and medicine.^{10,11}

The past 5 years have witnessed the discovery of new classes of nucleolytic ribozymes through comparative genomics¹⁹ and a doubling of the number of structurally characterized ribozymes since the first crystal structure almost 30 years prior.²⁰ These breakthroughs have fueled intense experimental and theoretical efforts directed toward elucidation of the detailed catalytic mechanisms of these ribozymes. The resulting whirlwind of research has sparked much debate and caused some confusion in the literature, creating barriers to progress. Recently, Breaker pointed out contributing factors to

these barriers and made a call to clean up the resulting “mechanistic debris” in order to help the community navigate to calmer conditions.²¹ The first factor identified by Breaker was that “researchers frequently use different terms to discuss the same catalytic effects”, and it was suggested that the community adopt the framework illustrated in Figure 1 for discussion of catalytic strategies for RNA cleavage.¹⁶

This framework has been useful in facilitating discussion of RNA cleavage reactions¹⁶ for over 15 years, but as more detailed mechanistic data emerge, there is need to accommodate increased specificity and resolution of our description of catalysis. Toward that end, we propose to develop an ontology for discussion of catalytic strategies of RNA cleaving enzymes that stratifies Breaker's original framework¹⁶ into different levels of contribution for each strategy in order to enable more precise interpretation of mechanism in the context of structure and bonding. Ontologies are used frequently in science to establish common taxonomy and structured vocabulary, as well as define conceptual entities and their inter-relationships within an application domain.²² For example, an RNA ontology consortium has been proposed to describe and characterize RNA sequences, secondary structure, 3D structure, and dynamics pertaining to RNA function.²³

3. CLARIFICATION OF TERMINOLOGY: “ROLE” VERSUS “EFFECT” AND NORMAL, INVERSE, AND RESCUE EFFECTS

In the following section, we define three “levels of contribution” (e.g., primary, secondary, and tertiary) for each catalytic strategy. These terms are applied to describe both “catalytic roles” and “catalytic effects”. Before proceeding to the different contribution levels, we clarify terminology that will be used to distinguish between “roles” and “effects”. These definitions pertain to catalytic strategies employed by a native enzyme in comparison to a mutant enzyme used to probe such catalytic strategies. Here a “role in catalysis” or “catalytic role” refers to the chemical mechanism by which an atom or functional group contributes to a specific strategy used by the native enzyme (e.g., the guanine N1 plays a primary role in γ catalysis by acting as a general base to deprotonate the

nucleophile). Alternatively, an “effect on catalysis” or “catalytic effect” is used to indicate the outcome of a measurement that results from probing a modification of the native enzyme (e.g., the guanine N1C chemical modification has a primary effect on γ catalysis by knocking out the general base heteroatom). In the presentation below, we frame our discussion in the context of catalytic “effects” that arise due to perturbations to the native system under a set of standard conditions, with the assumption that the translation of their meaning to “roles” in the native system is readily inferred, although exceptions can occur.

Underlying this discussion is the idea that catalytic effects can be probed experimentally by measuring the ratio (k/k'), where k and k' are the pseudo-first-order rate constants for the native and modified substrate/enzyme reactions, respectively, with no change in rate limiting step. Implicit in this analysis, and hence on the application of the proposed ontology that follows, is that chemistry is the rate-limiting step in the catalytic reaction, the considerations for which have been described in detail elsewhere.²⁴

Borrowing terminology from the kinetic isotope effect literature,²⁵ a *normal effect* is one that has a ratio greater than unity ($k/k' > 1$) and leads to a rate reduction, whereas an *inverse effect* is one that has a ratio less than unity ($k/k' < 1$) and leads to a rate enhancement. For example, removal of a key conserved functional group would impede catalysis and have a normal effect, whereas introduction of an enhanced leaving group or alleviation of an inhibitory interaction would promote catalysis and have an inverse effect. Finally, a *rescue effect* is one where the deleterious (normal) effect on rate (i.e., $k/k' > 1$) due to a single change to the native system (e.g., a mutation, chemical modification, or change in reaction conditions) is partially, fully, or overfully recovered by a second aggregate change (not necessarily of the same type) to the system.

4. DEFINITIONS OF PRIMARY, SECONDARY, AND TERTIARY CONTRIBUTIONS TO β , γ , AND δ CATALYTIC STRATEGIES

Our proposed ontology uses ideas and conventions from other areas of mechanistic enzymology and structural biology to extend the framework for discussion of catalytic strategies illustrated in Figure 1. In-line fitness²⁶ (α catalysis) imposes geometrical requirements needed to satisfy so-called Westheimer's rules for transition states in phosphate transesterification and hydrolysis.²⁷ Our ontology does not introduce additional stratification of α catalysis from Breaker's original framework.¹⁶ On the other hand, β , γ and δ catalysis strategies can be inherently associated with bonding or nonbonding interactions involving specific atoms. Using this association, we propose a decomposition of β , γ and δ catalysis into primary (1°), secondary (2°), and tertiary (3°) contributions. As mentioned above, when interpreting the outcomes of measurements (or calculations) used to probe mechanism, we will refer these contributions to a particular (β , γ , or δ) strategy as primary, secondary, and tertiary catalytic effects.

For each catalytic strategy, we first identify the chemical space of bonds that are either broken or formed along the reaction coordinate and the *primary atomic positions* associated with these bonds. Due to the strong influence of divalent metal ions in RNA cleavage,^{28,29} we include ionic bonding (direct inner-sphere coordination) as part of this chemical bonding space. Positions not associated with these bonds are *non-*

primary atomic positions. The designations of the following primary and nonprimary atomic positions are used to facilitate definitions of the primary, secondary, and tertiary catalytic effects.

The primary atomic positions are defined for each catalytic strategy as follows:

- **Primary β atomic positions:** the NPO positions themselves, any atoms directly involved in protonation of the NPOs (e.g., the proton itself and the heteroatom of the acid from which the proton was transferred), and any metal ion directly coordinated to the NPOs (atoms under the green oval in Figure 1); it does not include atoms on the acid not directly involved in a bond with the proton, nor does it include atoms that hydrogen bond to the NPOs.
- **Primary γ atomic positions:** the O2' position itself, any atoms directly involved in nucleophile activation (e.g., the O2' proton itself and the heteroatom of the base to which the proton is transferred), and any metal ion directly coordinated to the O2' position (atoms under the red oval in Figure 1); it does not include atoms on the base not directly involved in a bond with the proton, nor does it include nearby metal ions (not directly coordinated to the O2') that electrostatically influence the pK_a of the base.
- **Primary δ atomic positions:** the OS' position itself, any atoms directly involved in leaving group stabilization (e.g., the acid proton itself and the heteroatom of the acid from which the proton is transferred), and any metal ion directly coordinated to the OS' position (atoms under the purple oval in Figure 1). It does not include atoms on the acid not directly involved in a bond with the proton, nor does it include nearby metal ions (not directly coordinated to the OS') that electrostatically influence the pK_a of the acid.

With these definitions, we now adopt conventions used in the discussion of isotope effects to categorize catalytic effects as *primary* or *secondary*²⁵ and concepts from structural biology to introduce *tertiary* catalytic effects. Illustrative examples for each effect are given in a general context below and then are expanded in the section 5 to include specific examples of perturbations (with measurements and/or predicted values) that in some cases involve coupling of catalytic effects.

4.1. Primary (1°) Catalytic Effects. A *primary catalytic effect* is one that results from changes in the identity of the primary atomic positions, as defined above.

Illustrative examples include (1) disruption of the direct coordination of a catalytic divalent metal ion at the NPO position (e.g., thio substitution disrupting Mg^{2+} coordination) would give rise to a primary β effect, (2) a chemical modification that removes the general base heteroatom involved in activation of the nucleophile (e.g., guanine general base N1C knockout) would give rise to a primary γ effect, and (3) a chemical modification that removes the general acid heteroatom that donates a proton to the leaving group (e.g., adenine (N1) general acid N1C knockout) would give rise to a primary δ effect.

4.2. Secondary (2°) Catalytic Effects. A *secondary catalytic effect*, on the other hand, is the change in the electronic environment of the primary atom resulting from changes in the identity of nonprimary atomic positions (and thus is exclusively different from a primary catalytic effect).

Modifications leading to a secondary catalytic effect have an indirect influence on the bonding environment of the primary atoms without involving any change to their identity. This can occur through electrostatic, inductive, or stereoelectronic effects that perturb the underlying electronic structure of the bonds between primary atoms (e.g., through either remote chemical modification or short-ranged nonbonded interactions).

Illustrative examples include the following: (1) Elimination of a stabilizing hydrogen bond to the NPO (while otherwise not changing the structure of the active site), such as deletion of a nucleobase exocyclic amine that hydrogen bonds to the NPO,³⁰ would give rise to a secondary β effect. (2) Chemical modification at nonprimary atomic positions of the general base that changes the pK_a of the primary position (e.g., guanine general base N7C modification up-shifting the pK_a at the N1 position) would give rise to a secondary γ effect. (3) Replacement of a divalent metal ion acting as a general acid through a coordinated water molecule with a different metal ion that has a shifted pK_a would give rise to a secondary δ effect.

4.3. Tertiary (3°) Catalytic Effects. A tertiary catalytic effect reflects alteration of the position of the primary atoms resulting from modification of the structural scaffold or hydrogen bond network that organizes the enzyme active site. This alteration can lead to changes in (1) positions of key residues, functional groups, or protons in the active site, (2) interactions that support active conformations of the substrate itself, (3) binding modes or occupations of metal ions or other small molecules required for activity, or (4) orientation of solvent molecules that form hydrogen bond networks important for catalysis.

5. APPLICATION OF THE ONTOLOGICAL FRAMEWORK IN THE CONTEXT OF PERTURBATION STUDIES

Experimental studies where perturbations (mutations, site-specific chemical modifications, or changes in environmental conditions) probe specific catalytic strategies can provide deep insight into mechanism.^{24,31–33} As the nature of these perturbations and their coupling reach higher precision and greater complexity, there is need for the framework for mechanistic discussions to evolve and keep pace. The present ontology strives to achieve this.

We recognize that in some cases, hydrogen bonds and metal ion binding modes play both a structural and chemical role in catalysis, and it can be challenging to design experiments that allow their effects to be isolated. Nonetheless, theoretical methods can impose constraints that experimental methods cannot and enable quantitative deconstruction of catalytic effects according to elements of our proposed ontology, with additive or nonadditive contributions to catalysis.

We now illustrate the application of the proposed ontology using several examples that involve experimental measurements. We consider a perturbation that is introduced by a modification to the native system under a standard set of conditions. The measured outcome of a perturbation produces an overall catalytic effect, as discussed above, that can have many additive or nonadditive contributions. In the present ontology, these contributions can be deconstructed into primary, secondary, or tertiary β , γ , or δ effects. We thus use the terminology “a primary, secondary, or tertiary β , γ , or δ (catalytic) effect due to a perturbation” to describe one of

possibly many catalytic effects that result from a particular perturbation, rather than using the descriptors to characterize the perturbation itself (e.g., we would not say “a primary, secondary, or tertiary β , γ , or δ perturbation”). The specific examples below for β , γ , or δ catalytic effects are based on plausible catalytic strategies of the Varkud satellite (VSr), hammerhead (HHr), and twister (Twr) ribozymes, respectively, and are summarized in Table 1. In some cases, a

Table 1. Summary of Exemplary Primary, Secondary, and Tertiary Catalytic Effects

sys ^a	variant ^b	effects ^c	k/k' ^d	change ^e
VSr	A621:S(S _p)	1° β	$>10^3$	NPO thio substitution disrupts Mg ²⁺ binding
		2° γ		pK_a upshift of G638:N1 (due to loss of Mg ²⁺)
		3° γ		disrupt anchoring of G638 (due to loss of Mg ²⁺)
	G638I/A756(3cP)	2° β	140	NPO charge destabilization (loss of H-bonds)
		3° γ		disruption of G638:N1 (general base) positioning
		3° δ		disruption of A756:N1 (general acid) positioning
	G623(7cG)	3° β	$>10^3$	disruption of Mg ²⁺ binding pocket (G623:N7)
		2° γ		pK_a upshift of G638:N1 (due to loss of Mg ²⁺)
		3° γ		disrupt anchoring of G638 (due to loss of Mg ²⁺)
HHr	C17(dC)	1° γ	$>10^3$	nucleophile knockout (C17:O2' removal)
	G12(3cG)	2° γ	>200	pK_a upshift of G12:N1 (general base)
	A9G	3° γ	>300	disrupt anchoring of G12 (due to loss of H-bonds)
Twr	A1(3cA)	1° δ	$\gg 10^5$	general acid (A1:N3) knockout
	A1(1cA)	2° γ	0.05 ^f	pK_a upshift of A1:N3 (general acid)
	A1(2AP)	3° δ	$>10^5$	disruption of A1 position (due to loss of H-bonds)
		2° δ		pK_a down-shift of A1:N3 (due to loss of NPO interactions)

^aRibozyme systems: Varkud satellite (VSr), hammerhead (HHr), and twister (Twr) ribozymes. ^bIdentity of the chemical modifications to the wild-type (WT) ribozyme or substrate. ^cExemplary effects (discussed as “specific examples” in the text) are shown in bold and additional effects (indicated by “Note” in text) are light face. ^dRelative rates of the mutations to the native system for VSr,³⁴ HHr,^{35–37} and Twr.^{38,39} The k/k' value for A621:S(S_p) thio substitution for VSr is equivalent to k_O/k_S . ^eSummary of the change in the system associated with each catalytic effect. ^fBased on A19U variant.

perturbation may produce multiple catalytic effects (referred to as “functional blurring”). For these, the catalytic effects highlighted in the discussion are shown in boldface type under “effects” in Table 1, whereas the other catalytic effects, enumerated in the table but not discussed in the text, are shown in lightface type. Finally, the last subsection illustrates a more complex example involving the twister ribozyme (Figure 2 and Table 2) that has been a focal point of “mechanistic debris” in the recent literature.²¹

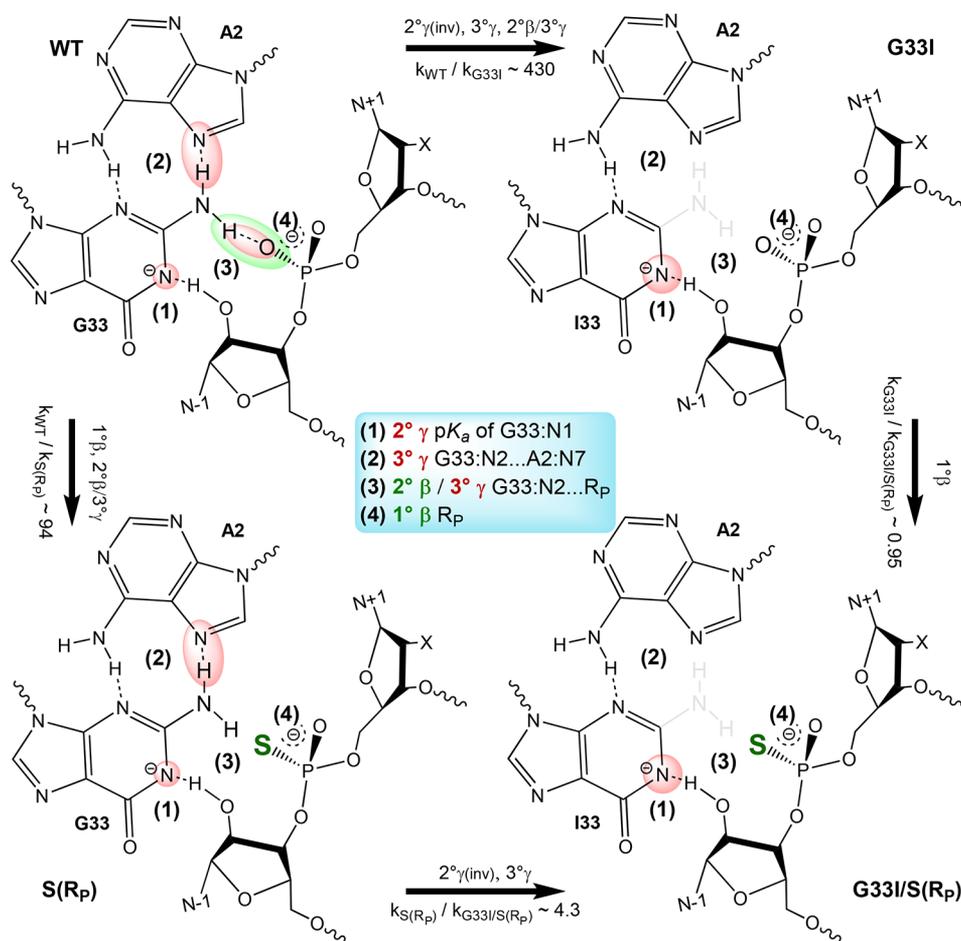


Figure 2. Application of ontology in describing the effects of G33I mutation and NPO thio substitutions of the substrate in the twister ribozyme (Twr). Summarized in the center are key interactions (1–4) that are highlighted in each variant or substrate by ovals in colors corresponding to their associated catalytic strategies, γ (red) and β (green). The wild-type (WT) Twr is shown in the top left, G33I mutant (top right), $S(R_p)$ substrate (bottom left), and G33I/ $S(R_p)$ (bottom right). Arrows indicate transformations between the different variants or substrates. Relative kinetic rates and catalytic effects resulting from each transformation are indicated with their associated arrows. Lack of color highlighting indicates an absence (or significant weakening) of an interaction present in the wild-type and has a normal catalytic effect, whereas emphasis of color or size of highlighting indicates an inverse effect (e.g., a larger red sphere at G33:N1 (1) indicates a favorable down-shifted pK_a).

5.1. Perturbations Affecting α Catalysis. Any mutation or chemical modification that impacts the in-line fitness of the reactive atoms (e.g., mutation that disrupts scaffold hydrogen bonds imposing the splay of $N-1$ and $N+1$ nucleobases flanking the scissile phosphate required for in-line fitness) is categorized as α catalytic effect. The present ontology does not distinguish tiers of α catalysis.

5.2. Perturbations Affecting β Catalysis. **5.2.1. Primary Beta ($1^\circ \beta$).** Substitution of the NPO by another atom (e.g., NPO thio substitution), alteration of conditions that disrupt or re-establish direct (inner-sphere) Mg^{2+} ion coordination to the NPO (e.g., titration with $Co(NH_3)_6^{3+}$ or thiophilic Cd^{2+} ions, respectively⁴⁰), or chemical modification of an acid donor heteroatom that prevents protonation of the NPO are examples of $1^\circ \beta$ effects.

As specific examples, VSr A621: $S(S_p)$ thio substitution³⁴ ($k_O/k_S > 10^3$) that leads to disruption of the direct coordination of a Mg^{2+} ion at the pro- S_p NPO of the scissile phosphate (A621) gives rise to a (normal) primary β effect, and recovery of the rate by titration with a thiophilic (Cd^{2+}) metal ion ($(k_O/k_S)^{Mg^{2+}} / (k_O/k_S)^{Cd^{2+}} > 60$) gives rise to a primary β rescue effect. Note (example of functional blurring),

this Mg^{2+} ion also plays secondary and tertiary roles in γ catalysis, tuning the pK_a of G638 (the implicated general base) and orienting it for nucleophile activation.

5.2.2. Secondary Beta ($2^\circ \beta$). Disruption of H-bond donation to the NPO from Mg^{2+} bound water or nucleobase functional group (e.g., removal of exocyclic amine of guanine, G:N2, or adenine, A:N6) constitutes a $2^\circ \beta$ effect.

As a specific example, VSr G638I/A756(3cP) double mutation³⁴ ($k/k' \approx 140$), where 3cP indicates 3-deazapurine, that leads to elimination of hydrogen bond donation from the exocyclic amines of G638 and A756 to the pro- R_p NPO of the scissile phosphate gives rise to a (normal) secondary β effect. Note (example of functional blurring), these functional groups also play a role in tertiary γ and δ catalysis, positioning the general base (G638) and acid (A756), respectively.

5.2.3. Tertiary Beta ($3^\circ \beta$). A change in structural scaffold or hydrogen bond network needed to support primary or secondary β catalysis (e.g., mutation of residue that anchors a nucleobase or disrupts a metal ion binding site in position to H-bond or electrostatically stabilize a scissile phosphate NPO) leads to $3^\circ \beta$ effects.

Table 2. Observed and Relative Rates for G33I Mutation and Substrate Thio Substitutions, $S(R_p/S_p)$, in Twister Ribozyme Compared to Wild Type (WT)

variant ^a	ions ^b	k_{obs} (min ⁻¹) ^c	k/k' ^d	k_O/k_S ^e	rescue ^f
WT		2.45 ± 0.04			
$S(R_p)$	Mg ²⁺	0.026 ± 0.002	94	94	
$S(S_p)$		3.3 ± 0.2	0.7	0.7	
WT	Mg ²⁺	0.8 ± 0.1	3.1		(metal)
$S(R_p)$	+	0.007 ± 0.002	350	114	0.8 ^g
$S(S_p)$	Mn ²⁺	3.7 ± 0.4	0.7	0.2	3.2 ^g
G33I		0.0057 ± 0.0005	430		(mutational)
G33I/ $S(R_p)$	Mg ²⁺	0.0060 ± 0.0003	410	0.95	99 ^h
G33I/ $S(S_p)$		0.004 ± 0.001	610	1.4	0.5 ^h

^aIdentity of the chemical modifications to the wild type (WT) ribozyme and substrate. ^bMolarity of each indicated divalent ion present is 10 mM. ^cAll experimental values from ref 38. ^d k/k' values are obtained using the WT in 10 mM Mg²⁺ conditions (first row). ^e k_O/k_S values are for oxo and thio substituted substrates for a given variant under a single set of ionic conditions. ^fRescue effects are shown for metal ion rescue, $(k_O/k_S)^{\text{Mg}^{2+}}/(k_O/k_S)^{\text{Mn}^{2+}}$, and mutational rescue, $(k_O/k_S)^{\text{WT}}/(k_O/k_S)^{\text{Mut}}$. ^gMetal rescue. ^hMutational rescue.

As a specific example, VSr G623(7cG) mutation³⁴ ($k/k' > 10^3$) that leads to disruption of a Mg²⁺ ion binding site gives rise to a (normal) tertiary β effect. Note (example of functional blurring), this Mg²⁺ ion also plays secondary and tertiary roles in γ catalysis, tuning the pK_a of G638 (the implicated general base) and orienting it for nucleophile activation.

5.3. Perturbations Affecting γ Catalysis. **5.3.1. Primary Gamma ($1^\circ \gamma$).** Modification of the nucleophile itself (e.g., 2'-deoxy, 2'-amino, 2'-O-methyl) or of the general base heteroatom that accepts the proton from the 2'-OH (e.g., 1cG) or direct (inner-sphere) coordination of a metal ion to the nucleophile to facilitate its activation (deprotonation) is considered a $1^\circ \gamma$ effect.

As a specific example, HHr C17(dC) mutation³⁵ ($k/k' > 10^3$) that leads to removal of the nucleophile gives rise to a (normal) primary γ effect.

5.3.2. Secondary Gamma ($2^\circ \gamma$). Remote (nonprimary) chemical modification or metal ion binding that affects nucleophile activation by proton transfer to a general or specific base constitutes a $2^\circ \gamma$ effect. This may include a pK_a shift of the general base itself (e.g., G → A, 7cG, 6sG, or G:O6-Mg²⁺) or a change in the acidity of the nucleophile through hydrogen bonding or outer-sphere metal ion coordination.

As a specific example, HHr G12(3cG) mutation³⁶ ($k/k' > 200$) that leads to up-shifting of the pK_a at the N1⁴¹ gives rise to a (normal) secondary γ effect.

5.3.3. Tertiary Gamma ($3^\circ \gamma$). Change in structural scaffold or hydrogen bond network needed to support primary or secondary contributions to γ catalysis (e.g., G → A mutation that disrupts the sugar/Hoogsteen edge base pair that positions G to act as the general base or competitive "over-determined" H-bonding that releases the 2'-OH from inhibitory interactions with the NPOs upon introduction of a pro-R_p thio group, thus giving rise to an inverse thio effect^{30,42,43}) leads to $3^\circ \gamma$ effects.

As a specific example, HHr A9G mutation³⁷ ($k/k' > 300$) that leads to disruption of the hydrogen bonding interactions

between the sugar edge of G12 and the Hoogsteen edge of A9 that anchors the general base (G12) gives rise to a (normal) tertiary γ effect.

5.4. Perturbations Affecting δ Catalysis. **5.4.1. Primary delta ($1^\circ \delta$).** Modification of the leaving group itself (e.g., OS' thio substitution) or the general acid atom that donates the proton to the OS' (e.g., 1cA or 3cA chemical modifications in the case these positions act as general acid), replacement or removal of a divalent metal ion that acts as a Lewis acid by making direct inner-sphere contact with the OS' leaving group, or elimination of a divalent metal ion that acts as a Brønsted acid by donating a proton from a metal-bound water molecule to the OS' (e.g., Mg²⁺ → Na⁺ or Co(NH₃)₆³⁺) constitutes a $1^\circ \delta$ effect.

As specific examples, Twr A1(3cA) mutation³⁸ ($k/k' \gg 10^5$) that leads to knockout of the adenine general acid gives rise to a (normal) primary δ effect, and (predicted) recovery of the rate by 5' thio substitution⁴⁴ would give rise to a primary δ rescue effect.

5.4.2. Secondary Delta ($2^\circ \delta$). Remote (nonprimary) chemical modification or metal ion binding that affects leaving group departure by proton transfer from a general or specific acid constitutes a $2^\circ \delta$ effect. This may include pK_a shift of the general acid itself (e.g., A → G or 7cA) or replacement (not removal) of a divalent metal ion that acts as a Brønsted acid by donating a proton from a metal-bound water molecule to the OS' leaving group (e.g., Mg²⁺ → Mn²⁺ or Cd²⁺), or action of buffer through a water wire to protonate the leaving group when protonation by the general acid (e.g., A:N3) is not productive (neutral and higher pH).⁴⁵

As a specific example, Twr A1(1cA) chemical modification³⁸ ($k/k' \approx 0.0013$) that leads to tuning of the pK_a at the N3 position ($\Delta\text{pK}_a = 1.1$ upshift) gives rise to a (inverse) secondary δ effect.

5.4.3. Tertiary Delta ($3^\circ \delta$). A change in the structural scaffold or hydrogen bond network needed to support primary or secondary δ contributions to catalysis (e.g., A → P mutation that eliminates key structural hydrogen bonds that position A to act as a general acid to donate a proton to the OS' leaving group) leads to a $3^\circ \delta$ effect.

As a specific example, Twr A1(2AP) mutation³⁹ ($k/k' > 10^5$) that leads to disruption of a critical anchoring interaction between A1:N2 and the NPOs of C16 and C17 gives rise to a tertiary δ effect. Note (example of functional blurring), these interactions also play a role in secondary δ catalysis by shifting the pK_a of A1:N3 toward neutrality in the ribozyme environment.

5.5. Specific Illustrative Example: The Twister Ribozyme. In this section, we explore in more detail chemical modifications in Twr to demonstrate application of the proposed ontology. Unlike the twister examples given in the previous sections, we now turn our attention to a more complex set of examples involving a mutation of the general base to inosine (G33I) together with stereospecific thio substitutions of the NPO positions of the scissile phosphate of the substrate (Figure 2). Kinetic data for these mutations, including under ionic conditions (10 mM Mn²⁺) that examine metal ion rescue effects, are summarized in Table 2. These effects have been measured experimentally and interpreted computationally,⁴⁶ and here we apply the ontology to discuss the interpretation of these results using a model whereby G33 is held in position by hydrogen bonds with A2 to act as a general base to activate the nucleophile through the N1

position, while the exocyclic amine (N2) donates a hydrogen bond to the pro- R_p NPO of the scissile phosphate.

WT \rightarrow G33I, $k/k' \approx 430$.³⁸ This mutation eliminates the exocyclic amine ($-\text{NH}_2$) of guanine and down shifts the pK_a at the N1 position. The expected catalytic effects include the following:

- Position 1, a slightly inverse $2^\circ \gamma$ effect due to the shift in pK_a toward neutrality from 9.5 (inferred from activity–pH profiles³⁸) to 9.1 (estimated from a $\Delta pK_a \approx -0.4$ shift of G to I⁴¹), which could be detected from measurement of the mutant activity–pH profile. This scenario assumes the N1 of G33 is the general base. The inverse effect due to pK_a shift has been predicted by computation to be attenuated by a very slight normal effect due to an increase in the barrier to proton transfer from the nucleophile to the N1 of G33.⁴⁴ Alternately stated, the thermodynamic gain of pK_a down-shifting outweighs the kinetic penalty of decreased basicity, leading to the overall slight inverse effect.
- Position 2, a normal $3^\circ \gamma$ effect due to the disruption of the G33/A2 base pair that helps to structurally position (anchor) the general base (G33:N1 in close proximity to O2') for nucleophile activation.
- Position 3, a normal $2^\circ \beta/3^\circ \gamma$ effect due to loss of H-bonding of G33:N2 with the pro- R_p oxygen that provides both electrostatic stabilization of the NPO ($2^\circ \beta$) and structural organization (positioning) of G33 ($3^\circ \gamma$).

WT \rightarrow S(R_p): $k_O/k_S \approx 94$.³⁸ This chemical modification introduces a thio substitution at the pro- R_p position of the substrate scissile phosphate and exhibits negligible Mn²⁺ rescue ($(k_O/k_S)^{\text{Mn}^{2+}}/(k_O/k_S)^{\text{Mn}^{2+}} \approx 0.8$). The expected catalytic effects include the following:

- Position 3, a normal $2^\circ \beta/3^\circ \gamma$ effect due to weakened H-bonding of G33:N2 with the thio-substituted S(R_p) nonbridge position of the substrate, similar to the $2^\circ \beta/3^\circ \gamma$ effect for WT \rightarrow G33I, as well as steric effects involving the larger sulfur atom that may further disrupt positioning of the general base.
- Position 4, a near-negligible $1^\circ \beta$ effect due to thio substitution at the NPO position.

S(R_p) \rightarrow G33I/S(R_p): $k/k' \approx 4.3$.³⁸ This mutation is identical to WT \rightarrow G33I except for the inclusion of the thio-substituted substrate, with expected catalytic effects as follows:

- Position 1, a slightly inverse $2^\circ \gamma$ effect due to the shift in pK_a toward neutrality from 9.5 to (estimated) 9.1 as in WT \rightarrow G33I.
- Position 2, a normal $3^\circ \gamma$ effect due to the disruption of the G33/A2 base pair as in WT \rightarrow G33I. Note: the $2^\circ \beta/3^\circ \gamma$ effects in the WT \rightarrow G33I mutation at position 3 due to loss of H-bonding of G33:N2 with the pro- R_p oxygen are largely absent with the S(R_p) substrate since this hydrogen bond is already disrupted due to the presence of the thio-substituted NPO with which it is interacting.

G33I \rightarrow G33I/S(R_p): $k_O/k_S \approx 0.95$.³⁸ This perturbation involves a S(R_p) thio substitution of the substrate for the G33I mutant, with no significant expected overall catalytic effects.

- Position 4, a near-negligible (inverse) $1^\circ \beta$ effect due to thio substitution at the NPO position. Note: with the

elimination of the N2 exocyclic amine of inosine, the H-bonding interaction that was disrupted by the S(R_p) substrate in the WT \rightarrow S(R_p) is already absent. The subtle differences in $2^\circ \beta/3^\circ \gamma$ effects in position 3 are expected to be small.

Theoretical methods⁴⁷ can, in principle, aid in the deconstruction of some mutational effects by isolating different contributions. For example, the normal $2^\circ \gamma$ effect at position 1 resulting from G33I mutation could be decomposed into its thermodynamic (pK_a down-shifting) component by measuring or calculating the microscopic pK_a shift at the N1 position due to G33I mutation and its kinetic (decreased basicity) component by calculating separately the relative free energy barriers for proton transfer to N1 for each mutation. Finally, the $3^\circ \gamma$ (position 2) and $2^\circ \beta/3^\circ \gamma$ (position 3) effects in the WT \rightarrow G33I mutation could be decoupled by removal of the exocyclic amine of G33 and calculating the new free energy barrier for the reaction under artificial structural restraints that maintain anchoring of its position to act as general base, followed by repeating the calculation releasing these restraints (requiring additional free energy to properly position the general base) to recover the aggregate effect.

6. SUMMARY

The end goal of experimental and computational work on a catalytic RNA system is to gain insight from the development of an atomically detailed model of mechanism that enables prediction. Thus, it is essential to have an ontology that enables interpretation of experimental and computational data, including that from precision chemical modifications and molecular simulations, in terms of specific interactions within an active site and their contributions to catalysis. Toward this end, the proposed ontology has several advantages. First, it adheres to the original framework introduced by Breaker¹⁶ that has served the community for the last 15 years. Second, it adopts familiar terminology from mechanistic enzymology (particularly isotope effects) and structural biology. Third, it allows different catalytic strategies to be further categorized into a tiered hierarchy of primary, secondary, and tertiary contributions (even though the coupling of such effects occasionally become blurred experimentally) that enable more precise characterization of mechanistic details. It is the hope that the community will adopt this ontology as a common framework for discussion of catalytic strategies of RNA-cleaving enzymes and this will help pave the path forward toward a consensus view of these fundamental and fascinating mechanisms.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful for financial support provided by the National Institutes of Health (R35-GM127064 to P.C.B., R35-GM127100 to M.E.H, GM131568 to J.A.P., and GM62248 to D.M.Y.). The authors further thank R. Breaker, D. Lilley, A. Veenis, K. Messina, and M. Meyer for helpful comments and suggestions.

KEYWORDS

Catalytic strategies: The precise chemical actions taken by enzymes to promote desired chemical transformations

Catalytic role: The chemical mechanism by which an atom or functional group contributes to a specific catalytic strategy used by the native enzyme

Catalytic effect: The outcome of a measurement resulting from a perturbation to the native enzyme under a set of standard conditions

Rescue effect: A catalytic effect that results when the deleterious (normal) effect on rate due to a single change to the native enzyme is partially, fully, or over-fully recovered by a second aggregate change (not necessarily of the same type) to the system

2'-O-transphosphorylation: The reaction of RNA strand cleavage that is initiated by the nucleophilic attack of a ribose 2'-oxygen atom on the adjacent phosphorus center to yield cleavage products carrying 2',3'-cyclic phosphate and 5'-hydroxyl termini

α catalysis: Abbreviation denoting an enzyme's use of in-line geometry (alignment of the 2'-O nucleophile, scissile phosphorus center, and 5'-O leaving group) as a catalytic strategy

β catalysis: Abbreviation denoting an enzyme's use of charge neutralization on a nonbridging phosphate oxygen as a catalytic strategy

γ catalysis: Abbreviation denoting an enzyme's use of general base catalysis to deprotonate the 2'-OH group and thereby generate a more reactive oxyanion nucleophile as a catalytic strategy

δ catalysis: Abbreviation denoting an enzyme's use of charge neutralization at the 5'-oxygen atom of a phosphoester linkage as a catalytic strategy to promote its departure as a leaving group

Primary (β , γ , δ) atomic positions: Atomic positions directly involved with the chemical space of bonds associated with a particular (β , γ , δ) catalytic strategy

Primary (β , γ , δ) catalytic effect: A catalytic effect that results from changes in the identity of the primary (β , γ , δ) atomic positions

Secondary (β , γ , δ) catalytic effect: A catalytic effect caused by a change in the electronic environment of the primary (β , γ , δ) atom resulting from changes in the identity of non-primary atomic positions (and thus is exclusively different from a primary catalytic effect)

Tertiary (β , γ , δ) catalytic effect: A catalytic effect caused by alteration of the position of the primary (β , γ , δ) atoms resulting from modification of the structural scaffold or hydrogen bond network that organizes the enzyme active site

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