

Tailoring D-Amino Acid Oxidase from the Pig Kidney to R-Stereoselective Amine Oxidase and its Use in the Deracemization of α -Methylbenzylamine**

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Abstract: The deracemization of racemic amines to yield enantioenriched amines using *S*-stereoselective amine oxidases (AOx) has recently been attracting attention. However, *R*-stereoselective AOx that are suitable for deracemization have not yet been identified. An *R*-stereoselective AOx was now evolved from porcine kidney D-amino acid oxidase (pkDAO) and subsequently used for the deracemization of racemic amines. The engineered pkDAO, which was obtained by directed evolution, displayed a markedly changed substrate specificity towards *R* amines. The mutant enzyme exhibited a high preference towards the substrate α -methylbenzylamine and was used to synthesize the *S* amine through deracemization. The findings of this study indicate that further investigations on the structure–activity relationship of AOx are warranted and also provide a new method for biotransformations in organic synthesis.

The efficient enzymatic synthesis of chiral amines, which are important building blocks for pharmaceuticals and agrochemicals, has been the focus of academia and industry. Lipase, an *R*- or *S*-stereoselective transaminase, has mainly been used to examine the enzymatic synthesis of chiral amines.^[1] The deracemization of a racemic amine to obtain the *R*-configured amine has been reported using an *S*-stereoselective amine oxidase and a chemical reductant.^[2] Amine oxidases (AOx) catalyze the oxidative deamination of amines to form the corresponding aldehyde, hydrogen peroxide, and ammonia via an imine intermediate. AOx can be classified into two groups based on the type of cofactor in the active site, namely copper-containing topaquinone (TPQ)-dependent AOx^[3] and flavin-dependent AOx.^[4] These AOx preferentially oxidize simple straight-chain primary amines, such as butylamine, phenylethylamine, and dopamine, rather than amines with a chiral center at the α -

position, such as α -methylbenzylamine (MBA).^[5] Several studies have examined the actions of AOx on chiral *S* amines.^[2] Turner et al. reported engineered *S*-stereoselective flavin-dependent monoamine oxidase (MAO) variants from *Aspergillus niger* for the deracemization of racemic amines to produce chiral primary, secondary, and tertiary amines.^[2a,b,e,f] The catalytic activity of these mutants towards chiral primary amines was shown to be higher than that towards simple primary amines. Leisch and co-workers successfully synthesized an *R* amine by deracemization using an *S*-stereoselective cyclohexylamine oxidase from *Brevibacterium oxydans* IH-35A.^[2c,d] More recently, Kohler et al. obtained secondary cyclic *R* amines from the corresponding racemic amines or imine precursors through a cascade reaction with a metalloenzyme under mild conditions.^[6a] The reaction consisted of two steps, namely amine oxidation by mutant MAO and reduction of the imine with an artificial transfer hydrogenase^[6b] instead of a harsh chemical reductant. However, *R*-stereoselective AOx that are suitable for deracemization have not yet been identified. TPQ-dependent AOx from *Escherichia coli* and *Klebsiella oxytoca* were shown to preferentially oxidize the *R* enantiomer of amphetamine with moderate enantiomeric ratios (corresponding to an *E* value of ca. 15),^[7] but they are not suitable for deracemization reactions because the cofactor TPQ and the intermediate imine formed a covalent bond. The purpose of this present study was to evolve a flavin-dependent porcine kidney D-amino acid oxidase (pkDAO) into an *R*-stereoselective AOx and apply it to the deracemization of racemic amines.

AOx with *R* stereoselectivity have not been reported previously, but *S*-stereoselective flavin-dependent AOx belonging to the AOx family proteins, including L-amino acid oxidase (LAO), polyamine oxidase, and spermine oxidase, have been described. We targeted the DAO family of proteins and pkDAO in particular as a starting enzyme for directed evolution, because when studying their primary structures, we observed that the DAO family of proteins, including DAO, glycine oxidase, and sarcosine oxidase, may have diverged from a common ancestral protein; therefore, the potential protein structure of *R*-stereoselective AOx should resemble that of DAO. We speculated that it may be possible to tailor-make AOx from typical DAO enzymes, such as pkDAO.

pkDAO was the first identified mammalian flavoprotein that catalyzes the oxidative deamination of α -amino acids with strict *R* stereoselectivity to form the corresponding α -keto acids, ammonia, and hydrogen peroxide, but it does not act on simple amines. The structure of flavin-dependent pkDAO complexed with benzoate as an inhibitor was

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previously revealed (PDB code: 1AA8).^[8] The overall structure of pkDAO was markedly different from that of the AOx family of proteins. On the other hand, the substrate-binding site of pkDAO and LAO may be considered as mirror images of each other, and their catalytic mechanisms have many similarities.^[4] The carboxylate group of the benzoate interacts with the guanidine moiety of Arg283, and the hydroxy oxygen atom of Tyr228 acts as the carboxylate binding site in cooperation with Arg283 in pkDAO. This arginine residue is conserved in several D- or L-amino acid oxidases from different sources. Therefore, the residues Tyr228 and Arg283 in the catalytic site were chosen as the target sites for mutation to improve substrate specificity. The pkDAO gene was synthesized by assembly PCR and expressed in *E. coli* bacteria. A first round of saturation mutagenesis of the Tyr228 and Arg283 residues was performed, and the resulting mutant libraries were screened in the oxidation of (*R/S*)- α -MBA ((*R/S*)-**1**). A positive clone was determined by a colorimetric assay that measured amine oxidase activity. The positive mutant could not be obtained from the saturation mutagenesis library of Tyr228. Twenty variants with oxidation activity toward (*R/S*)-**1** were obtained by screening the saturation mutagenesis library of residue Arg283. The residue Arg283 was found to have been replaced by either Gly, Ala, or Cys in the positive mutant enzymes that were obtained. The mutants R283G, R283A, and R283C catalyzed the oxidation of the *R* enantiomer of (*R/S*)-**1**. These mutants were used as parents for the second round of saturation mutagenesis of Tyr228 and screening. The resulting mutants, including Y228L/R283G, Y228L/R283A, and Y228L/R283C, were obtained by screening for an oxidative activity that is higher than that of the parent enzymes (Table 1). These mutants also showed highly stereoselective oxidase activity towards the *R* enantiomer of (*R/S*)-**1** only. Finally, the mutant pkDAO Y228L/R283G was selected for further investigations because it showed the highest activity towards (*R/S*)-**1**.

This mutant pkDAO (Y228L/R283G) was purified and characterized from recombinant *E. coli* bacteria (JM109). The specific activity of the purified enzyme was 21.5 U mg⁻¹ for (*R*)-**1** as the substrate, and the enzyme acted as a strictly *R*-stereoselective amine oxidase in the presence of (*R/S*)-**1**. This mutant lost its ability to catalyze the oxidation of *R*-

configured amino acids, such as phenylalanine, proline, methionine, and alanine. Its specific activity was four times higher than that of purified wild-type pkDAO (4.9 U mg⁻¹, with (*R*)-phenylalanine as the substrate). The reaction profile of the kinetic resolution of (*R/S*)-**1** (10 mM) to (*S*)-**1** using the purified enzyme is shown in Figure 1 a. The initially present

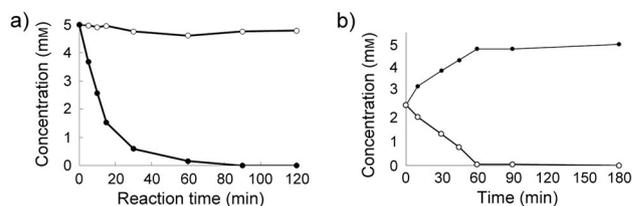


Figure 1. Time course of the kinetic resolution (a) and the deracemization (b) of (*R/S*)-**1** using the mutant pkDAO Y228L/R283G. a) The kinetic resolution of (*R/S*)-**1** (10 mM) was carried out with mutant pkDAO (1.5 U) in KPB (100 mM, pH 8.0) at 30 °C. b) Enzymatic conversion of (*R/S*)-**1** into (*S*)-**1** by deracemization. The reaction mixture (total volume 1.0 mL) was composed of KPB buffer (100 mM; pH 8.0), (*R/S*)-**1** (5 mM), NaBH₄ (100 mM), and the mutant enzyme (0.5 U) at 30 °C. The enzyme was added to initiate the reaction. The amounts of (*S*)-**1** and (*R*)-**1** are denoted by ● and ○, respectively.

(*R*)-**1** completely disappeared from the reaction mixture within two hours, and the enantiopurity of the remaining (*S*)-**1** reached 99% *ee*. This result indicates that the mutant enzyme is a novel *R*-stereoselective amine oxidase that could be applied to the production of chiral *S*-configured amines by kinetic resolution. Other mutants also showed completely *R*-stereoselective activity toward (*R/S*)-**1**. The mutant enzyme also exhibited a strong preference for MBA derivatives (especially for **1**, **8**, and **15**), whereas the enzyme hardly oxidized chiral aliphatic primary amines (**12** and **13**), simple primary amines (**20** and **21**), or chiral secondary *R* amines (**13**; Figure 2). However, *S* amines (**1–8**, **14**) were found to be unsuitable substrates. The stereoselectivity of the mutant pkDAO towards (*R/S*)-**1**, (*R/S*)-**3**, and (*R/S*)-**4** was determined by HPLC on a chiral stationary phase. Furthermore, both enantiomers of several amino acids, such as **22**, **23**, **24**, and glycine, could not be used as substrates for this enzyme. A comparison of the mutant enzyme with wild-type pkDAO showed that they had the same properties (preferring pH 9 and 45 °C) except for their heat stability: The mutant enzyme was stable at 55 °C, whereas the wild-type enzyme was stable only up to 45 °C.^[9]

Wild-type pkDAO has been used for the deracemization of α -amino acids to form *S*-configured α -amino acids.^[10] The Y228/R283G mutant enzyme was also capable of stereo-inversion of an *R*-configured amine using a chemical reductant such as NaBH₄. The mutant enzyme lost its activity under the harsh deracemization reaction conditions, which include the use of NaBH₄ at high temperature. The activity of the enzyme was almost lost when it was incubated at 45 °C for 30 minutes in the presence of NaBH₄ (100 mM). Milder and more stable chemical reductants, such as NaCNBH₃ and H₃N-BH₃, were previously shown to be suitable for use in deracemization reactions using AOx.^[2,10c,d] However, the

Table 1: Comparison of the activities of the pkDAO variants.^[a]

Variant pkDAO	Relative activity [%]	
	(<i>R</i>)-Phe	(<i>R</i>)-MBA
wild-type	100	0
R283G	0	26
R283A	0	14
R283C	0	14
Y228L/R283G	0	146
Y228L/R283A	0	20
Y228L/R283C	0	38
Y228L	0	0

[a] The activity of (*R*)-phenylalanine (Phe) that corresponds to 0.11 U mg⁻¹ was taken as 100%. The reaction mixture (total volume: 1.0 mL) was composed of KPB (100 mM; pH 8.0), the substrate (10 mM), and an appropriate amount of the cell-free extract.

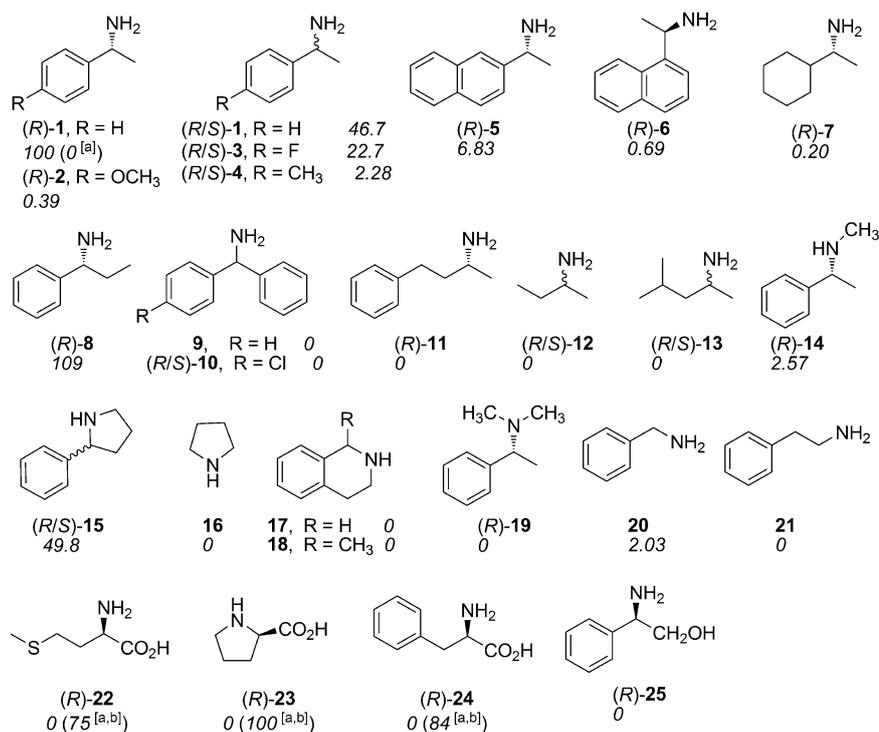


Figure 2. Substrate specificity of mutant pkDAO. Enzyme activities were measured as described in the Experimental Section. The activity of (R)-1 that corresponds to 21.5 U mg⁻¹ was taken as 100%. The italicized numbers below the structures indicate the relative activity. [a] Substrate specificity of wild-type pkDAO. [b] See Ref. [9].

conversion of (R)-1 (1 mM) into (S)-1 was lower with NaCNBH₃ (8.5% yield) and H₃N·BH₃ (48%) than with NaBH₄ (96%). The use of NaCNBH₃ or H₃N·BH₃ led to the formation of the undesired ketone, and an alcohol was then obtained as the main product by reduction of the ketone. On a preparative scale, the synthesis of (S)-1 from racemic 1 by deracemization was performed at 30 °C under the following optimized conditions: KPB buffer (100 mM; pH 8.0) containing (R/S)-1 (5 mM), NaBH₄ (100 mM), and purified enzyme (300 U). The typical time course of the deracemization

reaction is shown in Figure 1 b. Conversion of (R)-1 into (S)-1 was quantitative, and (R/S)-1 had been completely converted into (S)-1 (99% ee) with no detectable side product formation after three hours. Approximately 35% of the S amine was lost during the purification process, as it was isolated in 65% yield. To the best of our knowledge, this is the first study that examines the synthesis of S amines by a deracemization process using an R-stereoselective amine oxidase. This mutant enzyme is useful for the synthesis of the S enantiomer of amines, as it not only provides a method for kinetic resolution, but also catalyzes the deracemization process.

The exchange of a single amino acid to obtain the mutant R283G led to a marked change in the substrate specificity of the enzyme from amino acid oxidase to amine oxidase. We determined the crystal structure of the pkDAO mutant Y228L/R283G (PDB code: 3WGT). The crystal structure of the pkDAO mutant that binds (R)-1 was determined at a resolution of 1.88 Å (Supporting Information, Table S5), and the structure of the active site is shown in Figure 3 a. The electron density of (R)-1 was confirmed to be located on the re side of flavin adenine dinucleotide (FAD; Figure 3 a). A hydrophobic cavity, which has enough space to accommodate the phenyl ring of (R)-1 (Figure S5 a), was created near the xylene ring of FAD in the Y228L/R283G mutant. In this location, the benzene ring of the substrate was sandwiched between the xylene ring of FAD and the para-hydroxyphenyl group of Tyr224 and experienced π-π stacking interactions with the xylene ring and the para-hydroxyphenyl group of Tyr224 (Figure S5 b). This arrangement is different from that for the

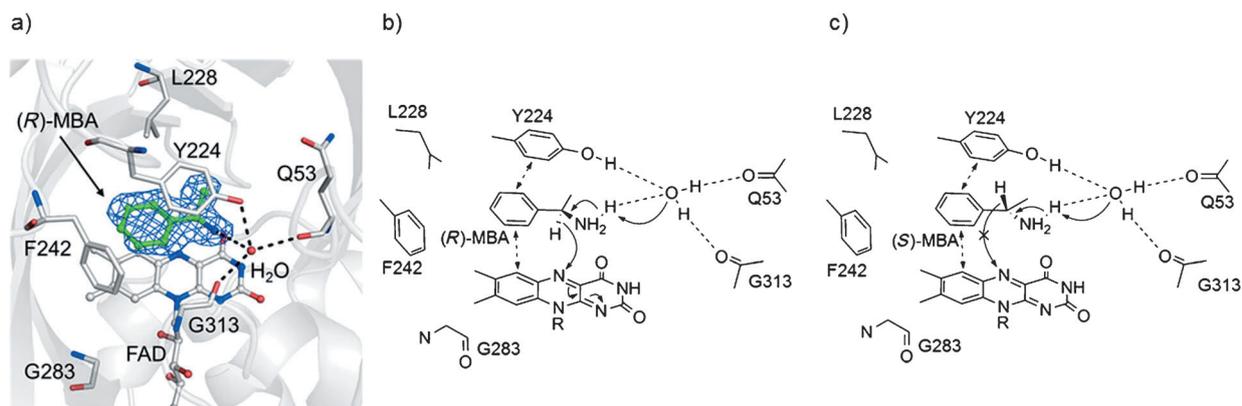


Figure 3. a) The active site of the pkDAO mutant Y228L/R283G with bound (R)-MBA. The carbon atoms of (R)-1 (or (R)-MBA) are shown in green. The carbon atoms of pkDAO and FAD are colored in gray. All hydrogen bonds were shorter than 3.4 Å. The 2F_o-F_c Fourier maps (blue) were contoured at 0.8 σ. b) Proposed mechanism for (R)-1 bound to the pkDAO mutant Y228L/R283G. c) Proposed mechanism for (S)-1 bound to the pkDAO mutant Y228L/R283G. The reaction mechanism for the mutant was proposed in analogy to the reaction mechanism that was already suggested for wild-type pkDAO.^[11]

binding of the inhibitor benzoate to wild-type pkDAO, which was observed by crystal structure analysis; in this case, the phenyl group of the benzoate was located above the uracil ring of FAD (Figure S5c). The marked change in the placement of the phenyl ring that is induced by the mutation (Figure S5a) renders the mutant a catalytically active and *R*-selective amine oxygenase that operates by the following mechanism: Regarding the binding of (*R*)-**1**, dehydrogenation can easily be performed because an α -hydrogen atom of the substrate is directed towards the N5 atom of FAD (Figure 3b). On the other hand, for (*S*)-**1**, the α -hydrogen atom is directed towards Tyr224. Therefore, the dehydrogenation of (*S*)-**1** could not be performed because the hydrogen atom was remote from the N5 atom of FAD (Figure 3c).

In conclusion, we have demonstrated that an engineered pkDAO enzyme catalyzes the oxidation of several *R*-configured amines to their corresponding imines with high stereoselectivity. This transformation could not be performed with wild-type pkDAO. Amino acid oxidases had previously not been described to catalyze the oxidation of amines, not even *L*-amino acid oxidases, which belong to the AOx protein family. On the other hand, the actions of AOx on amino acids have also not been examined to date. In spite of the different substrate specificities of the amino acid oxidase and AOx, a single point mutation (R283G) in pkDAO led to a marked change in the properties of the *D*-amino acid oxidase to give an amine oxidase, and the mutant Y228L/R283G showed improved catalytic activity towards (*R*)-**1**. The crystal structure of the mutant enzyme revealed the binding of (*R*)-**1** to the active site. The phenyl group of (*R*)-**1** may fit into a new hydrophobic cavity that is created by the mutation, so that the α -hydrogen atom of (*R*)-**1** points towards the N5 atom of FAD. We demonstrated that enantiopure (*S*)-**1** could be synthesized from racemic **1** by a deracemization process using an engineered enzyme that selectively oxidizes *R*-configured amines in the presence of a chemical reductant. This is the first study to identify a novel tailor-made flavin-containing *R*-stereoselective amine oxidase that is applicable to the production of chiral *S* amines by deracemization.

Experimental Section

Enzyme assay: The oxidase activity was routinely assayed at 30 °C by measuring the formation of a quinone pigment and following absorbance at 505 nm with an absorption spectrometer. The reaction mixture contained a K_2HPO_4/KH_2PO_4 buffer (KPB; 100 mM, pH 8.0), 4-aminoantipyrine (1.5 mM), phenol (2 mM), 2 units of horseradish peroxidase, substrate (10 mM), and enzyme.

Analytical methods: The enantiopurities of **1**, **2**, **3**, and **4** were analyzed by HPLC (Shimadzu, Kyoto, Japan) using a Crownpak CR(+) column (Daicel Co., Japan) with an aqueous solution containing $HClO_4$ (60 mM) and MeOH (5%) as the eluent. UV detection was performed at 210 nm.

The stereoinversion reaction of (*R*)-**1** was performed in KPB (100 mM; pH 8.0), with chemical reductant (20 mM), (*R*)-**1** (1 mM), and the enzyme.

Preparative-scale synthesis of (*S*)-1** by deracemization:** The identity of (*S*)-**1** that was formed by deracemization using the purified pkDAO mutant was confirmed by its isolation. The reaction mixture (400 mL) contained KPB buffer (40 mmol; pH 8.0), $NaBH_4$ (40 mmol), (*R/S*)-**1** (2.0 mmol; 0.24 g), and purified enzyme (300 U).

The mixture was then incubated at 30 °C for 3 h. The reaction mixture was adjusted to pH 12 with NaOH (6N), and the product was extracted four times with dichloromethane. The organic phase was evaporated in vacuo, and the remaining product was subsequently purified by column chromatography on silica gel (AcOEt/MeOH = 1:1). (*S*)-**1** was isolated with 99% *ee* and in 64.5% yield (0.156 g, 1.29 mmol); optical rotations were measured on a ATAGO AP-300 automatic polarimeter (Atago Co., Tokyo, Japan). $[\alpha]_D^{26} = -29.4^\circ$ ($c = 1.02$, MeOH; Ref. [12]), $[\alpha]_D^{20} = -25.7^\circ$ ($c = 0.98$, MeOH). 1H NMR ($CDCl_3$, 400 MHz): $\delta = 1.29$ (d, 3H, $J = 6.6$ Hz), 1.61 (s, 2H), 4.01 (q, 1H, $J = 6.6$ Hz), 7.11–7.23 ppm (m, 5H). MS (microTOF): m/z calcd for $C_8H_{12}N_1 [M+H]^+$: 122.0964; found: 122.0992.

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