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Priming ammonia lyases and aminomutases for industrial and therapeutic applications

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Ammonia lyases (AL) and aminomutases (AM) are emerging in green synthetic routes to chiral amines and an AL is being explored as an enzyme therapeutic for treating phenylketonuria and cancer. Although the restricted substrate range of the wild-type enzymes limits their widespread application, the non-reliance on external cofactors and direct functionalization of an olefinic bond make ammonia lyases attractive biocatalysts for use in the synthesis of natural and non-natural amino acids, including β-amino acids. The approach of combining structure-guided enzyme engineering with efficient mutant library screening has extended the synthetic scope of these enzymes in recent years and has resolved important mechanistic issues for AMs and ALs, including those containing the MIO (4-methylideneimidazole-5-one) internal cofactor.

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Introduction

Enzymatic routes to chiral amines such as α-amino and β-amino acids, are increasingly attractive options for industrial synthetic needs [1], especially after concerted initiatives that have emphasized the environmental importance of asymmetric amination reactions [2,3]. One such biocatalytic conversion is the amination of ketones by transaminases for production of chiral amines [3] such as β-amino acids [4]. However, the widespread application of transaminases is still in an infancy stage due to equilibrium and enzyme stability issues, as well as product separation challenges [3,5]. In addition to transaminases, hydantoinases [6] and Baeyer–Villiger monooxygenases [7] can be applied for the biocatalytic synthesis of β-amino acids. However, these enzymatic processes can be hampered by the requirement of additional (de)protection steps and the theoretical maximal yield limit of 50% when using preparative kinetic resolutions. An attractive alternative enzymatic route to enantiomerically pure amino acids involves the use of C–N lyases such as ammonia lyases (ALs) [8]. The ALs catalyze the reversible addition of ammonia and amines to double bonds of the corresponding α,β-unsaturated carboxylic acids, which are often cheap substrates. Aminomutases (AMs) play a role in the catabolism of lysine and the biosynthesis of β-amino acids, from which biotechnological applications have transpired according to a recent review [9]. Most of these enzymes contain radical-generating cofactors.

Biocatalytic and therapeutic applications of C–N lyases are on the rise due to the advancement of discovery and engineering technologies to improve functional properties of these enzymes [10•]. Such methods allow ALs to replace traditional chemical synthetic steps [11••,12], offer expanded synthetic scopes [13••,14], and indicate that ALs may serve in therapeutic applications for treating phenylketonuria (PKU) and cancer [15–17] (Figure 1). Furthermore, valuable mechanistic insight and engineering efforts have been reported in recent years for aspartase, 3-methylaspartate ammonia lyase (MAL), and C–N lyases containing the 4-methylideneimidazole-5-one (MIO) cofactor. This review highlights these breakthroughs, as well as the broadening of synthetic scopes that prime these enzymes for industrial applications.

Mechanistic aspects of ammonia lyases and aminomutases

Aspartase

Microbial aspartases (EC 4.3.1.1) catalyze the reversible deamination of L-aspartic acid to produce fumarate and ammonia (Figure 2a), an important reaction in nitrogen metabolism. The ubiquitous aspartases belong to the aspartase/fumarase (AF) superfamily, which comprises tetrameric proteins that have three main α-helical domains within each monomeric subunit [18,19]. Sequence identities among superfamily members that produce similar active site architectures may be as low as 15%. Most of these members convert a succinyl derivative. The structural similarities among the superfamily members suggest that all of them follow a mechanism in which a general base catalyzes proton abstraction and formation of an enediolate intermediate [19]. In the case of L-aspartase, the overall conversion follows an anti-elimination reaction (Figure 3a) that begins with abstraction of the *pro*-(R) proton from the

Cβ-position of L-Asp by the catalytic base (I), with subsequent intermediate stabilization (II). Fumarate is formed from a collapse of the intermediate, which may be assisted by a general acid (ambiguous for aspartases) that protonates the amino-moiety for deamination (III).

Recent work by Fibriansah *et al.* [20••] shed light on the precise mechanism of the AF superfamily members by identifying the catalytic base, reaffirming substrate-binding residues, and defining the role of the active site SS-loop containing two consecutive serines, of which one is the catalytic base. From an analysis of AspB structures in a L-Asp-bound (PDB: 3R6V) and unbound state (PDB: 3R6Q), it appeared that the substrate-induced closure of the SS-loop stabilizes the substrate in the active site, and that Ser-318 within the SS-loop is the catalytic base. The C-terminal domain was also implicated in controlling the SS-loop conformational change. To further support a general mechanism for AF superfamily members, a class II fumarase from *M. tuberculosis* (Rv1089c) was characterized by Mechaly *et al.* (PDB: 4ADL) [21•]. Rv1098c fumarase was shown to contain the same catalytic base as AspB within the SS-loop and also displayed the conformational changes of the SS-loop and C-terminal domain, which are associated with substrate binding.

Unresolved mechanistic issues [19] of the AF superfamily relate to the activation of the catalytic Ser. In addition, the identity of the catalytic acid in aspartase (seemingly a His-residue in most AF superfamily members) and the protonation state of the amino-leaving group, especially if no acid catalyst exists, remain elusive.

3-Methylaspartase ammonia lyase (MAL)

The physiological reaction catalyzed by MAL (EC 4.3.1.2) involves the deamination of L-*threo*-3-methylaspartate (major substrate, catabolic intermediate from L-Glu degradation) and L-*erythro*-3-methylaspartate (minor substrate, not depicted) to yield mesaconate and ammonia (Figure 2b) in *Clostridium tetanomorphum* H1 and other facultative anaerobes [18]. The TIM barrel fold of MAL associates it with the enolase superfamily. The catalytic cycle (Figure 3b) starts with abstraction of the Cβ proton (I) of L-*threo*-3-methylaspartate by Lys-331 (S-specific acid/base catalyst, depicted) or His-194 (R-specific acid/base catalyst), creating an enolate intermediate that is stabilized by Mg^{2+} coordination (II) [18]. The subsequent collapse of the intermediate releases ammonia and mesaconate (III). MAL lacks the moving SS-loop indigenous to AF superfamily members, resulting in a more fixed active site.

MIO-enzymes

Using the MIO internal cofactor (inset, Figure 2c), aromatic ALs deaminate phenylalanine (PAL), tyrosine (TAL), and histidine (HAL) for mostly catabolic roles in eukaryotes and prokaryotes. PAL has been extensively investigated (Table 1) [22–32], both for its critical role in plant physiology, including lignin biosynthesis, and for the synthesis of antioxidants [8]. The homologous AMs (phenylalanine aminomutase, PAM; tyrosine aminomutase, TAM) perform a 2,3-amine shift to interconvert α-amino and β-amino acids (Figure 2c) [9] to provide precursors of secondary metabolites (e.g. β-amino acids) in plants, fungi, and bacteria that are bioactive as antibiotics such as the anticancer drug taxol [8,9,33,34•]. The MIO cofactor is formed by an autocatalytic condensation of the Ala/Thr-Ser-Gly motif, creating a catalytic group atop a large helix bundle. Recent mechanistic evidence based on structural data [35••,36•,37••,38], kinetic isotope studies [14,39,40], computational work [41,42], the use of non-natural substrate probes [14,38,43••], and MIO inactivation studies [43••,44] support a MIO-amine covalent intermediate. This MIO-amine adduct facilitates the elimination of the vicinal NH_2/H pair with assistance from the catalytic base, which is a tyrosine located within a loop close to the N-terminus, to produce the corresponding α-unsaturated/β-unsaturated carboxylic acids. Although prior work [45] suggested the elimination to follow an E_1cB path (carbanionic intermediate), recent studies suggest that MIO-enzymes may adhere to an E_2 elimination step (concerted NH_2/H elimination) [36•,38,41].

Walker and colleagues have contributed significantly to our understanding of the MIO-AM mechanism by performing detailed biochemical and structural studies on a plant PAM from *Taxus canadensis* (*Tc*PAM, PDB: 3NZ4) [14,35••,40] and a bacterial PAM from *Pantoea agglomerans* (*Pa*PAM, PDB: 3UNV) [36•,39]. The mechanism of MIO-dependent AMs is illustrated with the *Tc*PAM catalytic cycle in Figure 3c, which begins with an electrophilic attack on (2*S*)-α-Phe by the MIO group (I) that produces an N-linked enzyme–substrate complex (II). The catalytic tyrosine abstracts the *pro*-(3S) proton with a concerted elimination of the amino group (III) to give the *trans*-cinnamic acid (*t*-CA) intermediate and the MIO-amine adduct. For re-addition of the NH_2/H pair (IV a, b) to conform to the stereochemical outcome, an intermediate flip must take place (III → IV a, b) before readdition of the ammonia and the proton to the opposite faces of Cα and Cβ (compared to the abstraction face) of the cinnamate (V a, b) to give (3*R*)-β-Phe (VI). An Arg-residue in both crystal structures of *Pa*PAM and *Tc*PAM was shown to form a mono-dentate or bi-dentate salt bridge with the carboxy-group of pathway intermediates, including α-Phe and β-Phe adducts in *Pa*PAM and *t*-CA in *Tc*PAM. The *t*-CA-*Tc*PAM complex confirmed *t*-CA as the reaction intermediate and led the authors to propose concerted 180° rotations about the C_1–C_α and C_β–C_{ipso} bonds (Figure 3c (III → IV b)) to explain the stereochemistry, with Arg remaining bound to *t*-CA throughout the catalytic