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Enzymatic Conversion of Unnatural Amino Acids by Yeast D-Amino Acid Oxidase

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Abstract: Unnatural amino acids, particularly synthetic α-amino acids, are becoming crucial tools for modern drug discovery research. In particular, this application requires enantiomerically pure isomers. In this work we report on the resolution of racemic mixtures of the amino acids D,L-naphthylalanine and D,L-naphthylglycine by using a natural enzyme, D-amino acid oxidase from the yeast *Rhodotorula gracilis*. A significant improvement of the bioconversion is obtained using a single-point mutant enzyme designed by a rational approach. With this D-amino acid oxidase variant the complete resolution of all the unnatural amino acids tested was obtained: in this case, the bioconversion requires a shorter time and a lower amount of biocatalyst compared to the wild-type enzyme. The simultaneous production of the corresponding α-keto acid, a possible precursor of the amino acid in the L-form, improves the significance of the procedure.

Keywords: D-amino acid oxidase; amino acids; biotransformations; enzyme catalysis; rational design

Introduction

The increasing interest of the pharmaceuticals and fine chemicals industries in the production of drugs and intermediates is an incentive to devise new chiral technologies and to improve enantioselective processes.[1] In fact, the worldwide sales of formulated chiral drugs in single-enantiomer dosage forms is growing at an annual rate of 13%.[2] On a commercial scale, the kinetic resolution approach allows one to separate enantiomers; in this context, the use of biocatalysts has found widespread application and is rapidly becoming a cheap approach.[3] A key factor for the successful development of biocatalytic processes is the possibility to employ on a large-scale an inexpensive enzyme with suitable properties (i.e. high activity, stability and selectivity). Here we provide an example for the combination of biological tools for the production of optically pure unnatural L-amino acids. D-Amino acid oxidase (EC 1.4.3.3, DAAO) is a highly stereoselective flavoenzyme which catalyses the dehydrogenation of the D-isomer of amino acids to give the corresponding α-imino acids and, after subsequent hydrolysis, α-keto acids and ammonia. Oxygen, the final redox acceptor, reoxidises the reduced FAD cofactor to give hydrogen peroxide (Figure 1). In particular, DAAO from the yeast *Rhodotorula gracilis* (RgDAAO) exhibits a very high turnover number, tight binding with the coenzyme FAD, a broad substrate specificity and it has an active site large enough to accommodate even bulky substrates, for example, cephalosporin C (for a review see[4,5]). The redox reaction of DAAO is exploited in a number of biotechnological applications, for example, 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.

In this work, we studied the enzymatic activity of wild-type RgDAAO towards racemic mixtures of unnatural aromatic amino acids (see Table 1) to achieve the resolution of the corresponding L-amino acid component. We also report on the use of rational design as a strategy to obtain an enzyme that is increasingly more suitable for this biotechnological application. Non-proteinogenic amino acids that either occur naturally or are chemically synthesised, and particularly synthetic α-amino acids, have played a significant role in the area of peptide research.[6] Furthermore, and due to their structural diversity and func-

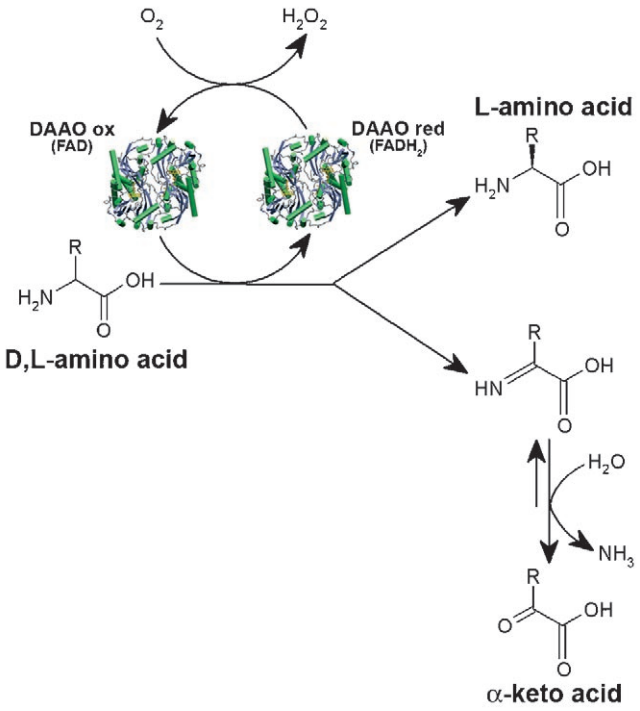


Figure 1. Reaction catalysed by D-amino acid oxidase.

tional versatility, they are widely used as chiral building blocks and molecular scaffolds in constructing chemical combinatorial libraries. Unnatural amino acids are of great value for the *de novo* design of peptides and proteins with a high propensity to fold with a predetermined secondary or tertiary structure[7] but are also valuable pharmaceuticals in their own right (e.g., L-DOPA), as well as components of numerous therapeutically relevant compounds (e.g., D-2-naphthylalanine is found in the peptide drug Nafarelin).[8] It is thus evident that unnatural amino acids are becoming indispensable tools in drug discovery efforts.

Results and Discussion

Kinetic Properties of Wild-Type RgDAAO on Naphthylamino Acids

Table 1 summarises the apparent kinetic parameters determined for wild-type RgDAAO towards unnatural amino acids **1–4** in comparison with the values obtained for the reference substrate D-alanine (the corresponding Michaelis–Menten plots are reported in Figure 2). The parameters were determined under steady state conditions at 25 °C, pH 8.5, and at an oxygen saturation of 21 % (the assay mixture was equilibrated with air). Wild-type RgDAAO shows a higher substrate affinity for all naphthylamino acids tested compared to D-alanine (in particular, significantly lower K_m values were determined for amino acids **1–3**). On the other hand, a appreciably higher specificity constant (as expressed by the ratio $V_{max,app}/K_{m,app}$) was only observed on D-2-naphthylalanine (**1**) which is mainly due to a higher substrate affinity (see Table 1); the low $V_{max,app}$ values (up to *ca.* 600-fold lower for D-2-naphthylglycine than for D-alanine) significantly affect the specificity constant with the substrates **2–4**. The Michaelis–Menten plot of the activity of wild-type RgDAAO on D-2-naphthylalanine (**1**)

Table 1. Comparison of the apparent steady-state kinetic parameters determined for wild-type and M213G RgDAAOs on different D-amino acids.[a]

Substrate		$V_{max,app}$ [min^{-1}]	$K_{m,app}$ [mM]	$V_{max,app}/K_{m,app}$ [min^{-1} mM^{-1}]
D-alanine	Wild-type	3900 ± 200	0.9 ± 0.1	4330
	M213G	770 ± 95	4.9 ± 0.5	160
D-2-Naphthylalanine (**1**)[b]	Wild-type	1965 ± 80	0.04 ± 0.003	49100
	M213G	1170 ± 72	0.06 ± 0.009	19500
D-1-Naphthylalanine (**2**)	Wild-type	125 ± 8	0.04 ± 0.007	3100
	M213G	870 ± 23	0.03 ± 0.003	29000
D-2-Naphthylglycine (**3**)	Wild-type	6 ± 0.1	0.01 ± 0.001	600
	M213G	31 ± 0.7	0.03 ± 0.002	1035
D-1-Naphthylglycine (**4**)	Wild-type	53 ± 4	0.33 ± 0.05	160
	M213G	92 ± 3	0.05 ± 0.006	1840

[a] *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.

[b] Because of the substrate inhibition effect, the kinetic parameters on compound **1** were determined using the activity values obtained in the 0–0.15 mM substrate concentration range.

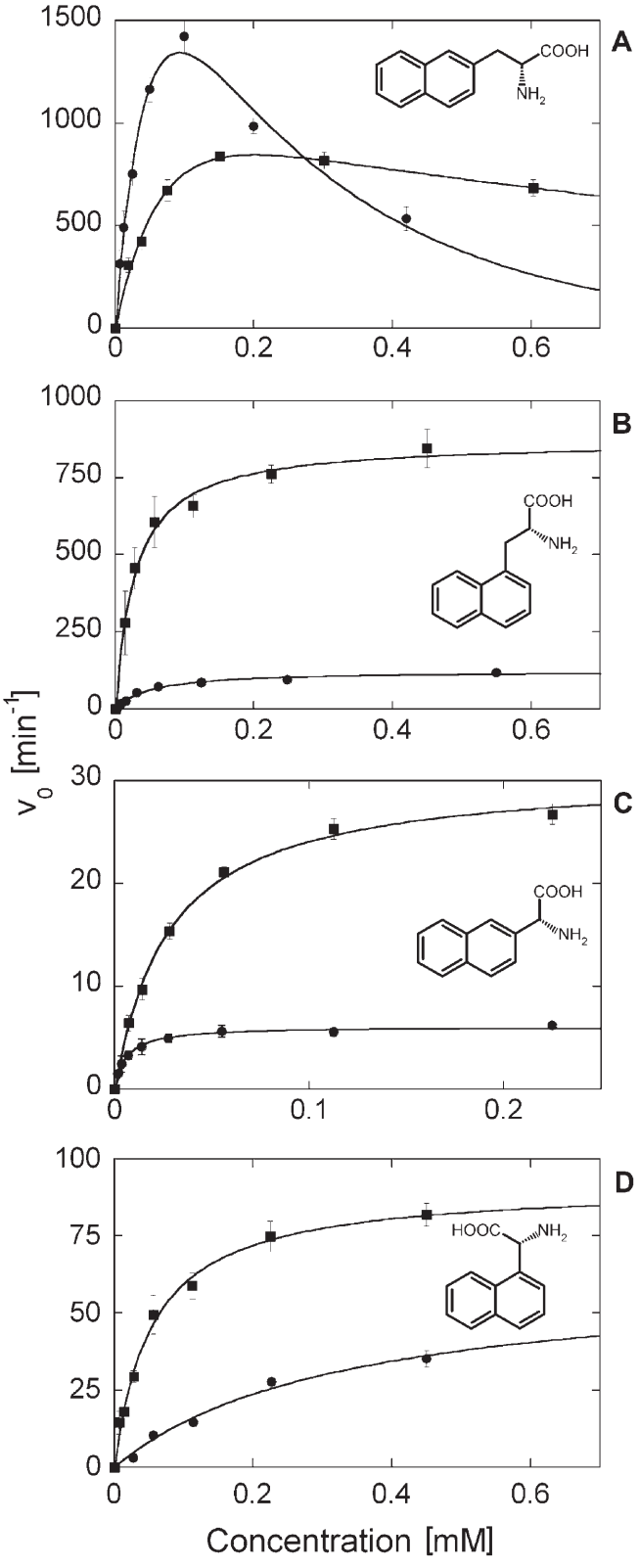


Figure 2. Michaelis–Menten plot of the activity values determined for wild-type (filled circles) and M213G (filled squares) RgDAAOs on compound (**1**, **A**), (**2**, **B**), (**3**, **C**), and (**4**, **D**). Bars indicate ±SEM for at least three determinations; where not shown, the error is smaller than the symbol used.

(Figure 2A) shows a substrate inhibition effect[9] that is evident at an amino acid concentration ≥ 0.1 mM. On the other hand, a similar effect is not observed using the compounds **2**–**4**. The determination of the kinetic parameters is not affected by the use of the racemic mixture instead of the pure D-isomer, thus confirming that the L-isomer is not an inhibitor of the DAAO reaction.[4,5] In conclusion, the investigation of the substrate specificity of wild-type RgDAAO towards these unnatural amino acids shows that D-2-naphthylalanine (**1**) is the best substrate.

Bioconversions using Wild-Type RgDAAO

We investigated the ability of wild-type RgDAAO in the resolution of racemic mixtures of unnatural aromatic amino acids **1–4** following the time course of bioconversion using a stirred reactor and the enzyme in the free form (Figure 3). The products of the bioconversion reaction were quantified by HPLC. In all cases, a higher rate and total conversion were obtained by bubbling air into the apparatus and in the presence of catalase (data not shown). After 300 min of reaction, the chromatograms show that over 90% of the D-enantiomer has been consumed using substrates **1**, **2** and **4** (Figure 3A, B, D). Under optimized conditions, the maximal conversion is reached using different amounts of enzyme depending on the substrate used (see legend of Figure 3 and Table 2). The conversion of D-2-naphthylalanine (**1**) requires the lowest enzyme concentration (40 U/L every hour), in agreement with the higher specificity constant of wild-type RgDAAO on this substrate (Table 1): a total conversion of the D-isomer (*ee* >99%) is reached in *ca.* 220 min. On the other hand, the conversion of compound **3** is only *ca.* 60% even if a large amount of wild-type RgDAAO has been used (*ca.* 6000 U/L).

Molecular Docking of Unnatural D-Amino Acids at the Active Site of RgDAAO

At first, the mode of interaction of D-2-naphthylalanine (**1**), the best substrate among the unnatural D-amino acids under investigation, at the active site of RgDAAO was studied using the software AutoDock 3.0.[10] This program employs a Monte Carlo simulated annealing strategy where solutions (ligand-protein complexes) possessing the lowest energy are the most probable ones. The crystal structure of RgDAAO in complex with D-trifluoroalanine (PDB entry 1c0L) was used as the macromolecular target.[11] As shown in Figure 4A and B, the D-2-naphthylalanine side chain points towards the entrance of the active site of RgDAAO engaging in strong hydrophobic and van

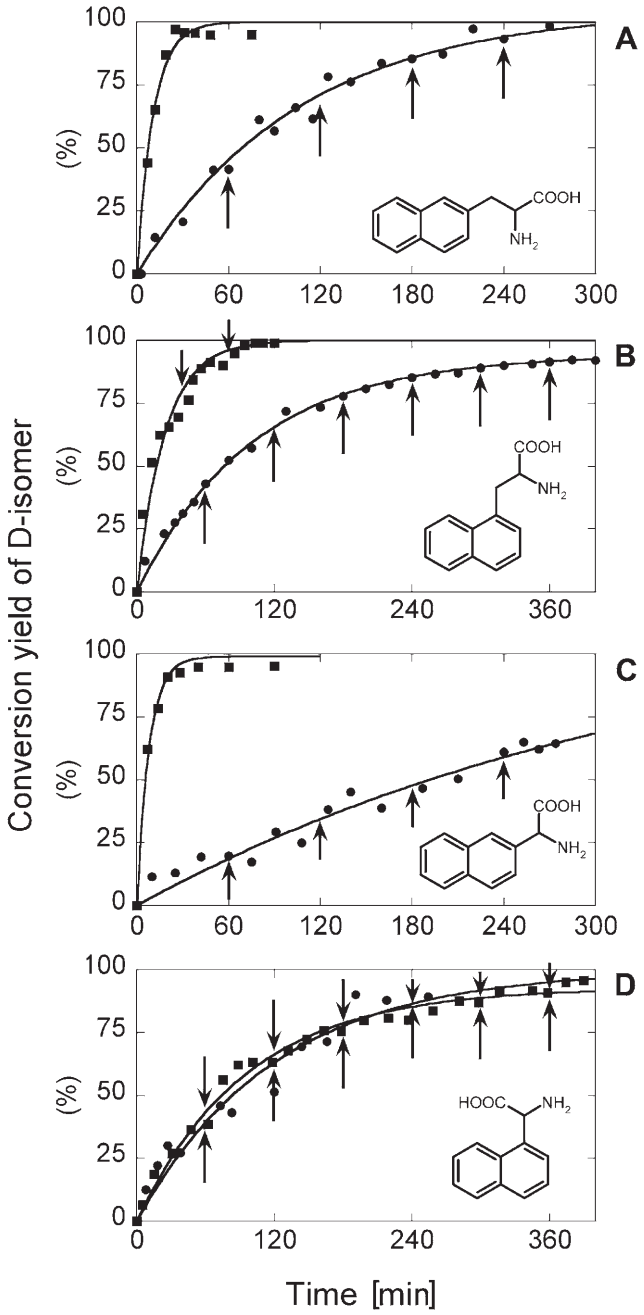


Figure 3. Comparison of the time course of bioconversion of compounds **1–4** in a stirred reactor using wild-type (filled circles) and M213G (filled squares) RgDAAOs at 25 °C, pH 8.5, and air bubbling. The arrows indicate the enzyme additions. **A**: To 1.2 mM of D,L-2-naphthylalanine is added 40 U/L (every hour) of wild-type RgDAAO or 20 U/L (at the beginning of the bioconversion reaction) of M213G RgDAAO. **B**: To 1.2 mM D,L-1-naphthylalanine is added 105 U/L (every 60 min) and 20 U/L (every 40 min) of wild-type and M213G RgDAAOs, respectively. **C**: To 0.75 mM D,L-2-naphthylglycine is added 1500 U/L (every hour) of wild-type RgDAAO or 100 U/L (at the beginning of the bioconversion reaction) of M213G RgDAAO. **D**: To 1.5 mM D,L-1-naphthylglycine is added 200 and 100 U/L wild-type and M213G RgDAAOs, respectively, every hour.

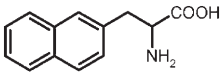
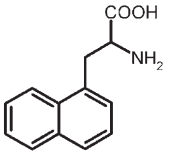
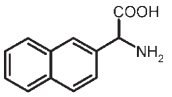
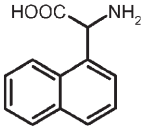
der Waals interactions with the aromatic side chains of F58 and Y238. Noteworthy, docking simulations show that these interactions modify the correct positioning of the ligand at the active site by increasing the distance between the N(5) position of the FAD co-factor and the α-carbon of the substrate (Figure 4C). Anyway, and in agreement with the high specific activity of RgDAAO on D-2-naphthylalanine (**1**), it is conceivable that random conformational motions of protein side chains in solution allow the substrate to reach a position suitable for oxidation. In fact, molecular modelling simulations show that minor movements of the F58 and Y238 side chains could modify the ligand positioning at the active site (not shown). Interestingly, binding of the substrate (as well as of the product) molecule between the side chain of these two latter residues results in a partial hindrance of the active site access (Figure 4B), giving the rationale of the observed substrate inhibition effect (Figure 2A).

In order to understand the rationale of the low activity measured for wild-type RgDAAO on the D-naphthylglycine derivatives, a similar docking analysis was performed using compounds **3** and **4** as ligands. The results show that, although D-naphthylglycine possesses a slightly shorter side chain, its major rigidity (due to the lack of a CH_2 group) does not allow a correct positioning of this substrate in the active site. Modelling of D-2-naphthylglycine at the active site of wild-type RgDAAO according to the positioning of D-2-naphthylalanine (see above) clearly shows a clash between the substrate side chain and the side chain of the active site residue M213 (see Figure 4A). Therefore, in an effort to improve the binding of compound **3** in the correct orientation at the active site of RgDAAO, the M213 residue was mutated in glycine. We have previously shown that the substrate specificity of yeast DAAO is modulated by the side chains of residues belonging to the hydrophobic binding pocket of the active site that interacted with the side chain of the substrate.[12] Among these residues, M213 appeared to be especially important and thus we have been successful in attaining an enzyme active on acidic D-amino acids using a structure-based rational design approach (M213R mutant).[12] The substrate D-2-naphthylglycine docked at the active site of the model of the M213G mutant RgDAAO is depicted in Figure 4D: it clearly shows that in this case the substrate reaches the correct position for oxidation, that is, the α-C–H bond of the substrate is in front of the N(5) flavin position (at a distance < 3.5 Å).

Purification and Properties of M213G RgDAAO

The M213G RgDAAO protein was expressed in *E. coli* and purified using the protocol for the wild-type

Table 2. Comparison of the bioconversion results of racemic mixtures of unnatural amino acids (**1–4**) under optimized conditions (and at 25 °C, air bubbling and pH 8.5).

Compound		Enzyme form	Concentration (D,L-form) [mM]	Conversion yield of D-form [%]	Conditions	
					Enzyme concentration [U/L][a]	Time [min]
D-2-Naphthylalanine (**1**)		Wild-type	1.2	>99	40 every 60 min (160 total units)	220
		M213G	1.2	>99	20	25
D-1-Naphthylalanine (**2**)		Wild-type	1.2	>96	105 every 60 min (735 total units)	400
		M213G	1.2	>99	20 every 40 min (60 total units)	100
D-2-Naphthylglycine (**3**)		Wild-type	0.75	≈60	1500 every 60 min (6000 total units)	240
		M213G	0.75	>99	100	30
D-1-Naphthylglycine (**4**)		Wild-type	1.5	>95	200 every 60 min (1000 total units)	300
		M213G	1.5	>99	100 every 60 min (500 total units)	300

[a] The enzymatic units are those determined on D-alanine as substrate.

RgDAAO containing an His-tag at the N-terminus.[13] Starting from 10 L fermentation broth, 30 mg of pure enzyme with a specific activity of 19.2 U/mg protein were achieved: an overall purification yield of 67% was obtained. The protein was about 95% pure and yielded a single band at 40 kDa in SDS-PAGE. Like the wild-type RgDAAO, the mutant enzyme is purified as a holoenzyme (the E_{278nm}/E_{455nm} ratio is 8.5) and is stable when stored at −20 °C for several months.

The maximal activity on the best substrates, D-alanine and D-2-naphthylalanine, is significantly reduced in the mutant enzyme (Table 1). On the other hand, an increase in $V_{max,app}$ is evident from compounds **2–4**. In the case of compounds **2** and **4**, this change is also accompanied by an increased affinity for the substrate and thus leads to a significant increase in the specificity constant $V_{max,app}/K_{m,app}$ value (up to 10-fold for D-1-naphthylalanine). Interestingly, the substrate inhibition effect with compound **1** is less evident for the M213G mutant than for wild-type DAAO (see Figure 2A).

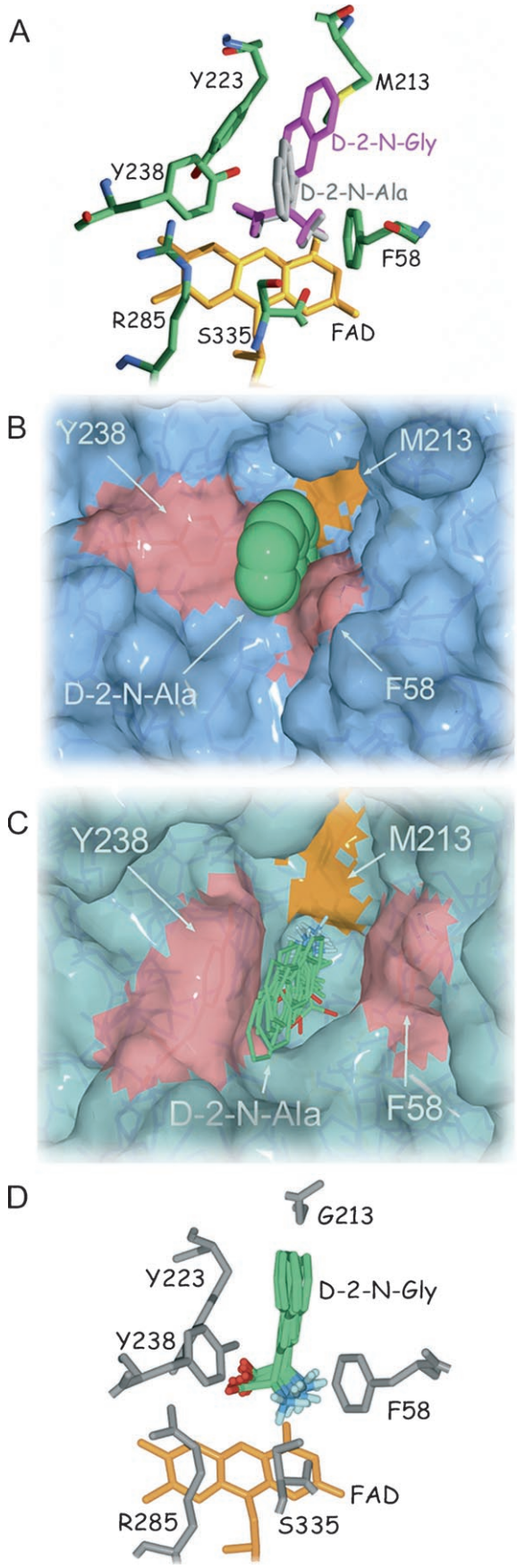
Bioconversions using M213G RgDAAO

The resolution of racemic mixtures of the unnatural amino acids **1–4** was newly performed using the M213G RgDAAO mutant and the same experimental conditions used with the wild-type enzyme (see above). In all cases, the complete resolution of the D,L-mixtures is obtained using a lower amount of enzyme (in terms of enzymatic units) and in a shorter time (see Table 2 and Figure 3). In particular, the conversion of the D-isomer of compound **3** is complete in only 30 min and using a 60-fold lower amount of enzyme than with wild-type RgDAAO (moreover, with this latter enzyme form only a partial conversion is obtained, Figure 3C).

As an example of the possibility to employ RgDAAO on a preparative scale, the bioconversion of 1 mM D,L-2-naphthylalanine (60 mg total, 240 mL, pH 8.5) was performed. By using 80 U/L of wild-type RgDAAO or 20 U/L of M213G mutant, the production of α-keto acid reached a plateau after 40 min and 20 min, respectively, corresponding to a 50% amino acid conversion. The residual amino acid was isolated from the produced α-keto acid by standard ion exchange chromatography on a Dowex column. In both cases the enantiomeric excess was >99.9% as judged by chiral chromatography and the overall yield was >90% (>27 mg of recovered product).

Conclusions

In the present work, we provide an example for the integration of an "*in silico*" analysis and a biological tool such as site directed mutagenesis in the development of resolution processes for the production of optically pure unnatural amino acids. The assay of wild-type RgDAAO on the unnatural D-amino acids **1–4** confirms its broad substrate specificity.[4] In fact, RgDAAO is active on all the compounds tested and



shows a higher specificity constant with naphthylalanine (compounds **1**, **2**), than with naphthylglycine derivatives (compounds **3**, **4**) (Table 1), probably because of the higher flexibility of the substrate side chain. In particular, compound **1** is the best substrate for the wild-type enzyme: the high specificity constant on this compound is mainly due to a higher substrate affinity (Table 1). On the other hand, using the wild-type RgDAAO only a partial resolution of D,L-2-naphthylglycine has been obtained, and for compounds **2** and **4** the complete conversion of the D-isomer required the addition of a huge amount of enzyme.

Models of RgDAAO-D-naphthylamino acid complexes were simulated by AutoDock software and showed that the low activity observed with naphthylglycine derivatives is largely due to the steric hindrance of the side chain of M213. The substitution of M213 to glycine does not result in gross perturbation of the FAD microenvironment but affected the kinetic properties of the enzyme. This substitution allowed a better interaction with compounds **2**–**4**, resulting in an increase in maximal activity and/or substrate affinity (see Table 1). Such a change in substrate specificity allows a complete resolution of the racemic mixtures of all the four unnatural D-amino acids using a significant lower amount of enzyme (see Table 2 and Figure 3).

In conclusion, the high activity on naphthylamino 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 overall conversion is not a simple kinetic resolution: in fact the α-keto acid produced is the substrate for an amino transferase or an amino acid dehydrogenase.[14] If enzymes with L-specificity are applied, the two convergent steps (oxidation of the D-enantiomer followed

Figure 4. **A**: Proposed model of the positions of substrates **1** and **3** at the active site of RgDAAO as predicted by docking analysis using the program AutoDock 3.0 (the position of substrate **3** was modelled based on the orientation obtained for compound **1**). This software combines a rapid, grid-based method for evaluation of the ligand-protein interaction energies with a Monte Carlo simulated annealing search algorithm for optimal conformation of ligands.[10,20] **B**: The same complex as in **A** for substrate **1** seen from outside the active site; protein surface is depicted in cyan, F58 and Y238 in red and M213 in ochre. The bulk naphthyl group of the substrate is shown in green using van der Waals representation. **C**: Alternative docking solutions at the active site of RgDAAO highlighting the interaction between the naphthyl moiety of **1** and the aromatic side chains of F58 and Y238. **D**: The model of M213G DAAO mutant was developed using Swiss PDB Viewer program; the docking of the D-2-naphthylglycine (**3**) at the active site was achieved as reported above (see legend of Figure 4 A).

by reductive amination of the produced α-keto acid to the L-amino acid) should produce a deracemisation of the starting amino acid. Moreover it has been recently shown that the *in situ* chemical reduction of the initially formed imine can also converge to a final complete conversion into one single enantiomer.[15]

Experimental Section

Enzyme Expression and Purification

The M213G mutant was generated by site-directed mutagenesis (QuikChange kit, Stratagene) using as template the cDNA coding for His-tagged wild-type DAAO.[13] The mutation was introduced using the following 37-mer mutagenic oligonucleotides M213G-up: GCAAGCGATGCACGGGAGACTCGTCCGACCCCGCTTC; M213G-dn: GAAGCGGGGTCGGACGAGTCTCCCGTGCATCGCTTGC. The introduction of the desired mutation was confirmed by automated DNA sequencing. Enzymatic DNA modifications were carried out according to the manufacturer's instructions and essentially as described.[16] Recombinant wild-type and M213G RgDAAOs were expressed in BL21(DE3)pLysS *E. coli* cells using the pT7-HisDAAO expression vector and purified by HiTrap Chelating chromatography (GE Healthcare Bio-sciences).[13] The best expression of the mutant enzyme was obtained by growing the cells at saturation, inducing the protein expression with 0.8 mM IPTG and collecting the cells after 3 h of growth at 30 °C. The pure enzymes possess a specific activity on D-alanine of 110 and 19.2 U/mg protein for wild-type and M213G RgDAAO, respectively. The enzyme concentration was determined spectrophotometrically by using an extinction coefficient of 12.6 $mM^{-1}cm^{-1}$.

Preparation of Compounds 1–4

D,L-2-Naphthylalanine (*rac*-1): To a suspension of 5 g of monoethyl (2-naphthylmethyl) acetamidomalonate[17] in 100 mL water-ethanol (1:1), 1 mL of 12 N HCl was added and the suspension was refluxed for 12 h under magnetic stirring. The reaction was monitored by TLC (AcOEt: *i*-PrOH:AcOH, 2:2:1). After this time 10 N NaOH was added dropwise under stirring until a pH of about 6.2 was reached. The white precipitate was collected by filtration and dried under vacuum to afford D,L-2-naphthylalanine; yield: 2.3 g (70%). ^{1}H NMR (DMSO-d_6, TFA-d_4): δ = 3.30 (m, 2H), 4.23 (s, 1H), 7.4–7.56 ((m, 3H), 7.75–7.92 (m, 4H), 8.37 (br s, 2H).

D,L-1-Naphthylalanine (*rac*-2): Obtained from Sigma Aldrich.

D,L-2-Naphthylglycine (*rac*-3): Prepared according to the literature.[18]

D,L-1-Naphthylglycine (*rac*-4): In a 50-mL flask, 20 mL of a 20% sodium hydroxide solution were mixed with 5 mL of acetone. 1 g of 1-naphthylglycine amide[19] was added and the solution was brought to reflux under magnetic stirring. The reaction was monitored by TLC (AcOEt:*i*-PrOH: AcOH, 2:2:1). After 24 h the mixture was cooled to room temperature, and treated with 20 mL of water. 8 mL of 37% HCl were added under stirring at 0 °C until a pH of *ca.* 6.3 was reached. The white precipitate was collected by filtration and dried under vacuum to give D,L-1-naphthylglycine; yield: 0.4 g (47%); ^{1}H NMR (DMSO-d_6): δ = 4.98 (s, 1H), 7.46–7.59 (m, 4H), 7.85–7.95 (m, 2H), 8.31 (d, 1H).

Activity Assay and Kinetic Measurements

DAAO activity was assayed with an oxygen electrode at air saturation (0.253 mM O_2) and 25 °C, using 28 mM D-alanine as substrate in 75 mM sodium pyrophosphate buffer, pH 8.5. 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 determined in 100 mM sodium pyrophosphate buffer, pH 8.5, and at 25 °C. The initial reaction rates were used to calculate the kinetic parameters employing the KaleidaGraph software (Synergy Software, PA, U.S.A.). The pure D-isomer of compound **1** and **2** was purchased from Bachem and Sigma Aldrich, respectively; the D-isomer of compounds **3** and **4** was isolated by chiral HPLC chromatography as detailed below but using the Crownpak CR SemiPrep column (10×150 mm, 5 μm).

Bioconversion

The time course of bioconversion was determined by high performance liquid chromatography (HPLC) assay. The reaction mixture contained (in a final volume of 35 mL) 100 mM sodium phosphate buffer, pH 8.5, 0.5–1.5 mM D,L-amino acid [highest solubility was: (**1**) 1.20 mM, (**2**) 1.20 mM, (**3**) 0.75 mM, (**4**) 1.50 mM] and different amounts of wild-type (160–6000 U/L) or M213G (20–500 U/L) RgDAAO and catalase (from Sigma Aldrich, 280 U/liter). Reaction mixtures were incubated at 25 °C and aliquots were drawn at different times (up to 500 min). Samples were separated by HPLC on a Zorbax SB-Aq (150× 4.6 mm, 5 μm; Agilent Technologies) using TFA 0.1%/acetonitrile (70/30), flow 1 mL min^{-1} for **1–2** and TFA 0.1%/acetonitrile (80/20), flow 1.1 mL min^{-1} for **3–4** , with benzoic acid as internal standard (UV detector at 254 nm). Enantiomeric purity of the final products was determined by means of chiral HPLC on a Crownpak CR$^+$ column (4×150 mm, 5 μm, Chiral Technologies) using aqueous perchloric acid/methanol (90/10) (except where stated otherwise). The following R_t values have been determined: compound **1** D = 31.6 min, L = 41.5 min (pH 1.5, flow = 1 mL min^{-1}); compound **2** D = 33.2 min, L = 40.5 min (pH 1.5, flow = 1 mL min^{-1}); compound **3** D = 13.4 min, L = 28.2 min (pH 2.0, flow = 1.2 mL min^{-1}); compound **4** D = 10.4 min, L = 20.3 min (aqueous perchloric acid, pH 2.0, flow = 1.0 mL min^{-1}). The isolation of the residual L-amino acid from the produced α-keto acid during a preparative bioconversion was performed by standard ion exchange chromatography on a Dowex 50× 8 column (the bound L-amino acid was eluted with 1 M ammonia).

Molecular Modelling Studies

Automated ligand docking was performed by AutoDock 3.0, a suite of automated docking tools based on a Monte Carlo simulated annealing approach.[20] The D-enantiomers of the compounds **1** and **3** were prepared applying a mirror sym-

metry to the coordinates of the corresponding L-forms, downloaded from the macromolecular structure database of EMBL-EBI (http://www.ebi.ac.uk/msd-srv/chempdb/cgi-bin/cgi.pl). Swiss PDBviewer 3.7 was used for protein three-dimensional structure visualisation and for the preparation of the model of the M213G mutant RgDAAO.[21]

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