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Deracemization and Stereoinversion of α-Amino Acids by L-Amino Acid Deaminase

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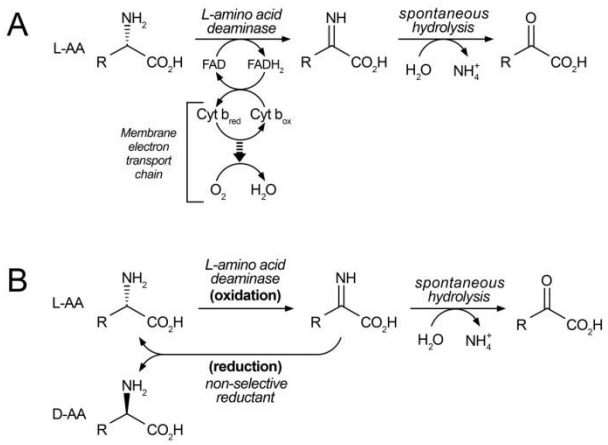
Abstract. Enantiomerically pure α-amino acids are compounds of primary interest for the fine chemical, pharmaceutical, and agrochemical sectors. Amino acid oxidases are used for resolving D,L-amino acids in biocatalysis. We recently demonstrated that L-amino acid deaminase from *Proteus myxofaciens* (PmaLAAD) shows peculiar features for biotechnological applications, such as a high production level as soluble protein in *Escherichia coli* and a stable binding with the flavin cofactor. Since L-amino acid deaminases are membrane-bound enzymes, previous applications were mainly based on the use of cell-based methods. Now, taking advantage of the broad substrate specificity of PmaLAAD, a number of natural and synthetic L-amino acids were fully converted by the purified enzyme into the corresponding α-keto acids: the fastest conversion was obtained for 4-nitrophenylalanine. Analogously, starting from racemic solutions, the full resolution (*ee* > 99%) was also achieved. Notably, D,L-1-naphthylalanine was resolved either into the D- or the L-enantiomer by using PmaLAAD or the D-amino acid oxidase variant having a glycine at position 213, respectively, and was fully deracemized when the two enzymes were used jointly. Moreover, the complete stereoinversion of L-4-nitrophenylalanine was achieved using PmaLAAD and a small molar excess of borane *tert*-butylamine complex. Taken together, recombinant PmaLAAD represents a L-specific amino acid deaminase suitable for producing the pure enantiomers of several natural and synthetic amino acids or the corresponding keto acids, compounds of biotechnological or pharmaceutical relevance.

Keywords: amino acids; biocatalysis; biotransformation; deracemization; stereoinversion

Introduction

Biocatalysis represents a well-suited method to produce enantiomerically pure amino acids for the pharmaceutical and agricultural industry. As a general rule, racemic mixtures of amino acids are less expensive than the corresponding single enantiomers. The resolution of racemates into the single enantiomer can thus be of significant economic interest. Here, the stereoinversion is also of economic interest since the D-enantiomer is often more expensive than the corresponding L-form, especially in the case of natural amino acids, which may be extracted as single isomers from natural sources or produced by fermentation (e.g., D-Phe costs 4-fold more than L-Phe). In past years, the FAD-containing enzyme D-amino acid oxidase (DAAO, EC 1.4.3.3) was used to produce L-amino acids from racemic mixtures by deaminating the D-isomer to the corresponding imino acid[1] and also by coupling chemical reduction, aimed at converting the imino acid back into a racemate, the cyclic deracemization proposed by Soda[2,3] and Turner.[4] An analogous approach can be used to produce D-amino acids when an amino acid oxidase with reverse stereoselectivity is employed. The flavoenzyme L-amino acid oxidase (LAAO, EC 1.4.3.2) catalyzes the stereoselective oxidative deamination of L-amino acids into the corresponding α-keto acids and ammonia; in the second half of the reaction, the reduced flavin is reoxidized by O_2 to generate H_2O_2.[5] In that study, however, the complications associated with overexpression of snake venom LAAOs in recombinant hosts and the narrow substrate specificity of microbial counterparts precluded using the L-selective

flavooxidase in biocatalysis.[5] Here, a suitable alternative is represented by L-amino acid deaminase (LAAD). This membrane-associated, FAD-containing enzyme catalyzes the O_2-dependent deamination of L-amino acids similarly to LAAO (and DAAO on the opposite enantiomer), in this case also yielding the corresponding α-keto acids and ammonia; however, the electrons are transferred from the reduced cofactor to a cytochrome-b-like acceptor without producing any hydrogen peroxide (see Scheme 1A).[6]



Scheme 1. A) Reaction catalyzed by PmaLAAD on L-amino acids. B) Stereoinversion of a L-amino acid into the corresponding D-enantiomer by PmaLAAD and a nonselective reduction.

When LAADs are separated from membranes, O_2 consumption decreases dramatically: the reduced FAD cofactor of LAAD from *Proteus myxofaciens* reacts very slowly with dioxygen with a rate constant of 0.08 s^{-1} at air saturation.[6]

LAADs are monomeric enzymes showing a low sequence identity with canonical homodimeric LAAOs or DAAOs.[6] LAADs have been identified in *Proteus* bacteria only. Two different types of LAAD are produced by each *Proteus* species, sharing high sequence identity (~ 57%) but differing in substrate specificity. The type-I LAADs preferentially oxidize large aliphatic and aromatic amino acids - the most well-known members are those from *P. mirabilis* (PmirLAAD) and *P. myxofaciens* (PmaLAAD) - while type-II LAADs – e.g., from *P. vulgaris* (pvLAAD) and *P. mirabilis* (Pm1LAAD) - show substantial activity towards charged (mainly basic) amino acids and L-Phe, the only hydrophobic amino acid which is also a good substrate.[6-9]

Very recently, the structures of two LAADs were solved, namely, the type-I PmaLAAD[6] and the type-II pvLAAD.[9] These proteins share a very similar tertiary structure: the root-mean-square deviation is 0.72 Å when the main-chain atoms are taken into consideration (370 residues). Canonical LAAOs consist of two main domains formed by several noncontiguous sequences: a FAD-binding domain and a substrate-binding domain. In addition to such regions, LAADs possess an N-terminal putative transmembrane α-helix (whose removal yields the deleted, fully soluble variant which is almost completely inactive),[6,9] and a small subdomain (named "insertion module") with an α+β structure, strictly interconnected to the substrate-binding domain. Details about the mode of FAD binding, the geometry of the active site, and the rationale underlying the substrate preference and stereoselectivity of LAADs have been recently reported in detail.[6,10]

In this work, we selected PmaLAAD as an optimal catalyst for deracemization and stereoinversion of selected L-amino acids of biotechnological relevance. Overexpressed in *E. coli* cells, the enzyme was easily purified by a single chromatographic step, recovered full activity when incubated with membranes and, notably, did not require exogenous FAD for maximal activity. The activity of PmaLAAD is highest at pH 7.5 and at 50 °C; indeed, the enzyme is quite stable.[6] PmaLAAD prefers bulky and hydrophobic substrates, such as L-Phe, L-Leu, L-Met, and L-Trp, followed by the polar amino acid L-Cys. It also shows the ability to deaminate small apolar or charged amino acids at a low rate.[6] Furthermore, PmaLAAD was also reported to be active on synthetic and biotechnologically relevant amino acids, such as L-3,4-dihydroxyphenylalanine (L-DOPA) and substituted alanines.[6]

Results and Discussion

Kinetic properties and docking analysis

The kinetic parameters of PmaLAAD on different substrates were determined under steady state conditions by the polarographic assay, at pH 7.5 and 25 °C, and at 21% oxygen saturation, see Table 1. The corresponding Michaelis-Menten plots are depicted in Figure 1. The enzyme showed a higher activity on L-Phe, L-Leu, L-Met, and L-4-nitrophenylalanine (L-4-Npa); the highest catalytic efficiency and apparent substrate affinity were observed on L-4-Npa and L-Leu (up to 4-fold in comparison to the reference substrate L-Phe), see Table 1.

Docking analysis was performed with Autodock Vina,[11] using the crystallographic structure of PmaLAAD in complex with the ligand (2-aminobenzoate) as receptor (PDB code: 5fjn). Docking analysis shows that all substrates tested can be accommodated in the active site in a catalytically competent conformation (i.e., with the αH pointing toward the N5 atom of the cofactor, see Supporting Information, Figure S1).[12]

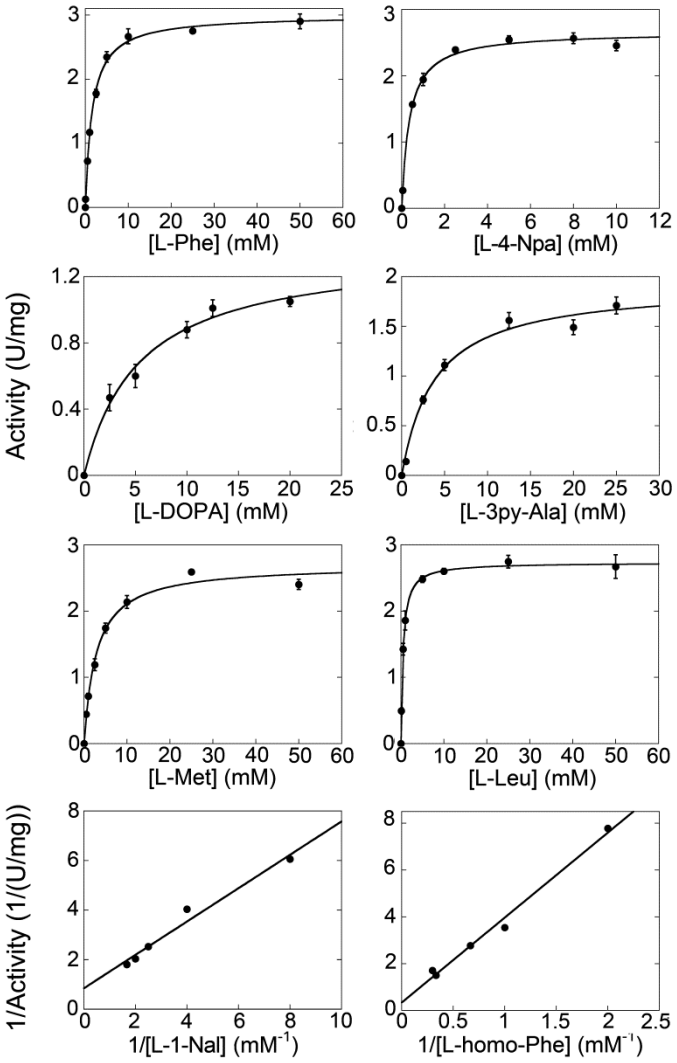


Figure 1. Michaelis-Menten plots of the activity values determined on different compounds. For L-1-Nal and L-homo-Phe the double-reciprocal plot is shown. Bars indicate SEM for at least three determinations; where not shown, the error bar is smaller than the symbol used.

Table 1. Kinetic parameters of PmaLAAD on different substrates.

Substrate	$V_{max,app}$ (U/mg protein)	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$
L-Phe	3.00 ± 0.04	1.60 ± 0.09	1.88
L-4-Npa	2.66 ± 0.04	0.36 ± 0.03	7.40
L-DOPA[a)]	1.36 ± 0.09	5.37 ± 1.05	0.25
D,L-3py-Ala	1.92 ± 0.09	3.82 ± 0.70	0.50
D,L-1-Nal	1.18 ± 0.38	0.79 ± 0.27	1.50
L-Leu	2.73 ± 0.02	0.46 ± 0.02	5.95
L-Met	2.70 ± 0.09	2.80 ± 0.34	0.96
D,L-homo-Phe	1.51 ± 0.39	2.54 ± 0.24	0.60

The activity was determined by the polarographic assay, at 25 °C, pH 7.5 and air saturation. The activity on the corresponding D-amino acid was negligible. [a)] The kinetic parameters were determined at pH 7.0 because of the higher stability of DOPA at this value.

These results demonstrate that there is enough space in the active site of PmaLAAD to accommodate even large substrates such as L-1-naphthylalanine (L-1-Nal). Only the substrate L-4-Npa was not docked in a conformation resembling the one observed for the canonical substrates such as L-Phe. Interestingly, a small conformation change of Phe318 allows binding of L-4-Npa in a conformation suitable for catalysis. Docking to the PmaLAAD with the altered Phe318 side chain orientation resulted in an alternative conformation of the bound substrate appropriate also for further substrates possessing large side chains (e.g., L-Phe or L-1-Nal, see Supporting Information, Figure S1A,B and Figure S1I,J). The binding energies between the two alternative modes of binding (as estimated by the Autodock Vina scoring function) are quite close (e.g., -8.1 and -6.9 kcal/mol for the L-1-Nal). However, we must point out that Phe318 (and Arg316) are located in a very flexible region of the active site entrance and, thus, it is plausible that conformational changes in these residues could improve binding of large substrates, contributing to the broad substrate specificity of the enzyme.[6]

When the activity of PmaLAAD on the tested compound was too low to be assayed by the oxygen-consumption assay (such as with 1-100 mM L-penicillamine, not shown), reduction of flavin cofactor under anaerobic conditions represents a very sensitive alternative detection method.[13] The absorbance spectrum in the UV-visible region of ~ 10 μM PmaLAAD was recorded in anaerobic cuvettes in the absence of *E. coli* membranes at 25 °C after adding the compound. Phenylglycine derivatives are amino acids of main biotechnological interest: here, 0.25 mM of L-phenylglycine did not reduce PmaLAAD. In contrast, the cofactor was slowly reduced by adding 0.25 mM L-4-methoxy-phenylglycine or L-4-methyl-phenylglycine. In the presence of L-4-methoxy-phenylglycine the cofactor was fully reduced in 90 min while in the presence of L-4-methyl-phenylglycine it was not fully reduced even after 210 min of incubation (see Supporting Information, Figure S2A,B). Despite the absence of any detectable O_2 consumption in the turnover enzymatic assay (up to 100 mM substrate), fully reduced cofactor was also observed after 2-hour incubation under anaerobic conditions in the presence of 10 mM L-penicillamine (Supporting Information, Figure S2C). Overall, PmaLAAD shows the ability to deaminate various L-amino acids in addition to the proteinogenic ones.

Bioconversions of different natural and synthetic L- and D,L-amino acids

The best reaction conditions for L-amino acid bioconversions were determined analyzing the time course of the deamination of 25 mM L-Phe using 0.1 mg/mL (corresponding to 0.3 U/mL) of PmaLAAD at

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25 or 37 °C, see Supporting Information. Accordingly, all bioconversion reactions were conducted at 25 °C and pH 7.5 (the only exception was L-DOPA).

A preliminary investigation of the deaminase activity on different natural and synthetic L-amino acids was performed spectrophotometrically measuring the formation of the corresponding ketone, see Supporting Information, Figure S3. Next, the bioconversion results were verified by measuring the residual L-amino acid through the ninhydrin method. Finally, under optimized reaction conditions, the time course of conversion of different L-amino acids and the resolution of different racemic mixtures by the deaminase enzyme were analyzed by HPLC: the products of the bioconversion reactions were separated and quantified by reverse-phase HPLC and the enantiomeric purity was evaluated by chiral HPLC.

As reported in Table S1, ≥ 90% of the substrate was converted to the corresponding ketone when L-Phe, L-4-Npa, and L-DOPA (12.5-25 mM) were incubated with the recombinant enzyme. Interestingly, the complete conversion of L-4-Npa was achieved in only 30 min (see Figure 2); the high yield and the high rate of conversion observed for this amino acid derivative (i.e., 6.8 µmol product/min x mg enzyme) well correlate to the highest kinetic efficiency and substrate affinity determined for the deaminase enzyme among the tested substrates (see Table 1).

When the racemic mixture was used, the chiral HPLC analysis of all reactions clearly showed that only the L-enantiomer disappeared in each case (as shown for D,L-1-Nal in Figure 3) and that the full conversion of the L-enantiomer was reached for all the tested substrates (see Table 2 and Figure 2).

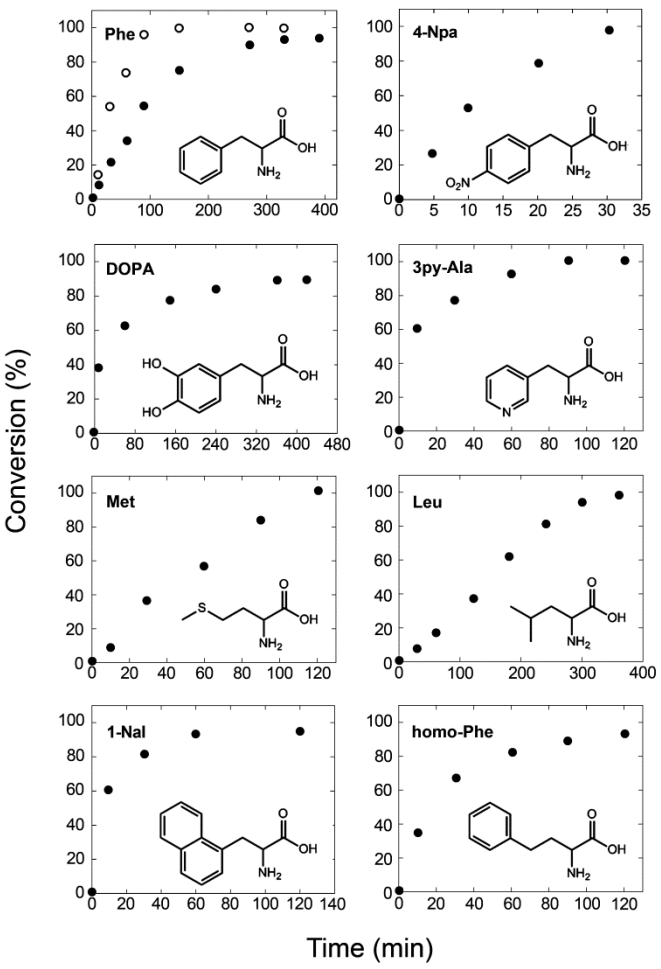

Figure 2. Comparison of the time course of bioconversion of L-isomer of different compounds using 0.1 mg/mL PmaLAAD, at 25 °C and air saturation. For L-Phe only, the bioconversion was also carried out by equilibrating the reaction mixture with pure oxygen (open symbols). For experimental conditions, see Supporting Information, Table S1 and Table 2.

Table 2. Resolution of racemic mixtures of different amino acids by PmaLAAD.

Compound	Initial concentration (mM)	Conversion yield of L-enantiomer (%)	Product formation at 90% conversion (µmol/min·mg enzyme)
D,L-3py-Ala[a)]	50	~ 75	-
	10	> 99	1.16
D,L-1-Nal[b)]	1.2	> 99	0.41
D,L-Leu[a)]	50	> 99	1.22
D,L-Met[a)]	50	~ 80	-
	10	> 99	0.68
D,L-homo-Phe[a)]	5	~ 95	0.37

The bioconversion was carried out at 25 °C, employing 0.1 mg/mL enzyme, under optimized conditions (2.5 mL, air saturation). The products of the bioconversion reactions were analyzed by chiral HPLC analysis (detection at 210 nm). HPLC conditions: [a)] H_2O/MeOH 4:6; [b)] H_2O/MeOH 2:8.

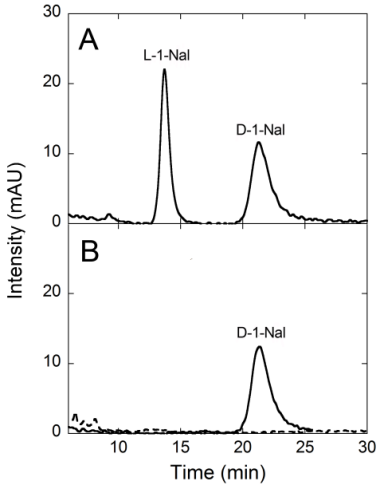

Figure 3. Chiral HPLC analysis of racemic D,L-1-Nal before (A) and following conversion by PmaLAAD (B, continuous line), and the subsequent addition of M213G DAAO variant (B, dashed line). Conditions: 1.2 mM substrate, 0.1 mg PmaLAAD/mL and 0.015 mg M213G DAAO/mL, pH 7.5, 25 °C (2.5 mL).

The highest rate of resolution (i.e. the amount of L-amino acid transformed at 90% conversion per minute, Table 2) was obtained for D,L-3-pyridylalanine (D,L-3py-Ala) and D,L-Leu as substrates. Interestingly, the complete resolution of D,L-3py-Ala and D,L-Met mixtures was reached when a lower starting amount of substrate was used (i.e., 10 mM vs. 50 mM): at the highest substrate concentration, only ~ 80% conversion was obtained for the enantiomeric solution of 3py-Ala and Met while full conversion was achieved for Leu. This observation is in line with the lower substrate affinity observed on the two previous substrates (see Table 1 and Table 2). Under optimized conditions, the lowest rate was observed for resolution of D,L-homophenylalanine (D,L-homo-Phe) (i.e., 0.37 μmol product/min x mg enzyme). The slow conversion can be explained based on the substrate concentration used: owing to the low substrate solubility, the reaction was performed using 5 mM of racemic mixture (i.e., 2.5 mM of each enantiomer), a value close to the K_m value of PmaLAAD for this substrate (see Table 1).

The conversion of L-Phe allows to produce phenylpyruvic acid, a compound employed as dietary supplement for patients with kidney diseases to reduce urea accumulation or as flavor enhancer in food formulations but whose use is hampered by the high cost of production. PmaLAAD better compares to the previous L-Phe bioconversions based on *E. coli* cells overexpressing selected LAAD or using purified enzyme preparations (maximal production of 0.51 μmol/min per mg of enzyme).[14] Notably, the L-Phe conversion by recombinant PmaLAAD was further increased 2.3-fold by substituting air with pure O_2: the full L-Phe deamination was reached in ~ 90 min vs. > 300 min when air was used (see Figure 2). The kinetic properties of PmaLAAD on L-Phe are significantly better than those of the D165K/F263M/L336M evolved PmirLAAD: the kinetic efficiency is 15.6-fold higher for the previous enzyme.[15]

A good production rate of the metabolic intermediate α-ketoisocaproate, a nitrogen-free substitute for Leu and a compound used to treat chronic kidney disease and hepatitis B virus infection, could be reached by using PmaLAAD: starting from 50 mM D,L-Leu, 1.22 μmol α-ketoisocaproate/min per mg enzyme was produced with a > 99% conversion (Table 2).

E. coli cells overexpressing pvLAAD, both the wild-type and the K104R/A337S variant, were previously used to convert L-Met into α-keto-γ-methylthiobutyric acid (an indirect inhibitor of tumor cell growth and a methionine supplement in livestock feed). The maximal conversion (~ 90%) was reached after 24 hours; the reaction time course is probably hampered by the high apparent K_m of this enzyme for L-Met (≥ 240 mM),[16] a value 100-fold higher than that of PmaLAAD. With the latter enzyme and using 10 mM D,L-Met, 0.68 μmol α-keto-γ-methylthiobutyric acid/min per mg enzyme were produced with a > 99% yield (see Table 2).

Exhaustive enzymatic deamination of D,L-1-Nal

Naphtylalanines are aromatic synthetic amino acids used to produce various drugs, such as the peptide drug nafarelin.[17] When the racemic mixture of D,L-1-Nal (1.2 mM, a concentration close to its maximal solubility) was incubated with PmaLAAD (0.1 mg/mL) the L-enantiomer was fully consumed, as shown by the HPLC chromatogram reported in Figure 3. Then, the DAAO M213G variant (0.015 mg/mL corresponding to 0.3 U/mL on D-1-Nal) was added to the reaction mixture produced by the deaminase enzyme:[18] the peak with a $t_{(R)}$ of 24.04 min disappeared showing the complete conversion of the racemic mixture into the ketone product. This result demonstrates the possibility to selectively trigger the resolution of a D,L-1-Nal solution into the L- or the D-enantiomer by alternatively using the DAAO M213G variant or the PmaLAAD enzyme, respectively, as well as the possibility to deaminate both enantiomers using the two flavoenzymes simultaneously. Notably, LAAD was also previously employed in multienzymatic reactions. For example, whole *E. coli* recombinant cells expressing PmirLAAD were used together with a commercial D-transaminase to resolve a racemic mixture of 2-amino-3(7-methyl-1-*H*-indazol-5-yl)propanoic acid, reaching a 79% yield;[19] phenylalanine ammonia lyase and PmirLAAD were used to convert *p*-nitrocinnamic acid into *p*-nitro-D-Phe, resulting in a 71% conversion;[20] lysed *E. coli* cells expressing PmaLAAD were used as the first step to convert L-amino acids into the corresponding α-keto acids, which were then asymmetrically reduced into (*R*)- or (*S*)-2-hydroxy acids in the following step by using L- or D-isocaproate reductase activities or converted into the D-amino acid by an engineered D-amino acid dehydrogenase.[21,22]

D,L-1-Nal is a good substrate for PmaLAAD but the volumetric conversion is hampered by its low solubility in aqueous solution. In order to improve the conversion yield and taking into consideration the higher solubility of the produced ketone, the reaction was carried out in aqueous dispersions of D,L-1-Nal corresponding to a nominal concentration of 2.4 or 6 mM, both of them larger than the substrate solubility. HPLC analysis of the reaction mixtures (i.e., the soluble part) showed that 1.2 and 2.9 mM ketone was formed after 90-120 min, respectively, thus demonstrating that the substrate virtual concentration in the reaction mixture can be significantly increased. This is not unusual since the use of a saturated solution was reported on the same substrate using DAAO to oxidize the D-enantiomer[23] as well as when PmaLAAD was employed on 200 mM L-Tyr (virtual

concentration since its solubility in buffer is ~ 2.5 mM).[21]

Chemoenzymatic stereoinversion of L-4-Npa

The generation of D-4-Npa (the starting point for the synthesis of plasmin inhibitors and anti-fibrinolytic drug production)[24,25] from the corresponding L-enantiomer was established by coupling the deamination of the L-amino acid to the corresponding achiral imino acid and its nonselective *in situ* reduction by the borane *tert*-butylamine complex (Scheme 1B). A 12.5 mM L-4-Npa solution was incubated with PmaLAAD under standard conditions and then a 5-fold molar excess of the reducing agent was added. Chiral HPLC analysis shows the formation of the D-amino acid: as shown in Figure 4, after 2 h of incubation, the complete conversion of L-4-Npa into the D-enantiomer was apparent (*ee* > 99%). The D-amino acid production stopped to ~ 70% stereoinversion in presence of 5-fold molar excess of the borane triethylamine complex as reducing agent.

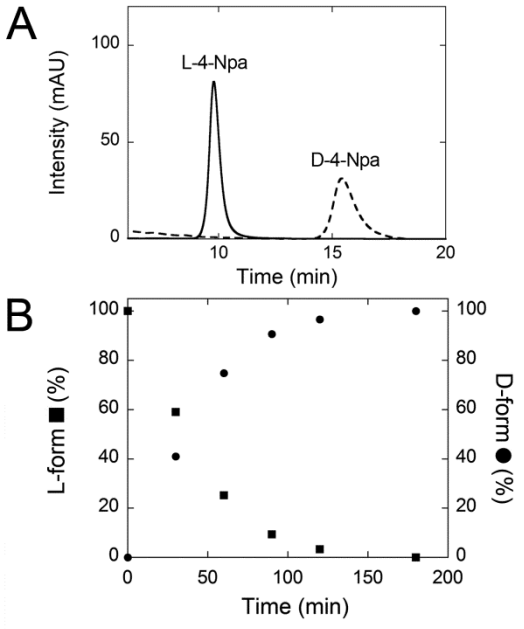

Figure 4. Stereoinversion of L-4-Npa by PmaLAAD and nonselective reduction (see Scheme 1B). A) HPLC chromatogram of 12.5 mM L-4-Npa before (continuous line) and 120 min after adding 0.1 mg PmaLAAD/mL and 62.5 mM borane *tert*-butylamine complex reducing agent (dashed line), at 25 °C and pH 7.5. B) Time course of D-4-Npa (●) production in the enzymatic conversion of L-4-Npa (■) reported in panel A. HPLC analysis on a C18 column confirmed the quantitative conversion of the L- into the D-isomer and the absence of keto acid.

In contrast, starting from 25 mM L-DOPA the D-enantiomer production stopped to ~ 20% amino acid stereoinversion: this result requires further investigation, see Supporting Information, Figure S4. Notably, in stereoinversion experiments we employed a low amount of reducing equivalents and a "milder, cheaper, and water-stable" reductant as compared to $NaBH_4$, a compound largely used but requiring higher ratios because of its instability in water.[20,26-28] Alternative reducing agents resulted in a lower conversion yield, see Supporting Information, page S6. Interestingly, no α-keto acid formation was observed in the L-4-Npa or L-DOPA stereoinversion reaction: a competition between chemical reduction of the produced imine and its hydrolysis at increasing substrate concentrations cannot be excluded. Moreover, no H_2O_2 is produced during the cofactor reoxidation. This feature is of utmost relevance: the enzyme stability is improved compared with oxidases and no byproducts stemming from oxidative decarboxylation are formed.[29]

Preparative scale

In order to demonstrate the possibility to employ PmaLAAD on a preparative scale, 130 mg of L-4-Npa (12.5 mM, 50 mL) was bioconverted. By using 0.1 mg/mL of enzyme and 62.5 mM *tert*-butylamine borane complex, the complete conversion into the D-enantiomer was apparent after overnight incubation, corresponding to *ee* > 99% as evaluated by chiral chromatography.

Conclusion

Enantiomerically pure amino acids, both natural and synthetic ones, are of increasing interest for the fine chemicals and pharmaceutical industries. Analogously, the demand for single-isomer agrochemicals is also growing due to environmental pressures. In past years, the deracemization of a D,L-amino acid solution to the L-isomer was achieved by using the flavoprotein DAAO, an enzyme used to generate a plethora of useful variants by protein engineering studies.[1,30] On the other hand, the isolation of enantiomerically pure D-amino acids was hampered by the scarce availability of suitable LAAO activities.[5] Searching for biocatalytic methods useful for resolving racemic amino acid mixtures by eliminating the L-enantiomer, the recombinant LAAD from *P. myxofaciens* was proposed in 2001 by Pantaleone et al.[31] Later on, *E. coli* cells expressing recombinant LAAD from various sources were used on different compounds,[14-16,20,21,26-28] thus demonstrating this to be a feasible approach. Here, we set up the bioconversion, the deracemization and the stereoinversion of natural and synthetic amino acids using the purified, recombinant PmaLAAD. The use of a purified enzyme preparation allowed a faster L-Phe conversion than with whole cells, as well as a faster production of α-ketoisocaproate from L-Leu and

of α-keto-γ-methylthiobutyric acid from L-Met. The faster conversion was also coupled to a higher conversion yield (> 99%).

In our opinion, novel competitive biocatalytic processes based on LAAD will largely benefit by generating further improved enzyme variants with altered substrate specificity. LAAD activity will also represent a well-suited tool to employ in multi-step cascade systems, especially in setting up original synthetic pathways.[32,33]

Experimental Section

Chemicals

L-Phe, phenylpyruvic acid (PPA), L-DOPA, D,L-1-Nal, D,L-Leu, D,L-Met, D,L-DOPA, D,L-homo-Phe, borane triethylamine complex, borane morpholine complex, bis(benzonitrile)palladium(II) chloride, and borane *tert*-butylamine complex were purchased from Sigma-Aldrich (Milano, Italy). L-Penicillamine was purchased from TCI Europe (Zwijndrecht, Belgium). L-4-Npa and D,L-3py-Ala were kindly supplied by Flamma S.p.A. (Chignolo d'isola, Italy). L-4-Methoxy-phenylglycine and L-4-methyl-phenylglycine were kindly supplied by Prof. Sandro Ghisla. All other chemicals were of analytical grade and were used as received.

3-(3,4-Dihydroxyphenyl)-2-oxopropanoic acid synthesis[34]

In a 50-mL round-bottomed flask, immersed in an oil bath and equipped with a magnetic stirrer and a water condenser, 2.76 g of 3,4-dihydroxybenzaldehyde (20 mmol) were poured together with 2.13 g of anhydrous sodium acetate (26 mmol), 3.04 g of *N*-acetylglycine (26 mmol) and 10 mL acetic anhydride. The resulting mixture was heated and stirred at 80 °C for 4 h, then at 100 °C for 1 h. The resulting dark brown solution was left at room temperature overnight. The next day, 10 mL of ice-cold water were added: the formed yellow crystals were crushed with a glass rod and filtered on a Buchner funnel, then rinsed with a few milliliters of cold water until the washings were no longer dark. The solid was then dried under vacuum, characterized, and used in the following steps. [4-((2-methyl-5-oxooxazol-4(5H)-ylidene)methyl)-1,2-phenylene diacetate: 4.57 g, yield 75.3%].

In a 50-mL round-bottomed flask, immersed in an oil bath and equipped with a magnetic stirrer and a water condenser, 3.0 g of the previous compound (9.9 mmol) were poured together with 10 mL water and 10 mL acetone. The resulting mixture was heated to reflux and left to react for 4 h. Then active carbon was added and the solution was hot-filtered through a Celite plug. The filtrate was left in the refrigerator overnight to allow complete crystallization of the product, which was suction-filtered the following day. After drying under vacuum, the light-yellow crystals were characterized and used in the following step without further purification. [(Z)-2-acetamido-3-(3,4-diacetoxyphenyl)acrylic acid: 2.71 g, yield 85.4%].

In a 100-mL round-bottomed flask, immersed in an oil bath and equipped with a magnetic stirrer and a water condenser, 2.0 g of the previous compound (6.2 mmol) were poured together with 40 mL of 2 M hydrochloric acid. The mixture was heated to reflux and left to react for 6 h. Then, active carbon was added and the solution was hot-filtered through a Celite plug. The solution was concentrated by means of a rotary evaporator, and the resulting mixture was left overnight in the refrigerator to completely crystallize. The following day, the crystals were collected by filtration on a Hirsch funnel, washed with ice-cold water, vacuum dried, and characterized. [3-(3,4-dihydroxyphenyl)-2-oxopropanoic acid: 869 mg, yield 71.2%]: ^{1}H-NMR (400 MHz, d_6-DMSO) δ ppm: 6.26 (s, 1H), 6.70 (d, J=8 Hz, 1H), 6.95 (dd, J=8 Hz, J=2 Hz), 7.35 (d, J=2 Hz, 1H), 8.72 (bs, 1H), 8.91 (bs, 1H), 9.02 (bs, 1H), 12.9 (bs, 1H).

Enzymes

Recombinant PmaLAAD (the -00N variant) was produced as stated in Motta et al.[6] The recombinant enzyme was purified in a single step by HiTrap chelating chromatography; from 1 L fermentation broth, 16 mg protein were produced. The amount of protein was estimated based on the absorbance at 457 nm using an extinction coefficient of 14.17 mM^{-1} cm^{-1}.[6] The recombinant enzyme was isolated as a single band at ~ 55 kDa with > 90% purity as judged by SDS-PAGE analysis. Following reconstitution with *E. coli* membranes, the specific activity was 2.9 U/mg protein on L-Phe as substrate.

Recombinant M213G DAAO variant was produced as stated in Caligiuri et al.[18] The recombinant enzyme was obtained with a yield of 3 mg of protein per liter of culture, with a specific activity of 20 U/mg protein on D-1-Nal as substrate. The enzyme (~ 95% pure) yielded a single band at 40 kDa in SDS-PAGE analysis; DAAO protein concentration was determined using an extinction coefficient of 12.6 $mM^{-1}cm^{-1}$ at 455 nm.[18]

Activity assay and kinetic measurements

The activity of PmaLAAD was determined on L-Phe as substrate in 50 mM potassium phosphate buffer, pH 7.5, with an oxygen electrode at 25 °C and at air oxygen saturation (0.253 mM). The enzyme was added with exogenous membranes prepared from *E. coli* cells not expressing PmaLAAD: membranes isolated from 0.3 mg of cells were added for each microgram of enzyme and the sample was sonicated (3 cycles of 15 s each, with a 15-s interval on ice). The enzymatic activity was determined after incubation at 25 °C for 30 min and defined as micromoles of oxygen consumed per minute. The activity and kinetic parameters on different compounds were similarly determined using a fixed amount of enzyme (prepared as stated above) and various substrate concentrations (0-50 mM). Activity vs. substrate concentration data were analyzed according to the classical Michaelis-Menten equation or modified to account for a substrate inhibition effect.[35]

Stability was assessed at 25 or 37 °C by incubating the enzyme in 50 mM potassium phosphate buffer, pH 7.5. At different times, samples were withdrawn and the residual activity was assayed using the polarographic method as described above.

Bioconversion procedures

The reaction mixtures contained 25 mM L-amino acid (if not stated otherwise) dissolved in 50 mM potassium phosphate buffer, pH 7.5, and the PmaLAAD enzyme at 0.1 mg/mL (corresponding to 0.3 U/mL on L-Phe as substrate) in a final volume of 2.5 mL. The reaction mixtures were incubated at 25 °C in a rotatory mixer and aliquots were withdrawn at different times for analysis. The time course of the bioconversion was determined by the ninhydrin assay (see below) and by HPLC analysis: 20 µL of reaction mixture was quenched by adding 180 µL of a 1:1 H_2O/MeOH solution containing 0.1% TFA, then centrifuged; the supernatant was analyzed by HPLC (see below). Product formation was also followed spectrophotometrically at 320-360 nm using a Jasco FP-750 spectrophotometer (Jasco Europe Srl, Cremella, Italy): an aliquot of the reaction mixture was transferred into a plastic cuvette containing 3 N NaOH to stop the reaction and allow the color to develop.

Ninhydrin assay

The residual amount of amino acid in the reaction mixtures was estimated using the ninhydrin assay. The aliquots taken from the reaction were diluted in 800 µL water and 200 µL of a 0.8% (w/v) ninhydrin solution in acetone added. The samples were boiled in the dark for 10 min and then placed in an ice water bath for 5 min. To each sample 200 µL of 50% (v/v) aqueous ethanol was added, the tubes were vortexed, and the absorbance value at 570 nm was recorded. In parallel, control samples (not containing substrate or enzyme) were also analyzed. The amino acid concentrations were calculated by referring to the corresponding calibration curve.

HPLC analyses

HPLC analyses were performed on a Jasco apparatus (Jasco Europe Srl) equipped with a PDA detector and using a Luna 5-µm C18(2) column, length/internal diameter = 150/4.6 mm (Phenomenex, Castel Maggiore, Italy). A binary system made of 0.1% TFA in H_2O (solvent A) and 0.1% TFA in MeOH (solvent B) was used, flow rate 1 mL/min, column temperature 35 °C. The retention times $t_{(R)}$ of L-Phe, PPA, L-4-Npa, 4-nitro-PPA, L-DOPA, and 3-(3,4-dihydroxyphenyl)-2-oxopropanoic acid were 3.20, 7.10, 3.39, 8.25, 4.64, and 6.52 min, respectively.

Chiral HPLC analyses were performed on an Agilent apparatus (Cernusco sul Naviglio, Italy) equipped with a UV detector set at 210 nm and fitted with an Astec Chirobiotic TAG column 5 µm (Sigma-Aldrich), length/internal diameter = 250/4.6 mm, eluent H_2O/MeOH, flow rate 0.8 mL/min, and column temperature 30 °C. The retention times were as follows: D,L-3py-Ala (L-, 10.38 min; D-, 21.17 min), D,L-1-Nal (L-, 13.71 min; D-, 21.17 min), D,L-Leu (L-, 5.67 min; D-, 11.18 min), D,L-Met (L-, 6.64 min; D-, 12.78 min), D,L-homo-Phe (L-, 7.48; D-, 16.84 min), and D,L-DOPA (L-, 6.97 min; D-, 24.04 min).

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FULL PAPER

Deracemization and Stereoinversion of α-Amino Acids by L-Amino Acid Deaminase

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