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Specificity Distorted: Chemical Induction of Biological Paracatalysis

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Abstract

We define paracatalysis as the acceleration of a reaction that appears abnormal or nonphysiological. With the high specificity of enzymes, side reactivity of this kind is typically negligible. However, enzyme paracatalysis can be amplified to levels that are biologically significant through interactions with a special class of small molecule “antagonist”, here termed a paracatalytic inducer. Compounds with this unusual mode of action tend to be natural products, identified by chance through phenotypic screens. In this Perspective, we suggest two general types of paracatalytic inducer. The first type promotes substrate ambiguity, where the enzyme’s ground state selectivity is compromised, enabling the transformation of non-native substrates. The second type involves transition state ambiguity, where the paracatalytic inducer changes the enzyme’s interactions with the activated substrate, giving rise to non-native bond making. Although they are unusual, small molecules that induce paracatalysis have established value as hypothesis-generating probes and a few substances, i.e., aspirin and the aminoglycosides, have proven to be translatable as medicines.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biochem.0c00643>.

Summary of enzyme paracatalysis induced by point mutagenesis and an associated table with selected examples (PDF)

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Specificity is a hallmark of biological catalysis, evident first in the ground state enzyme-substrate complex and then fully developed between the enzyme and the altered substrate in the chemical transition state. The discriminatory power of those interactions is remarkable and manifests in both the rate acceleration and mechanistic fidelity.¹⁻⁴ Fidelity to the native mechanism is particularly striking with enzymes, and this feature seems almost independent of the transformation's complexity.⁵

Even so, there are examples in which enzyme fidelity lapses. DNA polymerases on rare occasion incorporate a mismatched nucleotide or exhibit "slippage" during replication, the latter producing deletions in the daughter strand.⁶⁻⁹ Transcripts can also be misread by the ribosome, resulting in dysfunctional protein.^{10,11} Triosephosphate isomerase, an otherwise perfect enzyme, has fidelity spoiled by the sporadic partitioning of its enediol(ate)

intermediate into the toxic metabolite, methylglyoxal.¹² At the outer limits, there are enzymes that natively turn a single substrate into a product spectrum.¹³

This Perspective examines the possibility of breaking fidelity, or amplifying infidelity, through the binding by enzymes of non-substrate small molecules. Most antagonist-like compounds with this mode of action are natural products or their analogues. They have been applied as hypothesis-generating probes of enzyme specificity, and a few substances have advanced to clinical use. We suggest that these results provide the basis for an alternative strategy for manipulating enzyme action, termed paracatalytic induction.¹⁴ We define paracatalysis as the acceleration of a reaction that appears abnormal or nonphysiological.^a

Paracatalytic induction by a small molecule, or paracatalytic inducer (PI), would result in the diversion rather than the inhibition of enzymatic activity. In a paracatalytic pathway, the reaction product is distinct from the intended molecule, which could lead to metabolic waste or cellular toxicity. Compounds that stimulate this behavior may bind at the enzyme active site, and the interactions can be noncovalent or covalent. The single unifying feature is that paracatalytic inducers distort native enzyme specificity.

As a framework to illustrate two general cases of paracatalysis, we use the reaction coordinate diagrams in Figure 1. In the first diagram, the native pathway (green) is favored. The reaction begins with the Michaelis complex at the top left corner and travels over a stabilized transition state to the physiological product complex at the bottom right. Pathways to side reactions (blue and red) are present, but elevated barriers at the ground state and at the transition state restrict their access, resulting in product purity. In the middle diagram, the dark blue line represents the induction of paracatalysis by substrate ambiguity. Here, ground state selectivity is eroded. The enzyme takes up and transforms a non-native substrate (S_2), and a novel product accumulates (P_2). The dark red line in the third diagram represents the second mode of paracatalysis, transition state ambiguity. Here the native substrate undergoes a side reaction more readily, passing through a transition state that is lowered in energy, enhancing the yield of a new product (P^*). Examples in which these types of paracatalysis have been induced by small molecules are outlined below. Other PIs are listed in Table 1.

Acyl depsipeptides (ADEPs), a family of lasso-shaped antibacterials (Figure 2A) secreted by strains of *Streptomyces*, illustrate paracatalytic induction via substrate ambiguity. ADEPs were identified by Michael and Kastner through a phenotypic screen for novel antibiotics.¹⁶ The molecules are similar structurally to enopeptins, discovered later, also from *Streptomyces*.¹⁷ Members of this antibiotic family appear to kill bacteria by transforming the protease, ClpP, into a rogue degrader of vital cellular protein.

ClpP is normally activated to cleave misfolded proteins through sequential, reversible quaternary interactions. Fourteen ClpP subunits assemble first into a barrel-shaped “proto”-protease with limited function.^{18–20} Dipeptides are readily hydrolyzed, but longer peptides and full-length proteins escape degradation²¹ (Figure 2B, left). Substrate specificity is

^aAn early use of the term paracatalysis is found in the work of Christen, who described oxidative side reactions of thiamine-dependent enzymes that resulted in irreversible inhibition as a paracatalytic inactivation or paracatalytic modification.¹⁵

imparted by entry pores at either end of the ClpP barrel that restrict access to the (inner) proteolytic chamber.²⁰ Interaction with ClpX or ClpA, the ATP-powered unfolding enzymes, unleashes ClpP's full proteolytic activity. In a ClpX/ClpP complex, the entry pores of ClpP are opened and the unfoldase function of ClpX acts in an ATP-dependent manner to unwind and convey the substrate to ClpP for degradation.^{22,23}

ADEPs are small molecule mimetics of ClpX; their interactions with ClpP stabilize the multimeric form of ClpP and wedge open the entry pores. The targeting of ClpP by ADEP and the novel mode of action of ADEP were discovered through an elegant genetic selection in *Escherichia coli*.²¹ Subsequent X-ray structures of *E. coli* and *Bacillus subtilis* ClpP in their complexes with ADEP supported the pore-opening mechanism as a means to substrate ambiguity.^{24–26} Catalytically, the in vitro dipeptidase activity of ClpP is unchanged by ADEP; however, degradation of a decapeptide substrate is accelerated 50-fold by ADEP, and proteolysis of the 30-amino acid insulin β chain is enhanced >600-fold.²⁵ ADEP cannot induce ClpP to degrade folded proteins, suggesting that pore geometry still impedes the entry of globular substrates. In bacteria treated with ADEP, ClpP's paracatalytic destruction of an essential cell division protein, FtsZ, appears to best explain the molecule's antibacterial effect.²⁷

Medicinal chemistry efforts have advanced ADEP's clinical potential with analogues that show stronger affinity for ClpP,^{21,28} and there may be additional applications involving mitochondrial ClpP enzymes.²⁹

The induced loss of ClpP's substrate selectivity by ADEP recalls the influence of aminoglycoside antibiotics on the ribosome. Aminoglycosides like streptomycin and paromomycin function, at least in part, by undermining the fidelity of mRNA decoding by the ribosome.^{30,31} The fundamental chemical transformations of ClpP and the ribosome are unchanged by the respective paracatalytic inducers. ClpP when bound by ADEP still accelerates peptide bond hydrolysis; the ribosome with bound aminoglycoside continues to catalyze peptide bond formation. However, the ClpP/ADEP complex begins to destroy vital cellular protein, and the ribosome aminoglycoside complex incorporates amino acids that are inconsistent with the genetic code.³¹ The PI binding energy effectively pays the energetic cost of induced fit, and molecules normally discriminated against now serve as suitable substrates, i.e., substrate ambiguity.

Aspirin (acetyl salicylate) is a covalent PI of COX-2, inducing paracatalysis through transition state ambiguity. Following aspirin acetylation, the substrate specificity of COX-2 is left intact while the native chemical transformation is derailed.³²

COX-2, or prostaglandin H2 synthase isoform 2, converts arachidonic acid into PGG₂ and then PGH₂ in the committed step of pro-inflammatory and pro-clotting eicosanoid biosynthesis. COX-2 also represents the preferred pharmacological target of nonsteroidal anti-inflammatory drugs like aspirin.³² In the wild type COX-2 reaction, summarized in the top panel of Figure 3A, arachidonic acid undergoes a significant, multibond chemical transformation. In the achiral lipid substrate, COX-2 installs an endoperoxide and a hydroperoxide group through the formation of three C–O bonds, one C–C bond, and four

new stereocenters.^{33,34} COX-2 and its paralogue, COX-1, bring about these transformations by enlisting a phenoxyl radical at a conserved tyrosine side chain (Tyr358) and a redox active heme group.^{33–35} The positioning of those catalytic elements and a rigid, hydrophobic active site to constrain arachidonic acid appear to provide the main strategy for mechanistic fidelity.³⁶

COX-2 exhibits paracatalysis minutes after treatment with aspirin, induced by acetylation of the COX-2 residue, Ser530.^{37–40} Monoacetylation of COX-2 by aspirin represents a minor change to the enzyme's composition. Moreover, Ser530 does not appear to be catalytically essential by alanine mutation.⁴¹ Nonetheless, X-ray structures of COX-2 indicate that Ser530 packs against the ω end of enzyme-bound arachidonic acid (Figure 3B). The added bulk from side chain acetylation disrupts the native substrate conformation and by extension the interactions of the substrate with COX-2.^{41,42} The consequences of this subtle change are evident at the transition state where the discriminatory power of enzymes reaches its apex. Instead of PGG₂, the substrate is redirected toward the monohydroperoxide side products (15*R*)-hydroperoxy-eicosatetraenoic acid (15*R*-HpETE) and to a lesser extent 13*R*-HpETE (Figure 3A, bottom path). The phenoxyl radical at Tyr358 still forms in the Ser530-acetylated COX-2,⁴³ but the altered binding pose of the substrate changes the reaction trajectory. COX-2 is thereby converted from a cyclooxygenase to a lipoxygenase of arachidonic acid. The PI activity is unique to aspirin and its close salicylate analogues; other NSAIDs behave as reversible, competitive inhibitors.

Paracatalysis by aspirin-acetylated COX-2 can be phenocopied by point mutation that changes Ser530 to methionine or glutamine.^{44,45} The mutagenesis result is consistent with the idea that added bulk from Ser530 acetylation induces the altered mechanism. Similar observations in which a single mutation induces paracatalysis have been made with unrelated enzymes. We collect these examples in Supporting Note 1 and Supporting Table 1. Each entry in the table is an enzymatic transformation that has been redirected toward a new substrate, new mechanism, or both, by mutation at only one residue. Results like these suggest that enzymes have fidelity hot spots, and we speculate that those regions can be targeted with paracatalytic inducers, like the aspirin/COX-2 interaction. The other footnote relates to the hydroperoxide, 15*R*-HpETE, formed by Ser530-acetylated COX-2. This molecule is not a biological dead end. Recent work indicates that 15*R*-HpETE serves as a precursor to the biosynthesis of potent anti-inflammatory molecules, termed aspirin-triggered specialized pro-resolving mediators.^{46,47} Some of these secondary metabolites show a glimmer of anticancer activity.⁴⁸

We arrive at several takeaways from our survey of small molecule/enzyme interactions that induce abnormal activity, or paracatalysis. First, altering the specificity of biological catalysts can have unexpected consequences. With ADEPs, inducing substrate ambiguity compromises bacterial fitness leading to cell death. Conversely, promoting side reactivity through small molecule interactions can produce unforeseen benefits, as is the case with aspirin. Second, the structural basis of mechanistic fidelity appears to be encoded locally in the enzyme structure. This inference is supported by experiments in which PIs corrupt fidelity and by the point mutagenesis experiments in Supporting Table 1. Third, the role of paracatalytic inducers as probes for investigating biological catalysis tends to be hypothesis-

generating, which is distinct from the way mechanism-based analogue type inhibitors are applied for hypothesis testing. Lastly, we note that the existing database of PIs, although limited, consists largely of natural products and their derivatives (Table 1). This final observation suggests that molecules that induce paracatalysis, despite their utility, have not been considered for HTS campaigns with synthetic chemical libraries.

ADEPs and aspirin were identified by whole organism phenotypic screening rather than target-based screens. Their specific mode of action was not selected or anticipated. ADEPs were pursued because of their antibacterial properties; aspirin was developed by Bayer as a replacement for salicylic acid because acetylation of salicylic acid reduced gastrointestinal irritation. Only later, ~100 years later with aspirin, was the underlying biochemistry understood.

In a recent low-throughput in vitro screen by our lab, where we had the intention of finding conventional inhibitors of our target protein, we nearly overlooked a set of molecules that induce paracatalysis.¹⁴ Hedgehog activating compounds or “HACs” are PIs that we found by chance. We had not anticipated their existence. Through follow-up biochemical characterization, we observed that HACs induce substrate ambiguity. Allosteric binding of the molecules diverts a native cholesterol-mediated autoprocessing reaction of Hedgehog proteins toward an abnormal water-mediated hydrolytic pathway. Thus, one can stumble upon paracatalytic inducers through in vitro screens, as well.

The important role played by serendipity in these studies raises the question of whether it is possible to discover PIs through deliberate, target-centered chemical screening. If paracatalytic induction as a means to antagonize enzymatic activity has been established, pursuing second-generation derivatives through a targeted screen is logical and low-risk. This effort is underway in our lab for the HACs. The discovery and development of ADEP analogues follows a similar trajectory. After ClpP was identified as the ADEP target and the ADEP mechanism was sorted, biased screening campaigns for improved analogues were undertaken with a clear rationale.^{28,49,50} Instead of a whole cell phenotypic assay of bacterial killing, one screen used purified ClpP with a casein-FITC substrate, and that focused effort proved to be fruitful.⁵⁰ To the best of our knowledge, there are no reports of the riskier endeavor of large-volume library screening without first having a proof of concept.^b

There are some parameters that might be considered in assessing the viability of a paracatalytic induction strategy. If the enzymatic transformation proceeds through a high-energy intermediate (carbocation, carbanion, radical, metaphosphate-like, or activated acyl group), species like these are prone to off-pathway reactions and may therefore motivate screening. If the target enzyme undergoes a structural change like multimerization or induced fit that regulates catalysis, this transition could be deregulated by a small molecule to induce an abnormal transformation. If mutagenesis studies have identified a residue or hot

^bWhile our manuscript was under review, Siriwardena et al. reported the design and application of bivalent small molecules, called “PHICS”.⁵¹ PHICS appear to be paracatalytic inducers. PHICS were shown to redirect the substrate specificity of protein kinases toward non-native substrates. This behavior is in accord with our definition of paracatalytic induction via substrate ambiguity. PHICS represent a unique and exciting proof of concept for the rational design of paracatalytic inducers.

spot that influences mechanistic specificity, like in Supporting Table 1, this region might be targeted with small molecules provided that sufficient structural data were available. Apart from mutagenesis, if there were biochemical evidence that the native enzyme exhibits some degree of side reactivity, this too could motivate screening. In the COX-2 example, side production of 15*R*-HpETE by the native enzyme was known prior to the biochemical work with aspirin. Then, last, after selecting the enzyme and settling on the side reaction, we must confront an important practical question: is there a suitable assay for paracatalysis? These considerations provide a checklist for building a rationale and assessing risk in pursuing PIs.

We suggest the name paracatalytic inducer for non-substrate molecules that primarily divert rather than suppress biological catalysis. We identify two general types of paracatalysis: (1) promotion of apparently nonphysiological reactions of the native substrate, i.e., transition state ambiguity, and (2) loosening of enzyme selectivity to enhance the reactivity of a wider range of molecules, i.e., substrate ambiguity. The value of ADEPs to fundamental research as hypothesis-generating probes and of aspirin to applied biomedicine seems to be well established. The behavior of these compounds and related observations (Table 1 and Supporting Table 1) suggest that the mechanistic fidelity of biological catalysts is surprisingly fragile, more so than has been generally recognized.⁵² PIs do represent a niche category of antagonists, but that may be a consequence of the scarcity of focused discovery efforts. In addition to screening libraries for synthetic PIs, we also recognize the possibility of endogenous paracatalytic inducers, hidden in plain sight, that could be deployed adaptively by an organism to deflect enzymatic power toward an alternative mechanistic pathway. The compounds highlighted here provide a training set only; our challenge now is to build up a predictive understanding by identifying additional examples against a broader range of enzymes through targeted screening approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Errant paths. (A) Mechanistic fidelity. The wild type enzyme substrate complex (ES) yields a single physiologically useful product (EP). Although side reactions are possible, the larger activation barrier (red) prohibits transformation of the native substrate into an unnatural product (EP*). Likewise, the binding by the enzyme of a non-native substrate (ES₂) is suppressed by weak affinity and an elevated activation barrier (blue). (B) Substrate ambiguity. The native enzyme is induced to accept and transform a substance (S₂) that it would ordinarily reject. A new product (P₂) is generated. (C) Transition state ambiguity. The enzyme is induced to catalyze an abnormal reaction of the native substrate. A new product is generated (P*).

Figure 2. ADEP binding by ClpP induces substrate ambiguity. (A) ADEP-A. (B) ClpP/ADEP interaction. In the left panel, ClpP assembles into a 14-subunit barrel-like multimer. Catalytic activity limited to the hydrolysis of short peptides by access pore constriction. In the right panel, ADEP molecules bound by ClpP stabilize the multimer and open the access pores, allowing degradation of larger peptides.

Figure 3.

Aspirin acetylation of COX-2 induces transition state ambiguity. (A) The top pathway shows native partial transformation of arachidonic acid by COX-2. PGG₂ is reduced to PGH₂ in a subsequent step. The bottom pathway shows the paracatalytic reaction of arachidonic acid by aspirin-acetylated COX-2. (B) Active site of aspirin-acetylated COX-2. The heme group and Tyr358, site of phenoxyl radical, are labeled; the O-acetyl group of Ser530 are shown as spheres, and arachidonic acid is shown as purple sticks. Based on Protein Data Bank entry 5F19.

Table 1.

Small Molecules That Induce Paracatalysis

PI compound	biological catalyst	reported affinity	mode of action
ADEP1	ClpP	$4 \times 10^{-7} \text{ M}^{21}$	broadens the substrate range to include vital cellular proteins
Arg-Arg	cAMP-dependent protein kinase	$4 \times 10^{-3} \text{ M}^{53}$	enhances the hydrolysis of ATP at the expense of phosphorylation
aspirin	COX-2	$14 \times 10^{-3} \text{ M}^{54}$	accelerates the errant monooxidation of the native substrate
HAC8	hedgehog autoprocessing domain (HhC)	$9 \times 10^{-6} \text{ M}^{14}$	diverts the thioester intermediate toward hydrolysis
lyxose	hexokinase	$5 \times 10^{-3} \text{ M}^{55}$	enhances the hydrolysis of ATP at the expense of phosphorylation
paramomycin	ribosome	$1 \times 10^{-7} \text{ M}^{56}$	promotes misreading of the genetic code
PHICS	AMPK, PKC	$0.1-1 \times 10^{-6} \text{ M}^{51}$	redirects protein kinase activity toward new substrates