Enzyme Catalysis

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Mechanism-Inspired Engineering of Phenylalanine Aminomutase for Enhanced β-Regioselective Asymmetric Amination of Cinnamates**

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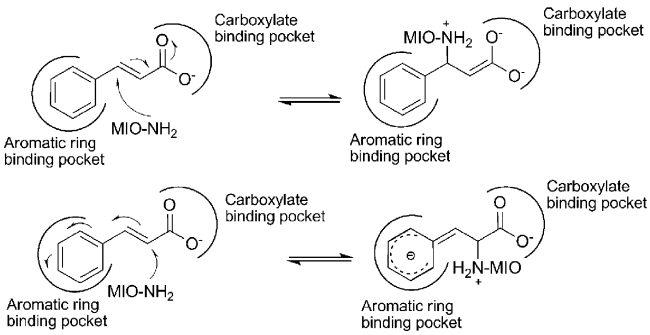
In the last decade, enormous progress has been made in the synthesis of optically pure β-amino acids, which occur as components of numerous bioactive compounds.[1] Although biocatalytic routes have been explored, they tend to use kinetic resolutions, implying a maximum yield of 50%.[2] Only a few enzymes, including aminotransferases[3] and aminomutases,[4] have been used in asymmetric synthesis of this class of compounds. The asymmetric addition of ammonia to cinnamic acid and its derivatives catalyzed by phenylalanine aminomutase (PAM) is an attractive route to enantiopure aromatic β-amino acids, because the substrates are readily available and PAM exhibits excellent enantioselectivity.[5] However, the final product mixture consists of both α- and β-amino acids owing to low regioselectivity.[6] Although selective separation procedures have been developed,[6] a much better option would be to employ a highly β-regioselective enzyme. Unfortunately, no such enzyme has been discovered to date. Herein we describe the successful alteration of *Taxus chinensis* PAM into a selective β-lyase through structure- and mechanism-based protein engineering.

The activity of PAM relies on the internal cofactor 4-methylideneimidazole-5-one (MIO).[7] The role of MIO has been debated,[8] but most recent evidence, including X-ray structures of aminomutases in complex with substrate analogues,[9] suggests that in the mutase reaction MIO reacts with the amine group of the substrate. Kinetic studies of PAM with different cinnamates indicated that the regioselectivity is dominated by electronic effects,[5c,d] with either the aromatic ring or the carboxylate group acting as an electron sink to facilitate α- or β-addition, respectively. The delocalization of negative charge to these groups must be stabilized by the protein environment (Scheme 1). Therefore we hypothesized that mutations that diminish the ability of the phenyl ring binding pocket to accommodate negative charge or mutations that enhance the electron deficiency of the carboxylate binding pocket will likely promote β-addition.



Scheme 1. Proposed electronic effects that influence the regioselectivity of PAM-catalyzed amination reactions.

To identify targets for mutations, we inspected structures of cinnamic acid bound PAM (PDB 3NZ4)[10] and the apoenzyme (PDB 2YII, see the Supporting Information). Overall, PAM is structurally similar to the *Streptomyces globisporus* tyrosine aminomutase (*sg*TAM).[9] The two loops of PAM covering the active site, which are also present in other MIO-dependent enzymes,[9,11] are in a closed conformation. The active-site residues can be divided in two distinct sets, which form the aromatic ring binding pocket (F86, L104, C107, L108, L179, Q459; Figure 1A) and the carboxylate binding pocket (N231, Q319, Y322, R325, N355, F371; Figure 1B), respectively.

The binding pockets were separately mutated using a pairwise saturation strategy.[12] The carboxylate-pocket residues were divided into three sets, each comprising two structurally neighboring residues. Three saturation libraries were constructed (Q319X/R325X, N231X/N355X, Y322X/

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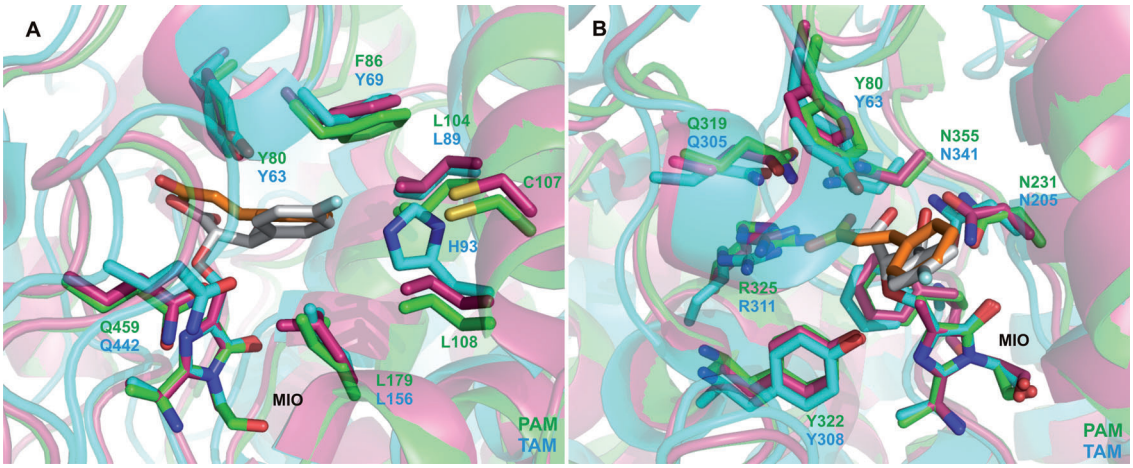


Figure 1. The binding pockets of PAM. A) Superposition of the aromatic ring binding pocket of PAM with that of *sg*TAM; B) Superposition of the carboxylate binding pocket of PAM with that of *sg*TAM. Blue: *sg*TAM in complex with substrate analogue α-di-F-β-Tyr (gray); green: apo structure of PAM; red: PAM in complex with *trans*-cinnamic acid (brown). C = Cys = cysteine, F = Phe = phenylalanine, H = His = histidine, L = Leu = leucine, N = Asn = asparagine, Q = Gln = glutamine, R = Arg = arginine, Y = Tyr = tyrosine.

F371X) and expressed in *E. coli* as N-terminal His-tag fusion proteins. The mutants were purified in a 96-well format using cobalt affinity resin plates (see the Supporting Information) and prescreened for deamination activity towards β-Phe by monitoring the formation of cinnamic acid at 290 nm. Whereas the mutants in libraries N231X/N355X and Y322X/F371X showed no detectable activity, two active mutants (Q319M and R325K; M = Met = methionine, K = Lys = lysine) were identified in library Q319X/R325X. The R325K mutant showed a 2.5-fold increase in deamination activity towards β-Phe and its β-regioselectivity (percentage of β-Phe in the product mixture) in the amination reaction of cinnamic acid had shifted from 49% (wild-type (wt) PAM) to 85%. The Q319M mutant exhibited fivefold enhanced β-Phe deamination activity and increased β-regioselectivity (88%), but an enzyme in which both mutations (Q319M and R325K) are combined was inactive.

Table 1: Kinetic parameters and regio- and enantioselectivity of PAM mutants in amination reactions.[a]

Enzyme	k_{cat} [$s^{-1} \times 10^3$]	K_m [mM]	k_{cat}/K_m [$s^{-1}\,M^{-1}$]	β [%]	ee_β (*R*)	ee_α (*S*)
wt PAM	12 ± 2	2.6 ± 0.6	6.3 ± 1.8	49 ± 1	> 99%	> 99%
R325K	8 ± 1	5.2 ± 0.4	1.5 ± 0.3	85 ± 2	> 99%	> 99%
Q319M	15 ± 1	1.2 ± 0.2	12 ± 1	88 ± 2	> 99%	> 99%
R325K + Q459E	> 0.15[b]	n.d.	n.d.	ca. 95	> 99%	n.d.

[a] Reactions were performed in saturated ammonium carbonate buffer at pH 9, and the cinnamate concentration was varied. [b] No saturation was obtained. Number represents the catalytic rate at the conditions used.

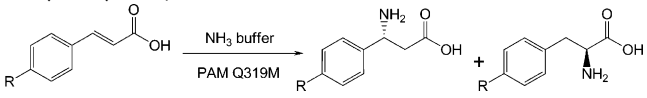
We then mutated the aromatic ring binding pocket for improved β-regioselectivity. Mutagenesis work was focused on residues C107 and Q459, to avoid changing the conserved nonpolar residues. From library C107X/Q459X/R325K mutant R325K/Q459E was discovered. This mutant retained 30% of the deamination activity towards β-Phe, and its deamination activity towards α-Phe was almost completely abolished ($V_{rel} < 1\%$ of wt PAM). In an amination reaction with cinnamic acid, the mutant appeared to be highly selective towards the β-position (up to 95% β-addition, Table 1), although the activity was low.

The enantioselectivity of the enzyme, both at the C_α and C_β carbon atom of the substrate, was retained in all mutants. The products of the amination reaction were enantiopure (*S*)-α-Phe and (*R*)-β-Phe. A time-course experiment with the efficient Q319M mutant showed that nearly 90% β-selectivity was maintained until at least 70% conversion (see the Supporting Information). Furthermore, we set up a laboratory-scale preparative experiment by incubating *trans*-cinnamic acid (20 mg) with Q319M mutant enzyme (5 mg) in ammonia solution (2 M, pH 9, 10% glycerol). After four days the conversion reached 60%, and after purification with a cation exchange resin (Dowex 50W X8) column, a 1:7 mixture of (*S*)-α-Phe ($ee > 99\%$) and (*R*)-β-Phe ($ee > 99\%$) was isolated with 50% yield. This outcome represents an almost twofold increase in the yield of (*R*)-β-Phe compared to the process in which wt PAM was used as a catalyst.[5d]

To investigate the applicability of the regioselective mutant for preparation of other aromatic β-amino acids, we used the Q319M mutant to convert several cinnamates. The results, compared to those obtained with wt PAM (Table 2), showed that in all cases the mutant is superior in terms of β-selectivity, thus allowing the preparation of various β-amino acids. Notably, ammonia addition to a cinnamic acid derivative with an electron-donating *p*-methyl substituent gave exclusively the β-product. Furthermore, the Q319M mutant has an improved affinity (lower K_m value) for all compounds described in entries 1–5 (Table 2); the improved affinity results in significantly higher catalytic efficiency (k_{cat}/K_m) than for wt PAM.[5c]

As expected, the introduction of an electron-rich, charged residue in the aromatic binding pocket (Q459E) enhanced β-selectivity, because the mutation reduces the electron-withdrawing effect of the aromatic ring. However, it was

Table 2: Kinetic parameters and regioselectivity of amination reactions catalyzed by the Q319M mutant PAM.



Entry	R	k_{cat} [$s^{-1}\times10^3$]	K_m [mM]	k_{cat}/K_m [$s^{-1}\,M^{-1}$]	β [%]	β [%] wt PAM[5c]
1	H	15±1	1.2±0.2	12±1	88±2	49
2	F	10±0.03	0.21±0.003	48±1	91±2	65
3	Cl	8.7±0.4	0.022±0.003	395±72	86±2	59
4	Me	12±0.3	0.072±0.004	167±14	>99	96
5	NO_2	98±8	0.3±0.06	326±92	8±1	2

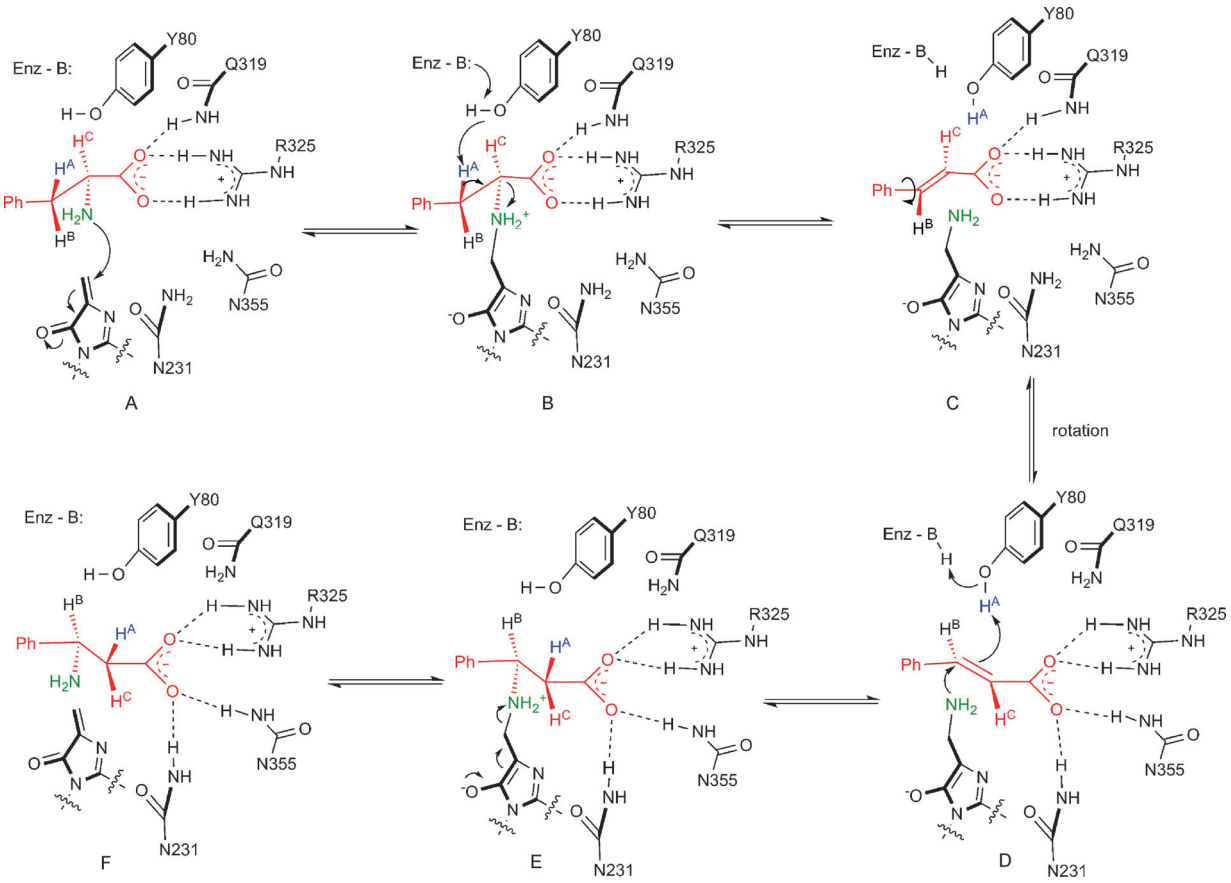
unexpected that the Q319M or the R325K mutations in the carboxylate binding pocket also enhanced β-regioselectivity, because electrostatic interactions that stabilize negative charge on the carboxylate group during conjugate addition would be weakened by these mutations. To unveil the mechanistic basis of the enhanced β-regioselectivity induced by the mutations in the carboxylate binding pocket, we analyzed the structural and biochemical data on MIO-dependent aminomutases.

It has been demonstrated that PAM catalyzes the conversion of (*S*)-α-Phe into (*R*)-β-Phe by exchanging the amine group ($C_\alpha\rightarrow C_\beta$) and the pro-3S hydrogen atom ($C_\beta\rightarrow C_\alpha$) with retention of configuration at the reaction termini.[13] A possible explanation is that the phenyl ring and carboxylate group of (*S*)-α-Phe are arranged in a *syn*-periplanar orientation with the amine group and the leaving pro-3S hydrogen atom positioned on the same side of the substrate, leading to the formation of *cis*-cinnamic acid. However, the evidence listed below suggests that *trans*-cinnamic acid is the real intermediate: 1) *trans*-cinnamic acid is released from the active center during the mutase reaction;[14] 2) only the *trans* form of cinnamic acid is a substrate for PAM;[5c] 3) the X-ray structure shows bound *trans*-cinnamic acid in the active site of PAM.[10] Accordingly, the stereochemistry requires a reorientation after deamination of (*S*)-α-Phe to cinnamic acid such that the *Re* face of the C_β and the *Si* face of the C_α carbon atom are positioned for amine addition and protonation, respectively.[10,13] In the structure of PAM with *trans*-cinnamic acid bound, the carboxylate group makes a strong salt bridge with residue R325.[10] Feng et al. postulated that this salt bridge is maintained, while both the C_1–C_α and the C_β–C_{ipso} bonds rotate to fit the stereochemical course of the PAM reaction.[10] However, this rotation is energetically burdensome, because it requires the loss of olefinic bond conjugation with both the aromatic ring and the carboxylate group. Furthermore, our mutagenesis work shows that interfering with the proposed stable salt bridge enhances β-addition.

We propose a mechanism for the mutase reaction that explains both the stereochemical features and the results of our mutagenesis experiments. When (*S*)-α-Phe enters the active site of PAM, the carboxylate group forms a bidentate salt bridge with residue R325 (Scheme 2 A, binding mode 1), as present in the cinnamic acid bound PAM structure. The carboxylate group further interacts with residue Q319. The amine group and pro-3S hydrogen atom (H^A, Scheme 2 A) are oriented in an *anti*-periplanar geometry that is suitable for the elimination step. A nucleophilic attack of the amine group of the substrate on the MIO group preceeds abstraction of the proton (H^A) by the general base Y80 (Scheme 2 B→C), possibly assisted by another basic group, and elimination of MIO-NH_2. This sequence leads to formation of *trans*-cinnamic acid, with the carboxylate group still bound to residue R325 (Scheme 2 C). The next step is the readdition of MIO-NH_2 to the β-position and of proton H^A to the α-position. This step requires exposure of the *Re* face of C_β to the MIO-NH_2 and the carboxylate group to function as a good electron sink. This 1,4-addition is hindered when the substrate makes a salt bridge with residue R325, owing to the high energy level of the LUMO (lowest unoccupied molecular orbital) of this ionized electrophile.[15] However, *trans*-cinnamic acid can undergo an internal rotation around the C_β–C_{ipso} bond to position the carboxylate group into an alternative binding mode where each oxygen atom is involved in two hydrogen bonds, that is, with residues N231, R325, and N355 (Scheme 2 D, binding mode 2). The sharing of hydrogen atoms between these H-bond donors and the carboxylate group of the substrate lowers the LUMO of the latter, thereby rendering the carboxylate a better electron sink and promoting conjugate addition at the β-position.[15b] Similar LUMO-lowering activation through hydrogen-bond interactions has been observed in other conjugate additions[16] and in Diels–Alder[17] enzymatic reactions. The re-added proton apparently is not always the pro-3S hydrogen atom from the substrate, because some hydrogen exchange was observed during the isomerization reaction.[13] The mutations (Q319M and R325K) in the carboxylate binding pocket would disturb binding mode 1, and consequently make binding mode 2 more favorable and promote β-addition.

Additional evidence for this proposal is provided by a *sg*TAM structure (PDB 2RJR),[9b] the active site of which is similar to that of PAM. The alternative binding mode 2 of the carboxylate group proposed for PAM has already been observed in the *sg*TAM structure with a substrate analogue bound in the active center. The carboxylate oxygen atoms are involved in a hydrogen-bonding network with residues N205, R311, and N341 (corresponding to N231, R325, and N355 in PAM).[9b] The aromatic hydroxy group forms hydrogen bonds with residues H93 and Y415, thereby triggering a different binding mode for the ring as compared to *trans*-cinnamate in PAM and placing the carboxylate group in an orientation that is suitable for β-readdition without rotation, leading to formation of (*S*)-β-Tyr instead of (*R*)-β-Tyr.

In summary, we have shown that the regioselectivity of a MIO-dependent enzyme can be tailored towards β-regioselectivity. A redesigned enzyme allows the synthesis of almost pure (*R*)-β-Phe and its derivatives by one-step asymmetric amination of cinnamic acid. Further engineering of PAM, aimed at improving activity while retaining regioselectivity and enantioselectivity, is underway. In combination with stereochemical,[13] synthetic,[5] and structural investigations,[9,10] the results provide a framework for explaining and engineering the regioselective and stereochemical properties of MIO-dependent aminomutases.



Scheme 2. Representation of the stereochemical course and reaction mechanism of PAM. The conformation of *trans*-cinnamic acid and the orientation of its carboxylate in (C) are based on the structure of PAM with bound *trans*-cinnamic acid (PDB 3NZ4). Enz-B: = enzyme base.

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