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Activity of yeast D-amino acid oxidase on aromatic unnatural amino acids

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Abstract

D-Amino acid oxidase is a FAD-dependent enzyme that catalyses the conversion of the D-enantiomer of amino acids into the corresponding α-keto acid. Substrate specificity of the enzyme from the yeast *Rhodotorula gracilis* was investigated towards aromatic amino acids, and particularly synthetic α-amino acids.

A significant improvement of the activity ($V_{max,app}$) and of the specificity constant (the $V_{max,app}/K_{m,app}$ ratio) on a number of the substrates tested was obtained using a single-point mutant enzyme designed by a rational approach. With *R. gracilis* D-amino acid oxidase the complete resolution of D,L-homo-phenylalanine was obtained with the aim to produce the corresponding pure L-isomer and to use the corresponding α-keto acid as a precursor of the amino acid in the L-form.

Keywords: D-Amino acid oxidase; Amino acids; Bioconversion; Enzyme catalysis; Multi-step enzyme catalysed reactions

1. Introduction

Unnatural amino acids are a growing group of compounds required for a number of biotechnological applications (from the pharmaceuticals and cosmetics to the agrochemicals field). Furthermore, synthetic α-amino acids are used as chiral building blocks and molecular scaffolds in constructing chemical combinatorial libraries [1], for the *de novo* design of peptides [2,3], and as valuable pharmaceuticals (in their own right or as components of numerous therapeutically relevant compounds) [4] and they are becoming crucial tools for modern drug discovery. The worldwide increase of the market for enantiopure raw materials, largely required to support the development of new pharmaceutical and agrochemical products, is also giving an increase in the use of biocatalysts for their production. Particularly, the market for amino acids in synthesis shows an annual rate of 5–7% [3]. In recent years, new chiral technologies and improved enantioselective processes have been developed [5,6]. We recently provided an example for the combination of biological tools for the production of enantiopure unnatural L-amino acids, both using a single enzyme [7] and a multienzymatic system [8]. In both cases we employed the FAD-containing flavoenzyme D-amino acid oxidase (EC 1.4.3.3, DAAO) from the yeast *Rhodotorula gracilis* (RgDAAO). DAAO catalyses the dehydrogenation of the D-isomer of amino acids to give the corresponding α-keto acids, ammonia and hydrogen peroxide. The overall reaction is shown in Eqs. (1a)–(1c):

$$\mathrm{E} \sim \mathrm{FAD_{ox}} + \text{D-amino acid} \leftrightarrow \mathrm{E} \sim \mathrm{FAD_{red}} + \text{imino acid} \quad (1a)$$

$$\text{imino acid} + \mathrm{H_2O} \rightarrow \alpha\text{-keto acid} + \mathrm{NH_3} \quad (1b)$$

$$\mathrm{E} \sim \mathrm{FAD_{red}} + \mathrm{O_2} \rightarrow \mathrm{E} \sim \mathrm{FAD_{ox}} + \mathrm{H_2O_2} \quad (1c)$$

RgDAAO is highly stereoselective (L-amino acids are neither substrates nor inhibitors), it exhibits a very high turnover number, tight binding with the coenzyme FAD, and a broad substrate specificity (for a review see [9]). These properties make RgDAAO a suitable enzyme for biotechnological applications, e.g. for the two-step conversion of cephalosporin C into 7-aminocephalosporanic acid, to detect and quantify D-amino acids, to produce α-keto acids from essential D-amino acids, and to resolve racemic mixtures of D,L-amino acids (for a review see [10]). Furthermore, the solution of the 3D structure

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Table 1
Apparent steady-state kinetic parameters determined for wild-type and M213G RgDAAOs on different phenyl- and naphthyl-D-amino acids

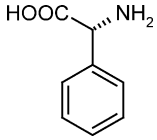
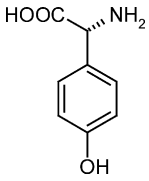
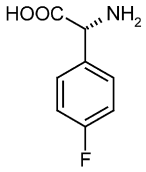
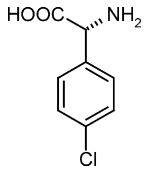
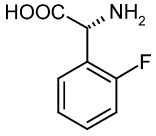
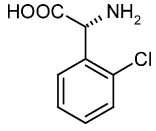
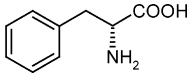
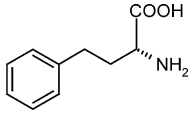
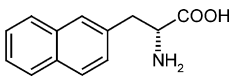
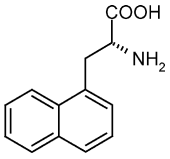
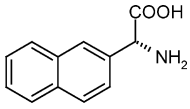
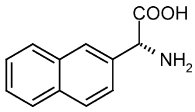
Substrate		$V_{max,app}$ (min^{-1})	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$ (min^{-1} mM^{-1})
 D-Phenylglycine	Wild-type	560 ± 25	3.5 ± 0.5	160
	M213G	530 ± 80	5.2 ± 0.8	100
 D-4-Hydroxy-phenylglycine	Wild-type	180 ± 10	2.1 ± 0.3	90
	M213G	950 ± 30	1.6 ± 0.2	600
 D-4-Fluoro-phenylglycine	Wild-type	480 ± 80	0.7 ± 0.2	690
	M213G	660 ± 90	0.6 ± 0.2	1,100
 D-4-Chloro-phenylglycine	Wild-type	17 ± 2	0.03 ± 0.01	570
	M213G	380 ± 15	0.2 ± 0.02	1,900
 D-2-Fluoro-phenylglycine	Wild-type	90 ± 15	0.5 ± 0.2	190
	M213G	65 ± 15	0.5 ± 0.2	130
 D-2-Chloro-phenylglycine	Wild-type	12 ± 1	0.13 ± 0.02	95
	M213G	100 ± 7	0.4 ± 0.04	250
 D-Phenylalanine	Wild-type	3990 ± 150	0.3 ± 0.05	13,300
	M213G	1770 ± 70	0.2 ± 0.03	8,850
 D-Homo-phenylalanine	Wild-type	800 ± 100	0.3 ± 0.1	2,670
	M213G	1100 ± 40	0.15 ± 0.01	7,330
 D-2-Naphthyl-alanine[a]	Wild-type	1965 ± 80	0.04 ± 0.003	49,100
	M213G	1170 ± 72	0.06 ± 0.009	19,500

Table 1 (*Continued*)

Substrate		$V_{max,app}$ (min^{-1})	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$ ($min^{-1}\,mM^{-1}$)
D-1-Naphthyl-alanine[a] 	Wild-type	125 ± 8	0.04 ± 0.007	3,100
	M213G	870 ± 23	0.03 ± 0.003	29,000
D-2-Naphthyl-glycine[a] 	Wild-type	6 ± 0.1	0.01 ± 0.001	600
	M213G	31 ± 0.7	0.03 ± 0.002	1,035
D-1-Naphthyl-glycine[a] 	Wild-type	53 ± 4	0.33 ± 0.05	160
	M213G	92 ± 3	0.05 ± 0.006	1,840

Reaction conditions: the activity was determined using the oxygen consumption assay, at 25 °C, pH 8.5, and at air saturation. The activity values were not modified by the use of the racemic D,L-mixture instead of the pure D-isomer.

[a] [7].

of RgDAAO in complex with different substrate analogues and ligands at high resolution [11,12] allowed a deep investigation of its structure–function relationships and the rational re-design of its substrate specificity [12,13,7].

Here, we continue on the use of DAAO as a biological tool for the deracemization of racemic materials, and in particular we studied the enzymatic activity of wild-type and M213G RgDAAOs towards racemic mixtures of unnatural aromatic amino acids to obtain the resolution of the corresponding L-amino acid component.

2. Experimental

2.1. Materials

The pure D-isomer of 1- and 2-naphthyl-alanine was purchased from Sigma–Aldrich and Bachem, respectively; D-phenylglycine, D-phenylalanine and D-4-OH-phenylglycine were purchased from Fluka; the D-isomer of 1- and 2-naphthyl-glycine was isolated by chiral HPLC chromatography as detailed in [7]. D-Homo-phenylalanine was obtained by subtilisin catalysed resolution of rac-*N*-Boc-homo-phenylalanine methyl ester.

2.2. Enzymes expression and purification

Recombinant wild-type and M213G RgDAAOs were expressed in BL21(DE3)pLysS *Escherichia coli* cells using the pT7-HisDAAO expression vector; the cells were grown at saturation, the enzyme expression was induced with 0.8 mM IPTG, and the cells were collected after 3 h of growth at 30 °C. The two DAAO forms were purified by HiTrap Chelating chromatography (GE Healthcare Bio-sciences) [7,14]. The pure wild-type and M213G RgDAAOs possess a specific activity on D-alanine of 110 and 19.2 U/mg protein, respectively. Their concentration was determined spectrophotometrically by using an extinction coefficient of 12.6 $mM^{-1}\,cm^{-1}$.

2.3. Activity assay and kinetic measurements

DAAO activity was assayed with an oxygen electrode at air saturation using 28 mM D-alanine as substrate. Condi-

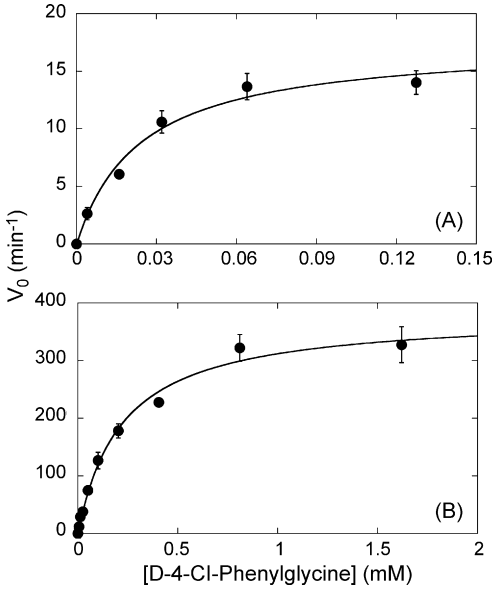


Fig. 1. Michaelis–Menten plot of the activity values determined for wild-type (A) and M213G (B) RgDAAOs on D-4-chloro-phenylglycine. Bars indicate ± S.E.M. for at least three determinations; where not shown, the error is smaller than the symbol used.

tions: 75 mM sodium pyrophosphate buffer, pH 8.5 at 25 °C (0.253 mM O_2). One DAAO unit is defined as the amount of enzyme that converts 1 μmol of D-amino acid per minute at 25 °C. The kinetic parameters of the DAAO reaction with different amino acids (both D- and D,L-forms) were analogously determined. The initial reaction rates were used to calculate the kinetic parameters employing the KaleidaGraph software (Synergy Software, PA, U.S.A.).

2.4. Preparation of compounds

D,L-2-Naphthyl-alanine and D,L-1-naphthyl-glycine were prepared as described in [7]; D,L-2-naphthyl-glycine was prepared according to [15].

2.5. Bioconversion of D,L-homo-phenylalanine

A (D,L)-homo-phenylalanine solution (1.09 mM) in 20 mL of sodium phosphate buffer (100 mM, pH 8.5) was prepared and incubated at 25 °C. One hundred and fifty microliters of DAAO solution (0.146 mg/mL) and 20 μL of catalase solution (Boehringer, 3250 U/mL) were added, and the time registration was started. Further aliquots (150 μL) of DAAO solution were added every hour.

The time course of bioconversion was determined by high performance liquid chromatography (HPLC) assay. Samples were analysed on a Zorbax SB-Aq column (150 mm × 4.6 mm, 5 μm; Agilent Technologies) [7]. Enantiomeric purity of the final products was determined by means of chiral HPLC chromatography on a Crownpak CR + column [7].

3. Results and discussion

3.1. Kinetic properties of wild-type RgDAAO on aromatic D-amino acids

DAAO from the yeast *R. gracilis* shows a broad substrate specificity: nonpolar and aromatic D-amino acids appeared to be the best substrates [16]. For aromatic natural amino acids the wild-type DAAO showed with phenylalanine a lower $K_{m,app}$ value than that determined for the reference substrate D-alanine (0.3 mM vs. 0.8 mM, respectively). Moreover $V_{max,app}$ value similar respect to D-alanine (≈4000 min^{-1} at pH 8.5, 21% oxygen saturation and 25 °C) [16]. Table 1 summarizes the apparent kinetic parameters determined for wild-type RgDAAO towards a number of substituted phenylglycines at position 2 and 4 and homo-phenylalanine in comparison with the values recently obtained for unnatural naphthyl-alanine and naphthyl-glycine derivatives [7]. Among the unnatural aromatic amino acids, the wild-type RgDAAO showed high substrate affinity for all compounds tested: a K_m value higher than 1 mM was observed only for 4-hydroxy-phenylglycine. Conversely, a low $V_{max,app}$ values (≤100 min^{-1}) significantly affected the specificity constant (as expressed by the ratio $V_{max,app}/K_{m,app}$) with a number of the substrates used (see Table 1). A substrate inhibition effect in the Michaelis–Menten plot [17] was evident only for D-2-naphthyl-alanine [7].

3.2. Kinetic properties of M213G RgDAAO on aromatic D-amino acids

We recently produced, by a rational design approach, a single point RgDAAO mutant (M213G) that allowed a complete resolution of the racemic mixtures of naphthyl-alanine and naphthyl-glycine derivatives using a significant lower amount of enzyme compared to the wild-type RgDAAO [7]. Models of RgDAAO-D-naphthyl-amino acids complexes obtained by simulations using AutoDock software showed that the low activity observed for the wild-type RgDAAO with large hydrophobic substrates such as naphthyl-glycine derivatives was largely due to the steric hindrance of the side chain of M213: its substitution to glycine allowed a better interaction with these compounds, resulting in an increase in maximal activity and/or substrate affinity (see Table 1 and [7]). The substrate specificity of M213G RgDAAO on aromatic D-amino acids was analysed as previously reported for the wild-type enzyme: on the natural aromatic D-amino acids the maximal activity, as well as the substrate specificity constant, was lower with the M213G

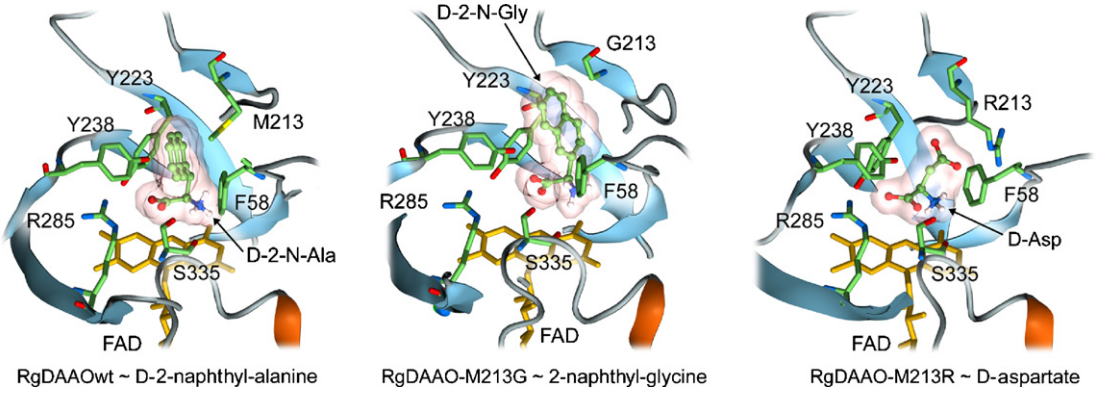


Fig. 2. Comparison of the active site of wild-type RgDAAO in complex with D-naphthyl-alanine, of M213G RgDAAO mutant with 2-naphthyl-glycine, and of M213R RgDAAO with D-aspartate. The docking of the ligands was predicted by docking analysis using the program AutoDock 3.0 and the models were developed using the Swiss PDB Viewer program as detailed in [13,7]. The crystal structure of RgDAAO in complex with D-trifluoroalanine (PDB entry 1c0l) was used as the starting structure.

mutant compared to the wild-type DAAO (see Table 1). Concerning the unnatural amino acids, the $V_{max,app}$ value was higher with the mutant DAAO compared to the wild-type enzyme (up to ≈22-fold with D-chloro-phenylglycine, see Fig. 1), the only exceptions being D-2-fluoro-phenylglycine and D-2-naphthyl-glycine. The apparent K_m for the D-amino acid was instead affected to a lower extent by the substitution of M213 (Table 1).

3.3. Bioconversion of D,L-homo-phenylalanine

We investigated the ability of wild-type RgDAAO in the resolution of racemic mixtures of homo-phenylalanine following the time course of bioconversion using a stirred reactor and the enzyme in the free form. The products of the bioconversion reaction were quantified by HPLC chromatography. In all cases, a higher rate and total conversion was obtained by bubbling air into the apparatus and in the presence of catalase. By using DAAO (600 U/L) and 1.8 mM D,L-homo-phenylalanine, the D-enantiomer was quite completely consumed after 300 min of reaction. Under optimized conditions, the maximal conversion is reached using different amounts of enzyme depending on the substrate concentration used: using 1.1 mM D,L-homo-phenylalanine a total conversion of the D-isomer (e.e. >99%) was reached in ≤200 min.

4. Conclusion

We have produced two single-point mutant enzymes using a structure-based rational design approach with an enlarged substrate specificity: the M213R mutant RgDAAO active on acidic D-amino acids [13] and the M213G mutant RgDAAO more active and unnatural naphthyl-amino acids [7]. A comparison of the active site of wild-type RgDAAO and of the model obtained for the M213G and M213R mutants proteins is depicted in Fig. 2.

In the present work, we analysed the substrate specificity of wild-type and M213G DAAOs on a number of unnatural aromatic D-amino acids. The assay confirms the broad substrate specificity of wild-type RgDAAO (it is active on all the compounds tested) and the increase in activity and specificity constant with the M213G mutant with many of the compounds tested (Table 1). As previously stated [7], the substitution of M213 to glycine does not result in gross perturbation of FAD microenvironment but affected the kinetic properties of the enzyme. This substitution allowed a better orientation of phenylglycine derivatives (as well as of homo-phenylalanine) with respect to the flavin redox site, resulting in an increase in maximal activity and specificity constant compared to the wild-type RgDAAO. Concerning the phenylglycine derivatives, the highest $V_{max,app}$ value was observed with the compound containing an hydroxyl-group at position 4 and the highest specificity constant was determined with the one containing a Cl-group at the same position (see Table 1). The wide substrate specificity of RgDAAO allows a complete resolution of the racemic mixtures of homo-phenylalanine, analogously to that obtained for unnatural naphthyl-alanines and naphthyl-glycines [7].

In conclusion, the high activity on naphthyl-amino acids and the high yield of D-isomer conversion observed with the M213G RgDAAO mutant appear to be a major advantage of the process we have successfully developed on a laboratory scale. The complete conversion of the D-isomer is not a simple kinetic resolution of the racemic mixture; the α-keto acid produced is the substrate for an amino transferase or an amino acid dehydrogenase [18–20]. We recently demonstrated that RgDAAO can be combined to L-aspartate amino transferase from *E. coli* to generate 2-naphthyl pyruvate from D-2-naphthyl-alanine (in the presence of cysteine sulphinic acid as an amino donor) [7]. The combined reactions afforded enantiomerically pure L-2-naphthyl-alanine in almost quantitative yield, and the biotransformation can be conducted in suspension to overcome the extremely low water solubility of the substrate. Furthermore, the *in situ* chemical reduction of the initially formed imine can also converge to a final complete conversion into one single enantiomer [21,22].

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