Reaction Mechanisms

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Phenylalanine Ammonia Lyase Catalyzed Synthesis of Amino Acids by an MIO-Cofactor Independent Pathway**

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Abstract: *Phenylalanine ammonia lyases (PALs) belong to a family of 4-methylideneimidazole-5-one (MIO) cofactor dependent enzymes which are responsible for the conversion of* L*-phenylalanine into trans-cinnamic acid in eukaryotic and prokaryotic organisms. Under conditions of high ammonia concentration, this deamination reaction is reversible and hence there is considerable interest in the development of PALs as biocatalysts for the enantioselective synthesis of non-natural amino acids. Herein the discovery of a previously unobserved competing MIO-independent reaction pathway, which proceeds in a non-stereoselective manner and results in the generation of both* L- *and* D*-phenylalanine derivatives, is described. The mechanism of the MIO-independent pathway is explored through isotopic-labeling studies and mutagenesis of key active-site residues. The results obtained are consistent with amino acid deamination occurring by a stepwise E_1cB elimination mechanism.*

Optically active amino acids are fundamental building blocks for the synthesis of a wide range of bioactive natural products, pharmaceuticals, and agrochemicals. For example, there is significant interest in the development of therapeutic peptides and proteins which can contain both natural and non-natural amino acids.[1] Non-proteinogenic amino acids have also been used independently as drug molecules, including Levodopa (L-dopa), the main drug used for the treatment of Parkinson's disease.[2] Furthermore, asymmetric synthesis frequently relies on amino acids as chiral starting materials and auxiliaries.[3] The increasing demand to access amino acids in optically pure form has led to the development of a number of biotechnological approaches.[4] A particularly attractive method for the synthesis of aromatic amino acids involves the use of phenylalanine ammonia lyases (PALs), a class of enzymes which catalyze the reversible regio- and stereoselective hydroamination of *trans*-cinnamic acid derivatives.[5] These reactions are compatible with aqueous and metal-free conditions and have the distinct advantage that no protecting group chemistry is required. Additionally PALs utilize readily available achiral cinnamic acid derivatives as starting materials and they do not rely on external cofactors or recycling systems, making them particularly suitable as industrial biocatalysts.[6] This feature is highlighted by the recent application of PAL in the synthesis of 2′-chloro-L-phenylalanine on a ton scale, by DSM Pharma Chemicals.[7]

To allow structure-guided rational engineering for the development of biocatalysts with desirable properties, a detailed understanding of the enzyme's catalytic mechanism is highly advantageous. However the PAL catalytic mechanism is still under investigation. PALs belong to a family of 4-methylideneimidazole-5-one (MIO) cofactor dependent enzymes, which also include histidine and tyrosine ammonia lyases (HALs and TALs) and aminomutases (AMs), the latter catalyzes the isomerization of α- and β-amino acids.[8] The MIO cofactor is formed by an autocatalytic condensation reaction of a conserved Ala-Ser-Gly motif. Mechanistic studies suggest the presence of a covalent intermediate formed by the addition of the substrate amine onto the exocyclic methylene group of the cofactor (Figure 1).[9]

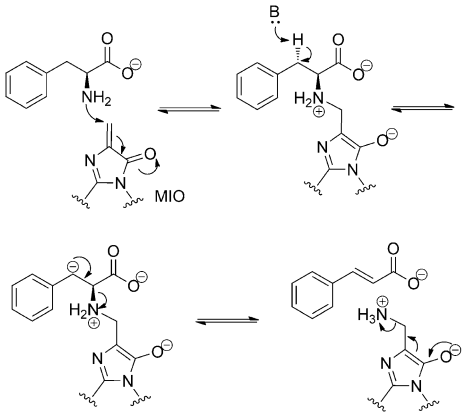

Figure 1. Proposed E_1cB mechanism for the PAL-catalyzed L-phenylalanine deamination reaction.

Subsequent elimination of the amine to form cinnamic acid is generally reported to occur by an E_1cB mechanism, although evidence in favor of an E_2 elimination pathway has also been presented.[10] An alternative Friedel–Crafts mechanism has been proposed on the basis that the elimination mechanisms involve unfavorable abstraction of a non-acidic proton from the β-position of the amino acid.[11] Nonetheless, there is considerable evidence to support the formation of an

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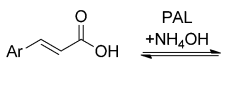
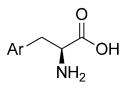
amine–MIO intermediate, and the evidence includes crystallographic data,[12] molecular mechanics calculations,[10,13] and kinetic isotope studies.[14]

The substrate range of eukaryotic PALs from the plant *Petroselinum crispum* (*Pc*PAL) and yeast *Rhodotorula glutinis* (*Rg*PAL) have been well characterized and show activity towards a range of substituted phenylalanine derivatives. The nature of the aromatic substituent has been shown to greatly influence activity, providing insights into the enzyme's catalytic mechanism.[15] To date, there have been few reported examples of bacterial PALs and they have not been previously exploited as biocatalysts for non-natural amino acid synthesis. Recently, the crystal structure of a double mutant of PAL from the bacteria *Anabaena variabilis* (*Av*PAL) was solved, representing the first structure showing the flexible catalytic loops well-resolved and in the active conformation for catalysis.[12c] Availability of a detailed crystal structure makes *Av*PAL an attractive candidate for directed evolution. To investigate the suitability of *Av*PAL as a biocatalyst for amino acid synthesis, the activity of wt *Av*PAL was determined alongside the eukaryotic *Pc*PAL and *Rg*PAL. The conversion of a panel of cinnamic acid derivatives (**1a**–**n**; (Table 1) into their corresponding amino acids were monitored over time using *E. coli* BL21(DE3) whole cells expressing the individual PALs. The activity of *Av*PAL was largely comparable to that of *Pc*PAL and *Rg*PAL, with all three enzymes catalyzing the conversion of the substrates **1a**–**n** with high to moderate conversions. All reactions initially proceeded with excellent enantioselectivity in favor of the L-amino acids (see the Supporting Information). Interestingly, it was observed that the *ee* values of the products **2b**, **2d**–**f**, and **2h**–**m** diminished significantly after prolonged reaction times with all three PAL biocatalysts. These products all possess electron-withdrawing substituents on the aromatic ring, suggesting that PALs are able to catalyze the formation of the D enantiomers of electron-deficient structures. D-amino acid formation is especially prominent with substrates which are able to stabilize a negative charge at the benzylic position, for example, in the cases of **1k** and **1m**, which possess *ortho* and *para* nitro substituents, respectively. The *Av*PAL-mediated amination reaction of **1b**, **1e**–**f**, **1h**–**j**, and **1l** demonstrated significantly higher enantioselectivity compared with that of *Pc*PAL and *Rg*PAL under the reaction conditions employed. Although the application of PALs as biocatalysts for the amination of cinnamic acid analogues has been described extensively in literature and implemented in industrial processes,[7] the formation of D-amino acids has not been reported. Furthermore to our knowledge the time-dependence of the *ee* value has not been described previously.

Table 1: The conversion and *ee* values of the cinnamic acid derivatives **1a**–**n** (5 mM) after 22 h.





Ar =
a C_6H_5 **h** 2-pyridyl
b 3F-C_6H_4 **i** 3-pyridyl
c 4F-C_6H_4 **j** 4-pyridyl
d 4CF_3-C_6H_4 **k** 2NO_2-C_6H_4
e 2Cl-C_6H_4 **l** 3NO_2-C_6H_4
f 3Cl-C_6H_4 **m** 4NO_2-C_6H_4
g 4Cl-C_6H_4 **n** 3HO-C_6H_4

	*Av*PAL		*Pc*PAL		*Rg*PAL	
	Conv.	*ee* [%]	Conv.	*ee* [%]	Conv.	*ee* [%]
1a	49	>99	58	>99	55	>99
1b	59	>99	75	6	71	2
1c	59	>99	60	>99	61	>99
1d	76	71	75	96	75	93
1e	91	>99	94	0	93	13
1f	62	>99	72	14	76	7
1g	53	>99	50	>99	51	>99
1h	61	76	49	−8	62	38
1i	80	>99	87	10	85	28
1j	77	75	89	0	89	1
1k	86	−9	87	−11	86	−14
1l	78	92	84	7	83	7
1m	79	−6	86	−4	85	5
1n	50	>99	50	>99	48	>99

Percentage conversion and product *ee* values (for the L-enantiomer) were determined by HPLC using a chiral stationary phase. *E. coli* BL21(DE3) whole cells (20 mg mL^{-1}) expressing each PAL were incubated with 5 mM substrate in 5 M NH_4OH pH 9.5 at 30 °C.

To gain insight into the origin of D-amino acid synthesis, the *Av*PAL-mediated amination of 4-nitrocinnamic acid (**1m**) was studied in detail. The reactions were repeated with purified PAL enzymes and confirmed that the variation in *ee* values over time did not occur as a result of background reactions taking place in the *E. coli* whole cells. Control reactions in the absence of enzyme showed a) no conversion into either the L- or D product and b) no racemization of either L- or D-amino acids, thus eliminating the possibility of non-selective background chemical reactions. The amination of **1m** was monitored over time using whole cell *Av*PAL biocatalysts (15 mg mL^{-1}). The reaction profile is consistent with initial (reversible) formation of the L-amino acid as the kinetic product followed by a slower process in which formation of the D enantiomer occurs (Figure 2). The observation that the same equilibrium composition (**1m**: 14 %, **2m**: 86 %, *ee* = −9 %) was obtained starting from each of the three components (i.e. **1m**, L-**2m**, D-**2m**) under standard amination reaction conditions supports this proposal.

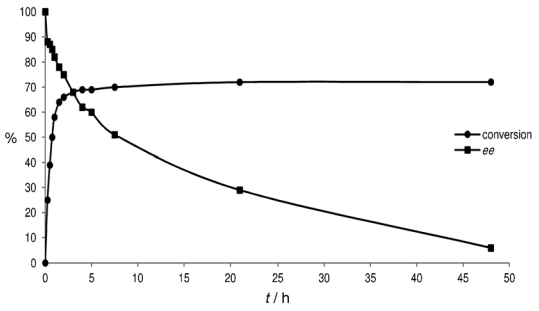


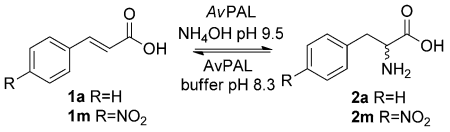
Figure 2. The amination reaction of 4-nitrocinnamic acid (**1m**) mediated by *E. coli* BL21(DE3) cells (15 mg mL^{-1}) expressing *Av*PAL in 5 M NH_4OH pH 9.5, 30 °C.

The active-site loop contains an essential Tyr78 residue, which is believed to be responsible for abstraction of the substrate's C3 benzylic proton.[12c] To assess the role of the catalytic base Tyr78 in D-amino acid formation, the Y78F variant of *Av*PAL was expressed and purified, and was shown

to be correctly folded by circular dichroism. Y78F showed no activity towards the deamination of either enantiomer of phenylalanine (**2a**) or 4-nitrophenylalanine (**2m**) and it was also inactive towards the amination of **1a** and **1m**. Our results confirm that Tyr78 plays a vital role in the *Av*PAL-catalyzed deamination of L-**2a** and also demonstrates that it is an essential catalytic residue for activity towards production of D-amino acids.

To examine the role of the prosthetic group in D-amino acid formation the *Av*PAL S168A variant was expressed, which can no longer form MIO by post-translational modification. The absence of the cofactor in this variant was confirmed by analysis of the UV difference spectra. The discrete maxima at about $\lambda = 310$ nm, associated with the presence of cofactor in wt *Av*PAL, is diminished in the S168A variant.[16] The kinetic parameters for S168A and wt *Av*PAL were determined. Surprisingly, the k_{cat} value for wt *Av*PAL-catalyzed amination of **1m** was 140 times greater than that for **1a** (Table 2). The S168A mutation resulted in complete loss of activity towards the amination of **1a** and deamination of L-**2a**. However, S168A retained low level activity towards the amination of **1m** and towards the deamination of L-**2m**, although a significant reduction (293- and 31-fold, respectively) in the k_{cat} values was observed. Interestingly, the k_{cat} value for the D-**2m** deamination was not significantly affected by the absence of the MIO cofactor.

Table 2: Kinetic constants for the amination reactions of **1a** and **1m** and the deamination reactions of **2a** and **2m** measured spectrophotometrically.



Substrate	wt *Av*PAL			S168A *Av*PAL		
	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/k_M [s^{-1} mM^{-1}]	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/k_M [s^{-1} mM^{-1}]
amination[a]						
1a	0.029	0.039	0.744	n.d.	n.d	n.d
1m	4.106	0.720	5.703	0.014	0.062	0.225
deamination[b]						
L-**2a**	0.491	0.298	1.648	n.d.	n.d	n.d
D-**2a**	n.d	n.d	n.d	n.d.	n.d	n.d
L-**2m**	1.380	0.478	2.887	0.045	0.137	0.328
D-**2m**	0.090	0.089	1.011	0.025	0.329	0.076

[a] Amination reaction in 5 M NH_4OH, pH 9.5, 30 °C. [b] Deamination reactions in 0.1 M buffer, pH 8.3, 30 °C. n.d = kinetic constants could not be measured.

These results suggest that the dominant pathway for the formation (or consumption) of L-amino acids is dependent on the MIO cofactor, whereas the analogous reactions involving the D enantiomers occur by a MIO-independent pathway. Comparable k_{cat} values are observed for the S168A-catalyzed deamination of both L-**2m** and D-**2m**, demonstrating that the cofactor-independent pathway proceeds in a non-stereoselective manner. Reduction of the MIO exocyclic methylene group represents an alternative method of cofactor inactivation.[11a] Treatment of wt *Av*PAL with $NaBH_4$ led to reduced enzyme which displayed similar activity to that of S168A (see the Supporting Information). A Y78F/S168A double mutant was expressed and displayed no activity towards **1m**, L-**2m**, or D-**2m**, demonstrating that the Tyr78 residue is essential for the cofactor independent reactions and that the non-selective pathway occurs in the active site rather than an alternative binding site.

It has been previously shown that PAL-mediated deamination of L-phenylalanine proceeds in a stereoselective manner with loss of the *pro-S* benzylic proton.[17] To investigate the stereoselectivity of D-amino acid formation and deamination, deuterated **3** (> 99 % deuterium incorporation) and deuterated D-**4** (99 % *ee* 83 % ^2H incorporation) and D-**5** (98 % *ee* 83 % ^2H incorporation) were synthesized by a chemoenzymatic strategy. Of particular interest was the application of *N*-acetyl amino acid racemase (NAAAR G291D/F323Y)[18] in combination with a commercially available D-acylase to invert the C2 stereocenter to the required *R* configuration for the synthesis of D-**5** (see the Supporting Information).

Amination of **3** was carried out in the presence of *Av*PAL, producing a mixture of L- and D-amino acids (*ee* = 6 %) which were isolated by ion-exchange chromatography. ^1H NMR analysis of this product shows the presence of a single diastereoisomer resulting from *anti* addition of ammonia across the double bond, as judged by comparison with chemoenzymatically synthesized standards **4** and **5** (Figure 3). Single enantiomers (*ee* > 99 %) of the deuterated biotransformation products (L-**4** and D-**4**) could be obtained

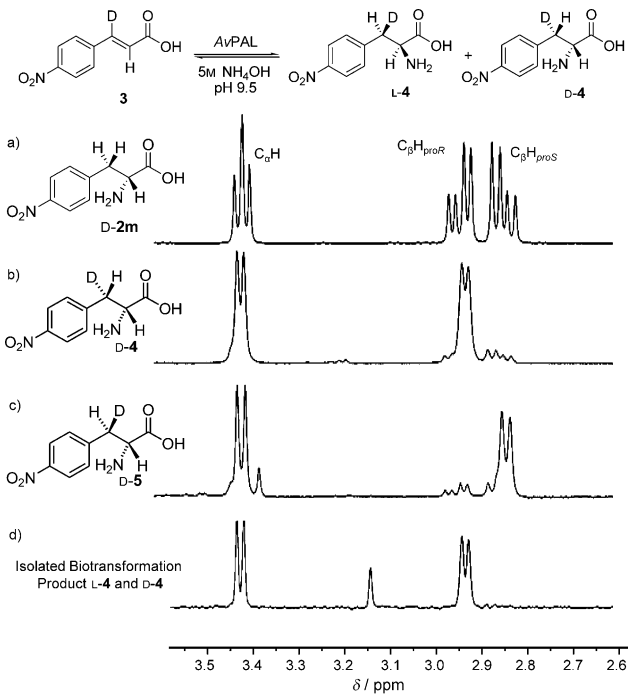


Figure 3. ^1H NMR spectra of a) the unlabeled 4-nitro-D-phenylalanine D-**2 m**, b, c) the deuterated 4-nitro-D-phenylalanine standards **4** and **5**, and d) the isolated product from the *Av*PAL-catalyzed amination of the 4-nitro-(3-^2H)-cinnamic acid **3**.

by kinetic resolution using commercially available D-amino acid oxidase from porcine kidney and L-amino acid oxidase from *Crotalus adamanteus*.

The stereoselectivity of *Av*PAL deamination reactions was also investigated using the isotopically labeled substrates D-**4** and D-**5**. Although substrate **5** yielded the single fully protiated product **1m**, consistent with the exclusive *anti* elimination of the 3*R* deuterium, substrate **4** gave a mixture of **1m** and **3**. The ratio of products produced is variable and appears to be influenced by subtle changes in reaction conditions. This result was somewhat unexpected considering that the (reversible) amination of 4-nitro-(3-^{2}H)-cinnamic acid **3** was completely stereoselective to produce isomer **4**. PAL-catalyzed deamination reactions are performed at pH 8.3 whereas reactions in the amine synthesis direction are conducted at pH 9.5, which leads to a more favorable equilibrium position. Deamination of **4** and **5** were repeated at pH 9.5 and in both cases were shown to result in the formation of a single product as a result of *anti* elimination of the 3*R* proton or deuterium, respectively. Deamination of **4** and **5** catalyzed by S168A (lacking the MIO cofactor) showed a similar trend to wt *Av*PAL. At both pH 8.3 and pH 9.5, **5** yielded fully protiated **1m** exclusively, whereas deamination of **4** gave a mixture of **1m** and **3**. The loss of deuterium incorporation was significantly higher at pH 8.3 than at pH 9.5.

These observations are consistent with the deamination of D-amino acids proceeding by a stepwise E_1cB mechanism, with initial stereoselective deprotonation of the pro-*R* benzylic proton catalyzed by Tyr78 and subsequent elimination of ammonia. At pH 8.3 non-stereoselective reprotonation of the carbanion intermediate from either bulk solvent or active-site residues competes with amine elimination, resulting in partial loss of the deuterium in the final product. The rate of reprotonation is pH dependent and is slower at pH 9.5 than at pH 8.3. This is consistent with studies regarding the mechanism of amino mutases *Cc*TAM and *Tc*PAM (from Taxus plants), which report a loss in the product deuterium incorporation and demonstrate that proton exchange with bulk solvent is pH dependent.[19] Docking L- and D-phenylalanine into the *Av*PAL active site (see the Supporting Information) demonstrates that only the *pro-S* benzylic proton of L-phenylalanine and *pro-R* proton of D-phenylalanine interact with the catalytic Tyr78 residue, and is consistent with the results of the isotopic labeling study.

Combined, the kinetic data obtained with wt *Av*PAL and selected variants along with isotopic-labeling studies and molecular modelling have provided significant mechanistic insights into a competing MIO-independent pathway. This pathway proceeds in a nonselective manner, leading to the formation of both L- and D-amino acids. Hydroamination has been shown to occur by *anti* addition of the amine and the benzylic proton. The key catalytic base (Tyr78) in the well-established MIO-dependent pathway remains an essential catalytic residue in the cofactor-independent pathway, demonstrating that this enzyme-assisted reaction occurs within the PAL active site. The deamination of D-amino acids proceeds by a stepwise E_1cB mechanism with initial deprotonation at the benzylic position catalyzed by Tyr78. We propose that electron-deficient substrates increase the stability of this intermediate carbanion with respect to reprotonation, allowing a slow MIO-independent amine elimination step to occur. Our observations further suggest that cinnamic acid derivatives are able to adopt two productive binding modes in the PAL active site (Figure 4). Although two binding modes of cinnamic acid have been reported to occur in the closely related mutases, a second binding conformation has not been described for lyases.[14,19a]

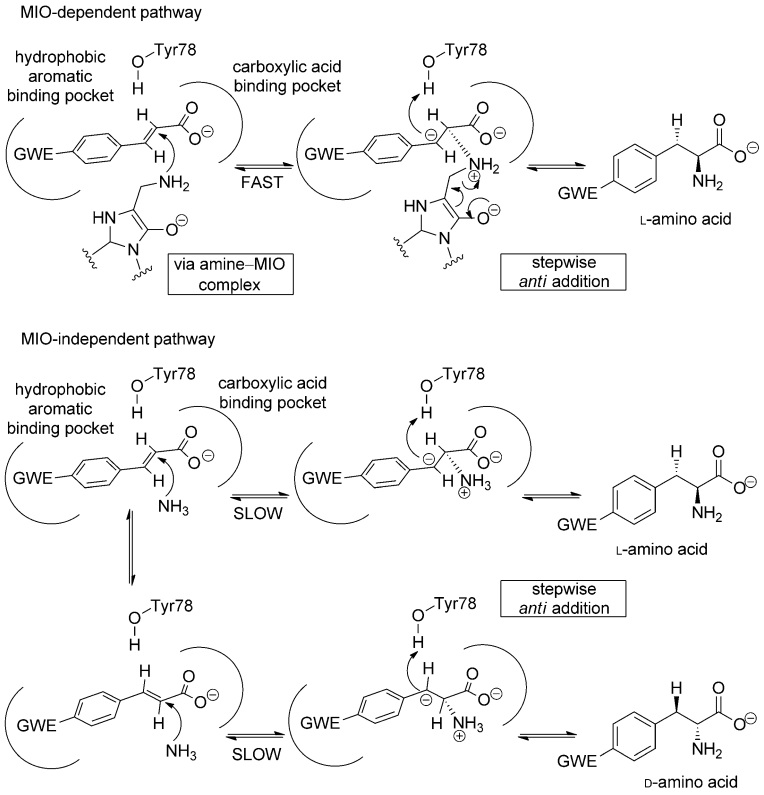


Figure 4. Proposed MIO-dependent and independent pathways. EWG = electron-withdrawing group.

In conclusion, amination reactions catalyzed by both bacterial *Av*PAL and eukaryotic *Pc*PAL and *Rg*PAL led to significant formation of D-amino acids in addition to the expected L-amino acid products. For substrates possessing an electron-deficient aromatic ring, a significant reduction in *ee* value was observed over time. Mutagenesis of key active-site residues and the determination of kinetic parameters has shown that D-amino acids are formed by an enzyme-assisted MIO-independent pathway. The mechanism of this pathway has been explored and the results are consistent with a stepwise E_1cB elimination process. The discovery of this alternative pathway may provide insights for future studies on

the mechanism of the MIO-dependent pathway. Moreover, these results may have broader implications for the future development of selective biocatalysts for the synthesis of non-natural amino acids.

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