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Reactive enamines and imines *in vivo*: Lessons from the RidA paradigm

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Abstract

Metabolic networks are webs of integrated reactions organized to maximize growth and replication while minimizing the detrimental impact reactive metabolites can have on fitness. Enamines and imines, such as 2-aminoacrylate (2AA), are reactive metabolites produced as short-lived intermediates in a number of enzymatic processes. Left unchecked, the inherent reactivity of enamines and imines may perturb the metabolic network. Genetic and biochemical studies have outlined a role for the broadly conserved Rid (YjgF/YER057c/UK114) protein family, in particular RidA, in catalyzing the hydrolysis of enamines and imines to their ketone product. Herein, we discuss new findings regarding the biological significance of enamine and imine production and outline the importance of RidA in controlling accumulation of reactive metabolites.

Keywords

2-aminoacrylate stress; enamine/imine metabolism; RidA; reactive metabolite

Metabolic networks must accommodate production of reactive metabolites

Metabolism consists of a network of biochemical reactions organized according to the chemical constraints of the cell and responsive to ever-changing environmental stimuli [1–3]. These networks are arranged to maximize the output of chemicals necessary for cell growth and survival, while minimizing the detrimental impact reactive metabolite accumulation can have on cell fitness [4–6],The biochemical pathways in central and secondary metabolism have been elucidated over the years by combinations of *in vitro* biochemistry and *in vivo* genetics. Importantly, a dichotomy exists where reactive metabolites act as obligatory catalytic intermediates, facilitating chemistry for synthesis of essential compounds. However, if left unchecked, their inherent reactivity can lead to detrimental disruption of the metabolic network. Examples of obligatory reactive intermediates include carbon monoxide in the Wood-Ljungdahl pathway of acetyl-CoA

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synthesis [7], acetaldehyde produced during ethanolamine catabolism [8], and nitric oxide generated by aerobic ammonia oxidation [9], among many others. Reactive metabolites can also contribute to **metabolic robustness** (see Glossary), leading to the emergence of non-native metabolic pathways. That is, the reactivity of certain metabolic intermediates or labile side-products, often acted upon by non-specific or **promiscuous enzymes**, can enable alternative methods of essential metabolite production [10, 11].

Enamines and **imines** are short-lived reactive metabolites produced as reaction intermediates by multiple enzymes central to amino acid metabolism [12–16]. Despite the prevalence of these reactive molecules in ubiquitous biochemical pathways, the potential for free enamine and imine species to persist *in vivo* was largely ignored prior to characterization of the RidA proteins as enamine deaminases [17–20]. This review summarizes the biochemical mechanisms for enamine and imine production, the *in vivo* consequences of enamine (specifically 2-aminoacrylate, or2AA) accumulation, and outlines the role of RidA in controlling enamine accumulation. We conclude with thoughts prompted by investigation of the RidA paradigm and propose challenges and opportunities that lie ahead in efforts to understand how reactive enamines and imines fit into emerging models of the cellular metabolic network.

Enamines and imines are reactive metabolites prevalent throughout metabolism

Enamines and imines contain unsaturated nitrogen often derived from the condensation of an aldehyde or ketone with a secondary or primary amine, respectively [21]. Peptide-bound enamines are produced biochemically via **β-elimination** of polypeptide residues, forming reactive peptide species that play an important role in pharmacology, enzyme engineering, and food science by facilitating formation of complex molecules [22–26]. Despite the practical use of peptide-bound enamines, the occurrence of free enamine (and imine) species *in vivo* and any physiological implications for the chemical make-up of the cell are poorly understood.

Biochemical studies show that many **pyridoxal 5'-phosphate (PLP)-dependent enzymes** generate enamine and/or imine intermediates from free α-amino acid substrates (Figure 1, Key Figure) [27]. These α-amino acid substrates contain electronegative side chains (e.g. L-serine, L-cysteine, 3-chloro-L-alanine, etc.), which are cleaved by dedicated (or promiscuous) α,β-eliminases to generate an enamine product. Some α,β-eliminases release an enamine directly into the reaction milieu [28, 29] (Figure 1A). This behavior is observed for a number of fold type II PLP-dependent α,β-eliminases (See Box 1 for a description of PLP enzyme fold type), such as biosynthetic serine/threonine dehydratase (IlvA, EC 4.3.1.19), cysteine desulfhydrase (EC 2.5.1.47), and diaminopropionate ammonia-lyase (EC 4.3.1.15) [15–17, 29, 30]. In some cases, the cofactor-bound enamine is protonated to an iminium ion prior to release from the enzyme (Figure 1B) [31, 32]. One example includes tryptophanase (TnaA; EC 4.1.99.1), a versatile fold type I PLP-dependent lyase responsible for tryptophan catabolism (via α,β-elimination) [33], cysteine detoxification (α,β-elimination) [34], and tryptophan synthesis (β-substitution) [35]. Iminium ions are also

produced as catalytic intermediates by FAD-dependent dehydrogenases (Figure 1C). In most cases, the iminium ion intermediates are enzymatically hydrolyzed within the active site, but a number of FAD-dependent dehydrogenases release the iminium ion into the cytoplasm [19, 36]. The biological relevance of unbound enamine and imine species has generally been overlooked due to their short half-lives determined *in vitro* (less than 3 minutes) [37, 38]. However, recent studies have shown that accumulation of enamines, specifically 2AA, can alter the physiological state of an organism, most notably through covalent damage of PLP-dependent enzymes (Figure 1E) [39–43], To that end, many organisms encode RidA, which facilitates the catalysis of enamines and imines *in vivo*, as described below (Figure 1D).

Characterization of RidA as a new enamine and imine deaminase

The Rid Superfamily and ability of RidA to hydrolyze enamine and/or imine species

The reactive intermediate deaminase (Rid) family of proteins (formally YjgF/YER057c/UK114) is a class of proteins identified by inter-subunit clefts made from seven well-conserved residues and found in all domains of life (Figure 2) [44, 45]. Recent phylogenetic analysis used **synteny** to split the Rid superfamily members into eight subfamilies: an archetypal RidA subfamily and seven other subfamilies (Rid1-Rid7) [36]. Rid1-Rid7 subfamily members are largely confined to bacteria and are often encoded in genomes also encoding RidA members [36]. Using the residue numbers from the *Salmonella enterica ridA* sequence, the most highly conserved residue across the Rid superfamily is glutamate 120 (E120). S30 and G31 are also common across all subfamilies and thus assumed to be significant in the function of Rid enzymes [36].

While multiple crystal structures of RidA homologs proved useful in identifying the putative active site of this class of proteins [46, 47], a number of genetic observations ultimately led to the identification of the biochemical function and physiological role for RidA [48–51]. Mentioned previously, *S. enterica* IlvA is an α,β-eliminase with activity on serine (K_M = 43 mM) or threonine (K_M = 6 mM) as substrates [52]. *In vitro*, IlvA alone produces α-ketobutyrate from threonine, or pyruvate from serine, showing that solvent water is sufficient for hydrolysis of the enamine to the corresponding keto acid [17]. The addition of purified RidA to the IlvA reactions increased the rate of ketoacid formation, showing that RidA catalyzes the hydrolysis of enamines derived from threonine (2-aminocrotonate; 2AC) and serine (2AA) [17]. These and other data culminated in the designation of RidA as an enamine/imine deaminase [49, 50]. Purified RidA orthologs from organisms across the tree of life displayed conserved enamine hydrolysis-enhancing activity *in vitro*, confirming the designation of this protein subfamily as enamine deaminases [17].

RidA has a broad substrate range, acting not only on 2AA and 2AC, but also on the imines of glutamine, histidine, leucine, methionine, or phenylalanine generated by FAD-dependent oxidases [19, 36]. Currently, a physiological role for RidA in the deamination of 2AC and 2AA has been identified in *S. enterica* and other organisms [39, 43, 53, 54], but the significance of its activity on other substrates remains to be determined.

Proposed mechanism of RidA-dependent enamine and imine catalysis

Efforts to define the catalytic residues involved in deaminase activity of RidA by site-directed mutagenesis identified a conserved arginine (R105) as the sole residue essential for function *in vivo*; variants altered at this site had greatly decreased activity *in vitro* [17]. RidA variants E120A and Y17F also had decreased deaminase activity *in vitro*, but retained the ability to complement *ridA* mutant phenotypes [17]. These data, the conserved residue observations above, and the crystal structure of the RidA homolog TdcF [46], suggested a mechanism for how RidA catalyzes the hydrolysis of iminium ion substrates. A representation of this mechanism using 2-iminiopropionate (2IP) as an example substrate is shown in Figure 2. In the mechanistic model, R105 forms a bidentate salt bridge with the carboxylic acid of the substrate. A substitution of this residue is expected to disrupt the critical interaction with the substrate carboxylic acid, consistent with the loss of activity of the $RidA^{R105A}$ variant [17]. R105 is absolutely conserved in RidA and Rid1-Rid3 subfamily members, predicting that members of these protein subfamilies act on amino acid-derived substrates [36]. Furthermore, Rid4–7 subfamilies lack R105 and no amino acid-derived enamine/imine deaminase activity has been detected for these subfamilies [19]. Based on the crystal structure from TdcF, the backbone carbonyl groups of R105 and G31 appear to stabilize the iminium ion (e.g. 2IP) formed from the substrate, while the backbone of C107 and the side chain of Glu-120 coordinate the water involved in hydrolysis of 2IP [46]. Although the TdcF crystal structure suggests the hydroxyl group of Y17 is too far away to hydrogen bond with the substrate, this residue may directly interact with substrate/product or play an important role in proper folding of the active site. The TdcF crystal structure also shows a number of solvent water molecules localized near Y17, making it reasonable that the Tyr hydroxyl group coordinates a water molecule near the imine nitrogen, where it acts as a proton donor for formation of ammonia [46]. Importantly, this modelproposes a mechanism for iminium ion hydrolysis. Consistent with RidA enzymes acting upon iminium ion substrates, when iminium ions are generated directly via FAD-dependent oxidases (Figure 1C), inclusion of RidA increases the rate of hydrolysis *in vitro* [19, 20]. However, it remains unclear whether RidA decreases enamine accumulation by binding the enamine and facilitating both iminium ion formation and subsequent hydrolysis, or whether RidA decreases enamine levels simply through increased consumption of its iminium ion tautomer [17].

Chemistry of free enamine species: from *in vitro* discovery to biological relevance

Inactivation of PLP-dependent enzymes by 2-aminoacrylate

Many **non-eliminase** PLP-dependent enzymes are promiscuous and capable of catalyzing adventitious α,β-elimination reactions using amino acids substrates with strong electronegative leaving groups (e.g.−Cl, $-SO_3^-$) bound to their β-carbon [27]. *In vitro* studies showed that the 2AA generated during these promiscuous elimination reactions can immediately and irreversibly damage the source PLP-dependent enzymes before 2AA escapes the active site [55–57]. The inactivation could be caused by 1) release of 2AA from PLP and subsequent nucleophilic attack of the electrophilic enzyme-bound PLP Schiff base

by the β-carbon of 2AA [56] (Figure 3A), or 2) attack of the 2AA/PLP adduct by active site nucleophilic residues [57] (Figure 3B). Both scenarios produce an irreversibly inactivated enzyme [58]. Critically, these *in vitro* systems contained a single enzyme that both generated, and was inactivated, by 2AA. The precedence for 2AA inactivation of PLP-dependent enzymes, coupled with biochemical characterization of RidA and genetic observations in *ridA* mutant backgrounds, led to a model of the biologically relevant function of RidA [17, 39]. An extrapolation of the *in vitro* results suggested that *in vivo*, PLP-dependent α,β-eliminases generated 2AA that could diffuse from the generator enzyme(s) and covalently modify susceptible PLP-dependent target enzymes [17]. The critical assumption was that, in the absence of RidA, 2AA would persist long enough in the cellular milieu to interact with and inactivate target enzymes. Given this assumption, several phenotypes of *ridA* mutant strains were predicted to be the consequence of 2AA-dependent inactivation of PLP-enzymes [39, 41, 42].

IlvA is the primary source of 2-aminoacrylate stress in S. enterica

The *ridA* mutant phenotypes were considered first with the purpose of identifying the proposed enzyme generator(s) of 2AA. *S. enterica ridA* mutants display a number of phenotypes, as shown in Table 1. It was particularly telling that the strain was sensitive to exogenous serine, and this sensitivity was reversed by the addition of isoleucine [48]. Isoleucine controls the activity of the serine/threonine dehydratase IlvA by feedback inhibition [59]. When the wild-type *ilvA* allele was replaced with an allele *(ilvA219)* encoding the feedback-insensitive $IlvA^{L447F}$ variant, isoleucine failed to reverse the serine sensitivity, indicating the effect of isoleucine was through allosteric regulation of IlvA [49, 50]. These and other data led to the key conclusion that multiple phenotypes of a *ridA* mutant in *S. enterica* were due to the IlvA-dependent generation of 2AA from serine [17, 39]. Although IlvA-dependent dehydration of L-serine is the predominant source of 2AA in *S. enterica* under standard growth conditions, other PLP-dependent α,β-eliminases are capable of generating substantial levels of free 2AA, when their substrates are supplied exogenously [15, 16] (Table 1).

Free 2-aminoacrylate inactivates multiple cellular PLP-dependent enzymes

While a serine/threonine dehydratase is the dominant 2AA generator in *S. enterica* and in most other organisms tested thus far [43, 54, 60], multiple enzymes are potentially damaged by 2AA. For instance, branched chain amino acid aminotransferase (IlvE) activity is decreased by 30–50% in *ridA* mutants; this decreased activity was confirmed to be due to covalent modification of the IlvE active site by 2AA [39]. Importantly, the generation of 2AA (by IlvA), inactivation of IlvE, and preventative quenching of 2AA by RidA were all reconstituted *in vitro* [39]. Growth phenotypes showed that multiple metabolic pathways were compromised in a *ridA* strain experiencing 2AA stress (Table 1). Thus far, assays have shown that serine hydroxymethyltransferase (GlyA; EC 2.1.2.1), aspartate aminotransferase (AspC; EC 2.6.1.1), and alanine **racemases** (Alr/DadX; EC 5.1.1.1), in addition to branched-chain amino acid aminotransferase (IlvE; EC 2.6.1.42), have lower specific activity in *ridA* mutant strains than in wild type [39, 41, 42, 53]. The mechanism(s) of inactivation has not been rigorously defined for target PLP-enzymes other than IlvE. Significantly, in a *ridA* mutant, the activity of multiple target proteins is compromised but

not eliminated, resulting in the perturbation of the metabolic network that can result in multiple **pleiotropic** phenotypes. For some of these phenotypes, (i.e., motility defect) the presumed target enzyme has not been identified [53, 60].

The work summarized above established the RidA paradigm in *S. enterica*, which in its simplest form states that 2AA produced by PLP-dependent α,β-eliminases can be deaminated by RidA. In the absence of RidA, the 2AA can 1) persist *in vivo*, and 2) diffuse into and inactivate distinct target PLP-dependent enzymes. This definition provided a starting point to explore the causes and implications of 2AA stress in a variety of organisms. Beyond *S. enterica*, enamine-related stress extends into other species, and RidA also plays an important part in controlling enamine stress for these organisms [19, 36, 43, 54, 60,61].

The conundrum of PLP enzymes being both generators and targets for 2AA.

Enzymes differ in their susceptibility to 2-aminoacrylate damage.

A dichotomy exists between PLP-dependent enzymes that generate 2AA, and those damaged by free 2AA. Presumably, 2AA generators are largely recalcitrant to 2AA-dependent damage, though these enzymes do not appear entirely immune to **mechanism-based inactivation** by 2AA [13, 62]. This form of inactivation involves the enzyme-catalyzed generation of 2AA from a 2AA precursor (β-substituted alanine species); 2AA is then displaced and nucleophilically attacks the enzyme used to generate it, rendering it permanently inactive. Some PLP-dependent enzymes can become sensitized to mechanism-based 2AA inactivation through site-directed mutagenesis [63]. This is exemplified by tryptophan synthase (EC 4.2.1.20), which catalyzes the last two reactions in the biosynthesis of tryptophan [64]. The PLP-dependent β-subunit of tryptophan synthase catalyzes both β-elimination and β-substitution reactions, but variant β-subunits bias reactions towards β-elimination relative to wild-type tryptophan synthase [65]. These same variants are susceptible to inactivation by 2AA produced from L-serine or 3-chloro-L-alanine, whereas the wild-type enzyme was undamaged by serine and less-damaged than the variant enzyme by 3-chloro-L-alanine [66]. These data showed that changing a single active site residue profoundly influenced susceptibility to 2AA inactivation, suggesting a fine line exists between substrate-based inactivation and successful elimination reactions. This work provides insights about how enzymes routinely exposed to 2AA could be protected from damage. The fact that enzymes proficient at α,β-elimination and 2AA generation are still susceptible tomechanism-based inactivation [13, 62], albeit less so than promiscuous eliminases [62], highlights the reactivity and potential toxicity of 2AA.

PLP-enzyme active site residues may determine susceptibility to 2-aminoacrylate damage

PLP-dependent enzymes are generally grouped into one of seven fold types according to structural similarities (Box 1) [67, 68]. Pairing 2AA interaction profiles with the relevant PLP-dependent enzymes revealed a correlation between fold type and 2AA production/inactivation; these data included four enzymes inactivated by 2AA and four that generated 2AA (Table 1). Generators, or enzymes most immune to 2AA damage, belonged to fold type II [15–17]. Conversely, enzymes damaged via the covalent addition of 2AA/PLP mechanism

(Figure 3B) were fold type I and IV enzymes [39, 42], and those damaged via the PLP/pyruvate adduct mechanism (Figure 3A) were fold type III racemases [39, 41, 42]. Reactions catalyzed by fold type I and IV PLP-dependent enzymes involve a **quinonoid intermediate** [69]. Enzymes belonging to these fold types satisfy this requirement through placement of a negatively charged amino acid adjacent to the pyridine N from PLP, which promotes the nitrogen's protonation and allows subsequent quinonoid formation [69]. Alternatively, L-alanine racemization (fold type III) and α,β-elimination (fold type II) do not require formation of a quinonoid intermediate [69]. Formation of a quinonoid intermediate is an essential step in the mechanism of damage via 2AA/PLP (Figure 3B), and may explain why the enzymes believed to be damaged in this way belong to fold type I and IV. In contrast, the PLP/pyruvate adduct damage mechanism (Figure 3A) requires the 4'C of PLP to act as an electrophile for successful attack by the 2AA-derived carbanion [58]. Therefore, the finding that fold type III racemases resist formation of a quinonoid intermediate may help explain why these enzymes are damaged via the PLP/pyruvate adduct mechanism and not the 2AA/PLP mechanism [70]. These data support a model where fold type II enzymes are more recalcitrant to 2AA damage via the 2AA/PLP mechanism due to their decreased propensity for forming a quinonoid species. In contrast, differences in the relative electrophilic strength of the C4' may account for fold type II enzymes being more immune than fold type III to the PLP/pyruvate adduct mechanism of 2AA-dependent damage. Further work using variant enzymes will be valuable in better defining features of the active site that determine the susceptibility of an enzyme to 2AA damage.

Understanding the global scope of enamine/imine reactivity

The global metabolic effects of 2-aminoacrylate are largely unexplored

A number of PLP-enzymes targeted by 2AA have been identified, primarily by work in *S. enterica* [39, 41, 42, 61]. However, in the context of metabolic network configurations, the large number of potential targets (>40 PLP-dependent enzymes are encoded in *S. enterica* [67, 71]) complicates efforts to define the global physiological consequences of 2AA stress. Moreover, the detectable physiological impact (e.g., phenotype) of 2AA stress varies by organism, reflecting how organism-specific metabolic network architecture dictates 2AA damage outcome [54, 60, 61]. As a first step toward understanding the global impact of 2AA stress in *S. enterica*, one study used differential gene expression in a *ridA* mutant as a downstream indicator of the metabolic perturbation caused by 2AA stress [53]. The resulting data identified a previously unknown 2AA-dependent motility defect, but failed to reflect dramatic changes in the expected metabolic pathways linked to 2AA production or damage. These initial global efforts haveemphasized the need for innovative technical approaches to better capture the complex metabolic environment in a living cell and identify the potentially subtle consequences of 2AA stress.

RidA-independent mechanisms for quenching 2-aminoacrylate

Studies of RidA highlight the importance of reactive metabolite control mechanisms *in vivo* despite the spontaneous lability of some reactive metabolites *in vitro*. Multi-copy suppressor analyses revealed alternative, RidA-independent mechanisms of reactive enamine control. For example, when overexpressed, cystathionine β-lyase (MetC; EC 4.4.1.8) and aspartate/

glutamate racemase (YgeA, MMP0739; E.C 5.1.1.13) quench 2AA in *S, enterica* mutants lacking RidA [30, 72]. This work suggests that these enzymes do not catalyze enamine hydrolysis but instead produce another reactive metabolite that reacts with and quenches 2AA. In the case of MetC, the data support a model where MetC acts upon a non-cystathionine sulfur-containing substrate to generate a free thiol group. The free thiol is then thought to generate a stable adduct with 2AA [30] Similar thiol-mediated reactions with enzyme-bound enamines, such as during lanthionine formation, are well established [24, 73, 74]. Alternatively, YgeA and MMP0739 contain a threonine in place of an active site cysteine residue found in well-characterized PLP-independent racemases [72]. The absence of this cysteine residue is proposed to allow formation of a carbanion intermediate that participates in a nucleophilic attack of 2AA, quenching it through derivatization [72]. This model is reminiscent of alkyl radical quenching of enzyme-bound dehydroalanine used for post-translational enzyme engineering [22, 23]. The discovery of these two alternative systems for quenching 2AA underscores a number of points. First, RidA may represent one of several strategies that have evolved to limit accumulation of reactive enamines/imines *in vivo*. In fact, a handful of non-RidA enzymes have been reported to quenchimines including aldehyde oxidase (AOX; EC 1.2.3.1) [75], pyrroline 5-carboxylate reductase (ProC; EC 1.5.1.2) [76], and glutamate dehydrogenase (GdhA; EC 1.4.1.4) [77]. Second, while 2AA has a demonstrated role in damaging PLP-dependent enzymes, this molecule may also perturb the metabolic network through direct interaction with other free reactive metabolites.

Concluding Remarks

The study of RidA and its role in catalyzing hydrolysis of enamines and imines has spurred a new interest in the prevalence and impact of these and other reactive species in the metabolic network. New questions have emerged concerning the strategies used by cells to control endogenous levels of enamines and imines as well as the metabolic fate and fitness impact if their accumulation is left unchecked (see Outstanding Questions). The prevalence and consequence of enamine and/or imine species in organisms remain largely unknown, and is an exciting area for future studies. The focus of this review was limited to the deleterious consequences of uncontrolled enamine accumulation. However, less-explored physiological benefits of enamine accumulation have been described [17, 78], suggesting there may be additional instances where enamines positively influence metabolic robustness. Future work probing the generation and consequences of enamines and imines in wild-type organisms, characterizing other Rid family proteins, and Rid-independent mechanisms of quenching enamines and imines will provide insights about the role of reactive metabolites and their control in the maintenance of a robust metabolic network.

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Conflict of Interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Glossary

α,β-eliminases
Also known as β-lyases; these enzymes abstract a proton bound to the α-carbon of amino acid substrates, leading to cleavage of a bond formed with the β-carbon, generating an enamine intermediate.

Aminotransferases
Also known as transaminases; this group of PLP-dependent fold type I and IV enzymes exchange an amino group on one molecule with a keto group from a different molecule.

β-elimination
A reaction where two atoms or molecular groups are cleaved from adjacent carbon atoms, resulting in formation of a new pi bond between the adjacent atoms.

Dehydrogenases
A group enzymes that oxidize a substrate by reducing an electron acceptor, which is often in the form of NAD+/NADP+or a flavin coenzyme.

Enamines
Unsaturated compounds containing a functional group made from an alkene covalently bound to an amine.

Exogenous
Originating outside an organism.

Feedback inhibition
The end product of a metabolic reaction inhibits an enzyme involved in the generation of that product by binding a site independent from the active site.

Imines
Also known as Schiff bases, imines are unsaturated compounds containing a functional group made from a double bond between carbon and an unprotonated nitrogen.

Iminium ion
A protonated imine, or an unsaturated compound containing a functional group made from a double bond between carbon and a protonated nitrogen.

Mechanism-based inactivation
Enzyme inactivation that involves the catalytically-induced bioactivation of a compound to a reactive molecule, which then covalently modifies an active site residue and/or a prosthetic group of the enzyme, rendering it inactive.

Metabolic robustness
A qualitative assessment of alternative/additional metabolic mechanisms in place enabling an organism to withstand genetic/environmental perturbations.

Pleiotropic

An effect where a single gene impacts multiple ostensibly distinct pathways, often resulting in multiple phenotypic outcomes.

Promiscuous enzymes
Enzymes harboring activities in addition to their dedicated activity/activities, where a dedicated activity is defined by its ability to provide an observable physiological benefit to the organism. A promiscuous enzyme is defined within a specific environmental and genetic context.

Pyridoxal 5'-phosphate
The active form of vitamin B6, this coenzyme is used in a large number of diverse enzymatic reactions.

Racemases
Also known as isomerase enzymes, these enzymes invert the stereochemistry around the asymmetric carbon of the substrate.

Synteny
The physical co-localization of genetic loci on a chromosome.

Quinonoid intermediate
A mesoionic zwitterion intermediate relating in structure to a quinone, and where the negative charge is delocalized due to the heterocyclic electron-accepting nature of the pyridine ring from PLP.

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Box 1

PLP-dependent enzymes are categorized according to fold type

PLP-dependent enzymes are grouped according to structural similarities, which are generally indicative of catalytic mechanism and evolutionary relatedness [27]. Seven different fold types of PLP-dependent enzymes have been identified (See reviews by Schneider et al., and Percudani and Peracchi for a thorough coverage of PLP-dependent enzyme fold type) [68, 79]. Briefly, fold type I (aspartate aminotransferase family) is the most diverse and contains the largest number of aminotransferases, as well as a number of decarboxylases, lyases, and other enzymes. Fold type II (tryptophan synthase β family) contains alkyltransferases, ammonia lyases (α,β-eliminases), and a number of racemases, while fold type III (alanine racemase family) consists of all other PLP-dependent racemases and decarboxylases. The other class containing aminotransferases is fold type IV (D-amino acid aminotransferase family), which includes D-amino acid aminotransferases, L-branched chain aminotransferases, and 4-amino-4-deoxychorismate lyases. The three remaining fold types (V, glycogen phosphorylases; VI, D-lysine-5,6-aminomutases; and VII, L-lysine-2,3-aminomutases) each contain members belonging to one reaction type. The chemistry behind the breadth of reactions catalyzed by PLP-dependent enzymes, structural differences between fold-types, and their biological and industrial significance have been outlined in a number of reviews [27, 80–83]. Reaction specificity between PLP-dependent enzyme fold types is mediated through stereoelectronic control (cleaved bond must be aligned perpendicular to the PLP plane), electrophilic strength of the Schiff base, and catalytic side chain placement [83]. To the second point, the residues coordinating the pyridine nitrogen and the O3’ phenol oxygen from PLP appear to be largely responsible for controlling the electrophilic strength differences of the Schiff base between fold type I-IV enzymes, and their identities separate well with fold type and the respective reactions these enzymes catalyze (Figure I) [67, 84].

Outstanding Questions

- What is the catalytic mechanism used by RidA to deaminate enamines and imines?
- Do RidA/Rid family proteins act on substrates other than amino acid-derived enamines and imines?
- Many organisms contain multiple Rid homologs; do these homologs serve as specialized deaminases within specific metabolic pathways, and in the case or Rid4-Rid7 enzymes, what is the catalytic function they have?
- What structural features in PLP-dependent enzyme targets of 2-amnoacrylate (2AA) dictate the mechanism of damage and what factors minimize 2AA damage of the PLP-dependent enzymes that generate it?
- Enzyme-bound enamines can interact with nucleophilic metabolites; does 2AA perturb the metabolic network through direct interactions with free metabolites?
- Do imines or iminium ions formed from PLP-, or FAD-dependent enzymes damage enzymes or react directly with other metabolites *in vivo*?

Highlights

- Multiple PLP-dependent α,β-eliminases use α-amino acid substrates to generate and release enamine intermediates, specifically 2-aminoacrylate (2AA).
- In the absence of RidA, 2AA accumulates and damages PLP-dependent enzymes through covalent modification.
- Rid proteins are conserved in all domains of life and split into an archetypal RidA subfamily and seven other subfamilies (Rid1-Rid7). Rid4-Rid7 proteins are missing an active site arginine essential for the enamine/imine deaminase activity seen in the other subfamilies, suggesting additional uncharacterized roles for Rid enzymes.
- Non-RidA enzymes, such as *S. enterica* cystathionine β-lyase, MetC, and the putative aspartate/glutamate racemase from *S. enterica* (YgeA) or *Methanococcus maripaludis* (MMP0739) quench 2AA *in vivo*, likely by producing a reactive intermediate that interacts with free 2AA.

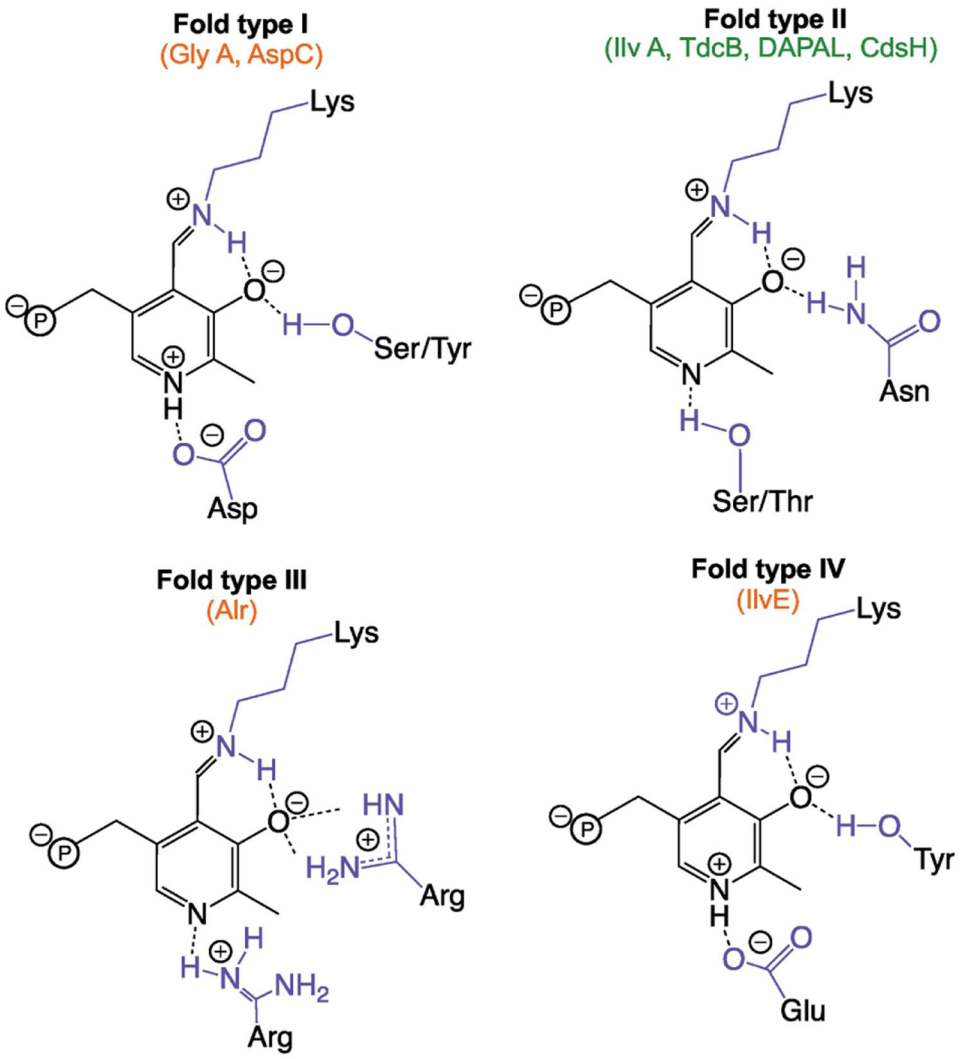

Figure I.

Generalized scheme showing coordination of pyridine N and 3'O from pyridoxal 5'-phosphate (black) for the four main fold types of PLP-dependent enzymes. Since fold types V-VII contain members belonging to only one reaction type, these were excluded from the scheme. Enzyme active site residues are shown in blue and the phosphate group of PLP is shown with 'Ⓟ'. Beneath the fold type label, generators of free 2AA are highlighted in green and enzymes damaged by free 2AA are shown in red. Note, the identified residues are provided using the listed enzymes as templates; for example, fold type III decarboxylases coordinate the pyridine N with a glutamate residue replacing the listed arginine residue. A comprehensive list of PLP coordinating residues is provided by Singh et al. [86].

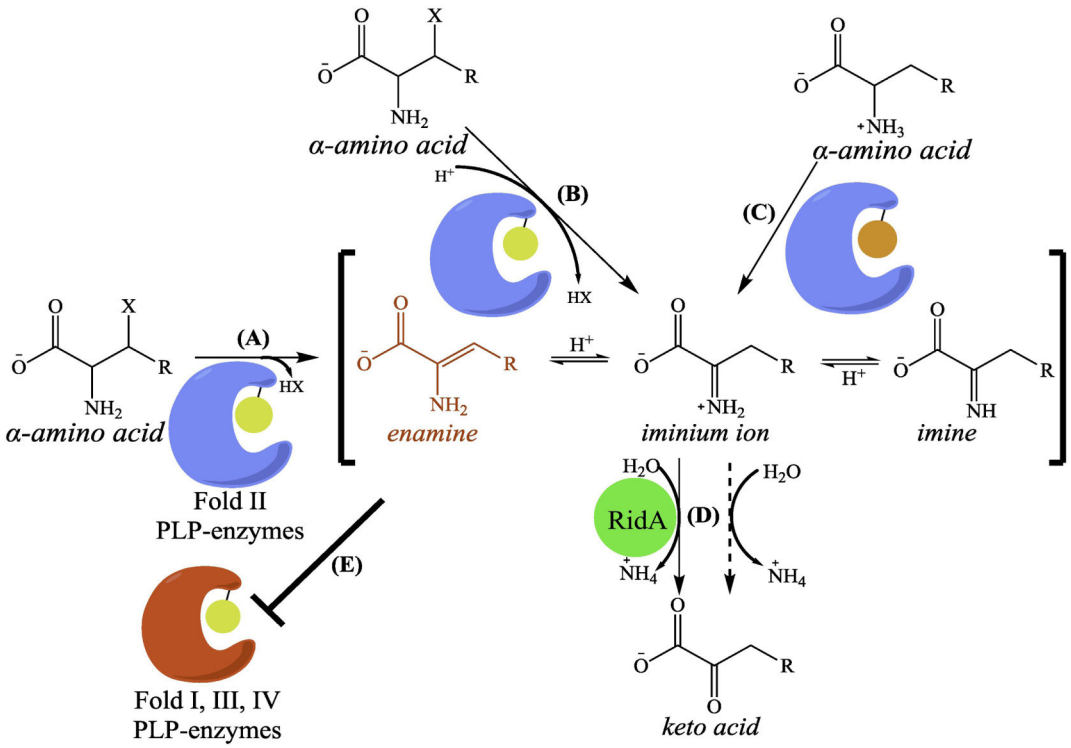

Figure 1, Key Figure - Enzymatic production of free enamines and imines.

Various fold-type II PLP-dependent enzymes produce and release a reactive enamine intermediate following α,β-elimination of an amino acid precursor (**A**). The enamine is protonated and tautomerizes to form an iminium ion intermediate. A subset of PLP-dependent α,β-eliminases enzymatically facilitate iminium ion formation prior to its release from the active site (**B**). FAD- dependent enzymes can also produce an iminium ion directly (**C**). Equilibrium between enamine, imine, and iminium ion forms is largely dependent upon pH. *In vitro*, the iminium ion can react with free water, generating a stable keto acid product (**D**). RidA serves an important role in catalyzing the deamination of enamines and imines *in vivo*. Elimination of RidA leads to accumulation of free enamines, most notably 2-aminoacrylate (2AA) (R-group = H). Free 2AA can covalently modify and inactivate a number of PLP-dependent enzymes belonging to fold-type I, III, and IV (**E**). Enamine/imine generator and target enzymes are denoted using blue and red, respectively. Relevant coenzymes are denoted as bound circles, where PLP is colored yellow and FAD is orange.

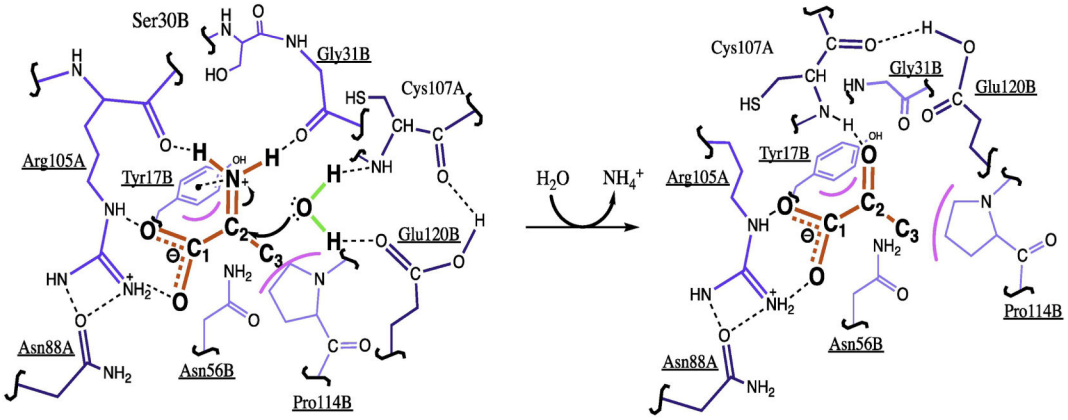

Figure 2 –. Proposed reaction mechanism for RidA-mediated hydrolysis of 2-iminiopropionate (2IP).

A schematic for the proposed reaction mechanism of RidA using data extrapolated from the crystal structure of the RidA homolog, TdcF, with bound serine (2UYK) and 2-ketobutyrate (2UYN) [46]. The seven residues conserved among Rid family members are underlined. The substrate/product are shown in red, water is depicted in green, and RidA residues are shown in blue. The active site is at the interface of two subunits and residues from one subunit are labeled with an “A” while those from the other are labeled “B”. Darker blue colors indicate residues in the foreground and paler blue colors are used for those in the background. The carboxyl group of 2IP forms a salt bridge with the R105 side-chain, while the protonated imine is stabilized through hydrogen bonding with the backbone carbonyl groups from R105 and G31 (left). The imine is also stabilized through a cation-pi interaction with the phenyl group from Y17. The secondary amine from the C107 backbone and the carbonyl group from the E120 side chain activate water for its nucleophilic attack on C2 of the bound iminium ion. Following a rearrangement, pyruvate is produced in the active site, before it is released along with ammonium (right).

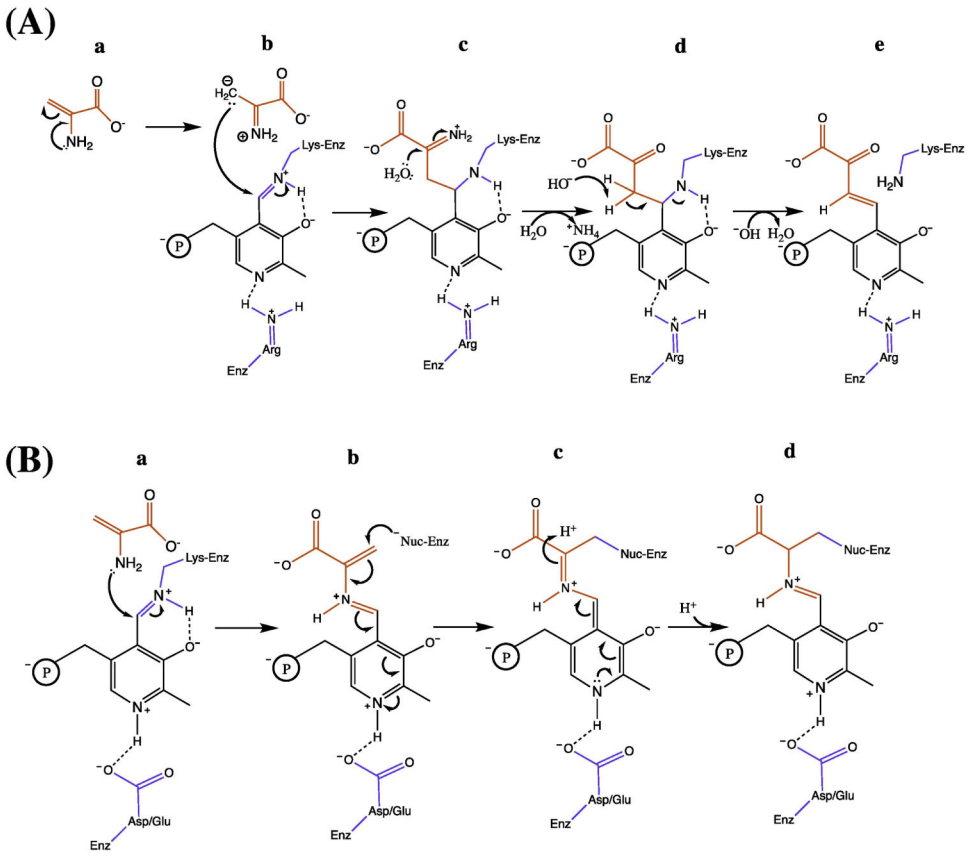

Figure 3 –. Free 2-aminoacrylate inhibits PLP-dependent enzymes by two mechanisms.

2AA is highlighted in red and enzyme active site residues are shown in blue. The phosphate group of PLP is shown with '℗'. (**A**) A PLP/pyruvate adduct is formed following addition of 2AA via a C-C linkage. (a) Tautomeric rearrangement of 2AA generates a carbanion intermediate that (b) acts as a nucleophile to attack the 4'C of PLP. (c) Hydrolysis of the imine releases ammonium and creates a PLP/pyruvate adduct covalently bound to the active site lysine residue. (d) The adduct is susceptible to base-catalyzed formation of (e) a free PLP/pyruvate adduct. (**B**) 2AA/PLP covalently modifies a nucleophilic active site residue. (a) 2AA forms an external aldimine with PLP. (b) The alkene from 2AA participates in the conjugated π-bond system with PLP, strengthening the electrophilic nature of Cβ and allowing nucleophilic attack by an active site residue. (c) This forms a quinonoid intermediate, which can be rearranged and protonated to produce (d) a 2AA/PLP external aldimine covalently bound to a nucleophilic active site residue.

TABLE 1

Characterized generators and targets of 2-aminoacrylate stress in *Salmonella enterica*

A

Endogenous 2AA generators	Gene Product	Stress[a]	Reference
Anabolic Ser/Thr dehydratase [EC: 4.3.1.19]	IlvA	Serine	[49]
Catabolic Ser/Thr dehydratase [EC: 4.3.1.19]	TdcB[b]		
Cysteine desulfhydratase [EC: 2.5.1.47]	CdsH	Cysteine	[15]
Diaminopropionate NH4 lyase [EC: 4.3.1.15]	DAPAL	Diaminopropionate	[16]

B

Endogenous 2AA targets	Gene Product	Phenotype[c]	Reference
Serine hydroxymethyl transferase [EC: 2.1.2.1]	GlyA	Gly limitation in minimal medium	[85]
BCAA Aminotransferase [EC: 2.6.1.42]	IlvE	Ile requirement on pyruvate carbon source	[49]
Alanine racemase [EC: 5.1.1.1]	Alr	Ala is a poor nitrogen source	[42]
Alanine racemase [EC: 5.1.1.1]	DadX	Ala is a poor nitrogen source	[42]

[a] In the absence of RidA, if the listed metabolite is added to minimal glucose growth medium, the relevant generator enzyme uses it to produce 2AA and prevent cell growth.

[b] TdcB is only produced anaerobically in response to threonine and does not contribute to 2AA stress under standard aerobic growth conditions.

[c] 2AA-dependent damage of the relevant enzyme is responsible for the listed growth phenotype in a *S. enterica ridA* mutant. Abbreviations: Gly:glycine, Ile:isoleucine, Ala:alanine