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Engineering of Amine Dehydrogenase for Asymmetric Reductive Amination of Ketone by Evolving *Rhodococcus* Phenylalanine Dehydrogenase

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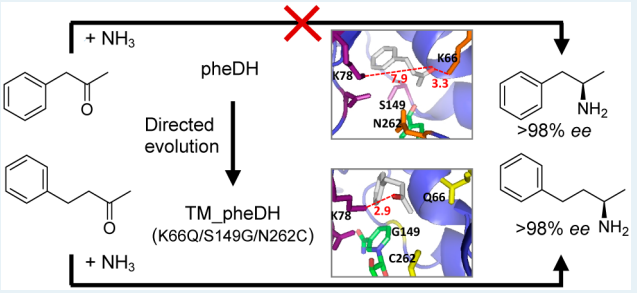
Supporting Information

ABSTRACT: Triple mutant K66Q/S149G/N262C (TM_pheDH) of *Rhodococcus* phenylalanine dehydrogenase (pheDH) was engineered by directed evolution as the first enzyme for the highly enantioselective reductive amination of phenylacetone **1** and 4-phenyl-2-butanone **3**, giving (*R*)-amphetamine **2** and (*R*)-1-methyl-3-phenylpropylamine **4** in >98% *ee*, respectively. The new amine dehydrogenase TM_pheDH with special substrate specificity is a valuable addition to the amine dehydrogenase family with very limited number, for asymmetric reductive amination of ketone, an important reaction in sustainable pharmaceutical manufacturing. Molecular docking provided insight into the role of key mutations of pheDH, being useful for engineering new amine dehydrogenases with higher activity and unique substrate scope.

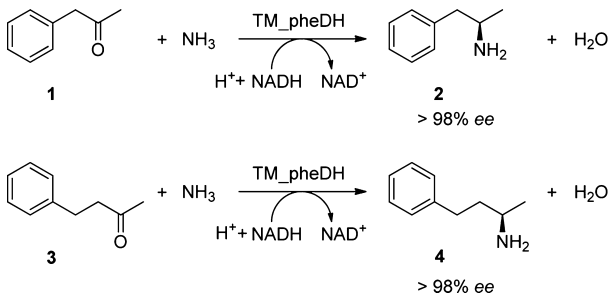


KEYWORDS: *amine dehydrogenase, biocatalysis, biotransformation, chiral amine, directed evolution, reductive amination*

Enantiopure amines are important building blocks for pharmaceutical manufacturing.[1] Enzyme catalysis provides green and selective tools for the enantioselective syntheses of this class of chiral molecules. Many enzymes such as lipase,[2,3] amine oxidase,[4,5] imine reductase,[6,7] transaminase,[8,9] and amine dehydrogenase[10−13] are reported for chiral amine synthesis. Among them, amine dehydrogenase is attractive, because it catalyzes the asymmetric reductive amination of ketone, a highly wanted reaction in green and sustainable pharmaceutical manufacturing,[14] and it uses cheap ammonia as the reagent and generates only water as byproduct, with high atom efficiency. However, only one wild-type amine dehydrogenase (from *Streptomyces* Sp) was reported, with low enantioselectivity.[15] Recently, directed evolution has become a useful tool for engineering enzymes with new substrate acceptance and improved catalytic performance.[16−18] By using this method, Bommarius et al. engineered two new amine dehydrogenases from *Bacillus stereothermophilus* leucine dehydrogenase and *Bacillus badius* phenylalanine dehydrogenase for the preparation of several enantiopure amines.[10−13] To further expend the synthetic scope of amine dehydrogenase, we explored the evolution of phenylalanine dehydrogenase (pheDH) from *Rhodococcus* sp. M4[19] to create new amine dehydrogenases with different substrate specificity for the asymmetric reductive amination of ketones (Scheme 1).

The asymmetric reduction of phenylacetone **1** and 4-phenyl-2-butanone **3** to produce (*R*)-amphetamine **2** and (*R*)-1-methyl-3-phenylpropylamine **4**, respectively, are selected as the target reactions. (*R*)-Amphetamine **2** is a useful intermediate for the preparation of (*R, R*)-formoterol, a potent bronchodilator,[20] and tamsulosin, a prostate drug.[21] (*R*)-1-Methyl-3-phenylpropylamine **4** is a precursor of the antihypertensive dilevalol.[22] The asymmetric synthesis of (*R*)-**2** and (*R*)-**4** via amination of ketone **1** and **3** with ammonia is highly desirable,

Scheme 1. Asymmetric Amination of Phenylacetone 1 and 4-Phenyl-2-butanone 3 To Produce (*R*)-Amphetamine 2 and (*R*)-1-Methyl-3-phenylpropylamine 4, Respectively, with New Amine Dehydrogenase TM_pheDH Evolved from *Rhodococcus* Phenylalanine Dehydrogenase



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but no amine dehydrogenases have been reported for these reactions. *Rhodococcus* pheDH was selected as starting template for the evolution, because the enzyme structure (PDB: 1C1D) and catalytic mechanism[23,24] were reported, which provides a solid basis for identifying a very limited number of key amino acid residues for saturation mutagenesis; in addition, this enzyme shares only 32% identity with *Bacillus badius* pheDH,[25] thus providing the potential to generate new amine dehydrogenase with different substrate specificity than the one engineered from *Bacillus badius* pheDH. In comparison with *Bacillus stereothermophilus* leucine dehydrogenase, *Rhodococcus* pheDH accepts a nature substrate which is structurally more similar to the target ketone substrate **1** and **3**.

As the targeted ketone substrates **1** and **3** do not contain a carboxyl group, the amino acid residues Lys 66 and Asn262 of pheDH, known to interact with the carboxyl group of the natural substrate, were selected for simultaneous double-site saturation mutagenesis. The saturation mutagenesis was performed by PCR using NNK degenerate codons, the mutated genes were transformed in *E. coli*, and the cells were grown on agar plate. As the theoretical number of enzyme mutants is 400, 4230 clones were picked up for screening to ensure the coverage of possible mutants of >95%.[26] A formazan-based colorimetric assay[10] was used to screen enzyme activity for the deamination of racemic amine **2** and **4**, respectively, with the cell-free extract of *E. coli* expressing pheDH mutant. Forty-one positive clones were identified, and six double mutants of pheDH were confirmed with the deamination activity toward amines **2** and **4**, respectively. The reversed reactions, asymmetric amination of ketone **1** and **3**, were then investigated with these double mutants, and the amine products were analyzed by HPLC. As shown in Table 1, four double mutants showed the amination activity for ketone **1** and four double mutants for ketone **3**. Among them, two double mutants K66Q/N262L and K66Q/N262C accepted both ketones **1** and **3** as substrates, giving amine **2** and **4** with a specific activity of 3.9−3.6 and 5.2−6.2 U/g protein, respectively. These activities are higher than those obtained with other double mutants. In comparison with the wild-type pheDH, all positive mutants contain relatively more hydrophobic amino acid residues at position 66 and 262 (S, M, Q more hydrophobic than K, and C, F, I, L more hydrophobic than N). Interestingly, the best double mutants K66Q/N262C and K66Q/N262L are not among the reported 21 double mutants at similar positions of pheDH from *Bacillus badius*.[11]

Table 1. Directed Evolution of PheDH for Asymmetric Amination of Phenylacetone 1 and 4-Phenyl-2-butanone 3, respectively

round	no. of clones screened	positive mutants	specific activity[a] U (g protein)$^{-1}$ **1** to **2**	specific activity[a] U (g protein)$^{-1}$ **3** to **4**
1[b]	4230	K66M/N262I	0	0.4
		K66S/N262I	1.7	0
		K66Q/N262I	0	2.8
		K66Q/N262F	3.2	0
		K66Q/N262L	3.9	5.2
		K66Q/N262C	3.6	6.2
2[c]		K66Q	1.1	0.9
		N262L	0	0
		N262C	0	0
3[d]	3760	K66Q/S149G/N262C	5.0	8.8

[a]Reactions were performed in NH_4OH/NH_4Cl buffer (0.5 M; pH 9.6) containing 5 mM substrate **1** or **3**, 2 mM NADH, and cell-free extract (1 g protein/L) of *E. coli* (pheDH mutant) at 30 °C and 250 rpm. Specific activity was determined for the first 30 min. [b]Simultaneous double-site saturation mutagenesis of K66 and N262. [c]Single mutation. [d]Single-site saturation mutagenesis at 20 selected amino acid residues.

To investigate the importance of single mutation at each of the two selected positions, three single mutants K66Q, N262L, and N262C were prepared. Mutant K66Q showed amination activity toward both ketones **1** and **3**, but the activity is significantly lower than those with the two best double mutants (Table 1). This suggests the importance of the K66Q mutation and a synergetic effect of the double mutants.[27] Two other single mutants N262L and N262C did not show any activity for the amination of **1** or **3**. This further confirmed the mutation K66Q as the key point of the success in changing substrate acceptance from a keto acid to a ketone.

To further enhance the amination activity, we performed another round of evolution with K66Q/N262C (DM_pheDH) as starting enzyme. Twenty amino acid residues located within 6 Å of phenylalanine bound in the catalytic center of the pheDH (Figure S2), except K78 and D118 which are essential for the catalysis,[24] were selected for single-site saturation mutation.[28,29] The mutant library was built by using primers containing NNK codons (Table S3). After transformation of the plasmid in *E. coli* and cell growth on an agar plate, 3760 clones were picked up for screening with 95% coverage of the possible mutants. A screening assay based on UV detection of NADH formation in the deamination of racemic **2** and **4**, respectively, was used, and only one triple mutant K66Q/S149G/N262C (TM_pheDH) was identified with higher amination activity of **1** and **3** than the double mutant (Table 1; 5.0 and 8.8 versus 3.6 and 6.2 U/g protein). The mutation S149G replaced a hydrophilic amino acid by a hydrophobic one.

The product *ee* of the asymmetric amination of ketone **1** and **3**, respectively, with the more active mutants K66Q/N262L, K66Q/N262C (DM_pheDH) and K66Q/S149G/N262C (TM_pheDH), respectively, was checked by HPLC with a chiral column. No peak of (*S*)-enantiomer existed in the chiral HPLC chromatograms suggested >98% *ee* for (*R*)-**2** and (*R*)-**4**, respectively, for each of these three mutants. The product *ee* values of other less active mutants were not determined.

To obtain insight into the role of the mutations on accepting ketone **1** and **3** as the substrate and influencing the enantioselectivity of the reductive aminations, the reported in silico modeling and substrate docking method[30,31] were used to establish the structure models of the mutants based on the structure of pheDH[24] and generate the active binding pose of the substrate in the structure model of the mutants or structure of pheDH. In the obtained active binding pose of phenyl pyruvate in pheDH (Figure S4), the orientation of the substrate resembles that of L-phenylalanine in the enzyme crystal structure,[24] thus confirming the reliability of the modeling method. In this pose, the carbonyl-O atom of phenyl pyruvate formed hydrogen bond with Lys78. In comparison, the binding poses of substrates **1** and **3** in pheDH (Figure 1a,b) were totally different from the active conformation of natural substrate in pheDH, and the carbonyl group of the substrate is far away from Lys78 and Asp118 that are responsible for the formation of imine intermediate based on the reported mechanism of

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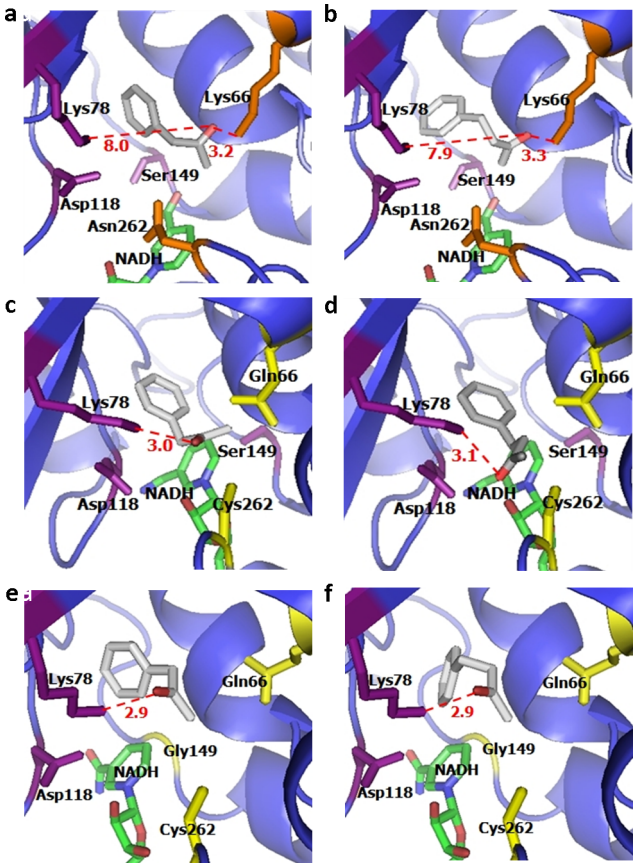

Figure 1. Enzyme–substrate binding pose for (a) wild type enzyme with substrate **1**, (b) wild type enzyme with substrate **3**, (c) mutant K66Q/N262C with substrate **1**, (d) mutant K66Q/N262C with substrate **3**. (e) TM_pheDH with substrate **1**, (f) TM_pheDH with substrate **3**. Mutated residues are shown in yellow. Distances (in angstrom) are denoted by dashed lines.

pheDH for natural substrate.[24] Such catalytically unfavorable poses were caused by the formation of hydrogen bond between the carbonyl-O atom of the substrates and Lys 66 (with a distance of 3.2–3.3 Å). To make active enzyme for the desired reductive amination of ketone, Lys 66 has to be mutated.

As shown in the poses of substrate **1** and **3** in the catalytic pocket of DM_pheDH and TM_pheD (Figure 1c–f), no binding between the carbonyl-O atom of the substrates and the amino acid at 66 due to the K66Q mutation. The induction of Asn262Cys mutation made the orientation of the terminal methyl group of the substrate close to Cys262. The substrates in the poses of Figure 1c–f showed similar position to that of phenyl pyruvate in pheDH, with a distance of 2.9–3.1 Å between substrate carbonyl-O atom and Lys78 due to hydrogen bond. Based on the reported catalytic mechanism from the natural substrate,[24] Lys78 and the nearby Asp118 catalyze the addition of ammonia to the carbonyl group of the substrate to form the imine intermediate. A proton from NADH was then added to the carbon atom of the imine group to give (*R*)-configuration (Figure 1c–f), and further addition of a proton to N atom gave amine (*R*)-**2** or (*R*)-**4**.

In comparison with DM_pheDH, TM_pheDH has the additional S149G mutation which decreased side chain length of the amino acide at 149 by ~2 Å to enlarge the binding pocket entrance, allowing for easier substrate access into the binding pocket. Docking simulations gave binding energy of −4.3 kcal/mol for substrate **1** and −5.0 kcal/mol for substrate **3** in TM_pheDH and binding energy of −3.1 kcal/mol for both substrate **3** and **4** in DM_pheDH. These resuls suggested lower K_m values of TM_pheDH and thus also higher catalytic efficiency and specific activity for TM_pheDH.

The growth curve of *E. coli* (TM_pheDH) in TB medium with the induction by IPTG is shown in Figure 2a. A cell density of around 4.5 g cdw/L was easily achieved at 9–11 h. The amination activity of **3** with the cell-free extract of the cells taken at different time points during growth was also shown in Figure 2a. The highest specific activity (8.8 U/g protein) was observed at 9 h in the late exponential growth phase.

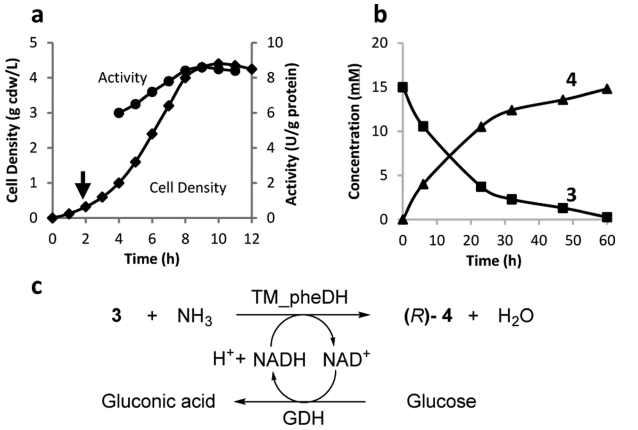

Figure 2. (a) Curves of cell growth of *E. coli* (TM_pheDH) in TB medium at 22 °C and 250 rpm and specific enzyme activity for the amination of **3** to (*R*)-**4**. Arrow indicates the addition of IPTG (0.5 mM) for the induction of TM_pheDH. (b) Time course of the amination of **3** (15 mM) to (*R*)-**4** with TM_pheDH (4 mg protein/mL), GDH (40 U/mL), NAD^+ (0.005 mM), and glucose (100 mM) in NH_4OH/NH_4Cl buffer (0.5 M; pH 9.6) at 30 °C. (c) Scheme of asymmetric amination of 4-phenyl-2-butanone **3** to (*R*)-**4** with cofactor recycling by using His-tagged TM_pheDH and GDH.

His-tagged TM_pheDH was then produced by growing *E. coli* cells expressing his-TM_PheDH and purified using a Ni-NTA column. The kinetic data of TM_pheDH were obtained for the asymmetric amination of ketone **1** and **3**, respectively (Table 2). The enzyme has 3-fold higher catalytic efficiency with substrate **3** than that with substrate **1**. The k_{cat} values are 0.70–0.72 s^{-1}.

Asymmetric amination of 4-phenyl-2-butanone **3** to produce (*R*)-1-methyl-3-phenylpropylamine **4** with the recycling of NADH was performed with TM_pheDH and glucose dehydrogenase (GDH) (Figure 2b,c).[32,33] Reaction of 15 mM **3** gave 14.3 mM (*R*)- **4** at 60 h, with 95.2% conversion and total turnover number of recycling NAD^+ of 2800 (Figure 2b).

Table 2. Kinetic Data for the Asymmetric Amination of Phenylacetone 1 and 4-Phenyl-2-butanone 3 with Purified His-Tagged TM_pheDH, Respectively

substrate[a]	K_m mM	V_{max} mM min^{-1}	k_{cat} s^{-1}	k_{cat}/K_m s^{-1} mM^{-1}
1	4.0	0.042	0.70	0.18
3	1.4	0.043	0.72	0.50

[a]Reaction was performed in NH_4OH/NH_4Cl buffer (0.5 M; pH 9.6) containing 0.25–10 mM substrate **1** or **3**, 2 mM NADH, and 1 μM TM_pheDH at 30 °C for 10 min. Product concentration was determined by HPLC analysis.

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In summary, a triple mutant K66Q/S149G/N262C (TM_pheDH) was successfully engineered by directed evolution of *Rhodococcus* pheDH via simultaneous randomization of two amino acid residues K66 and N262, followed by single site saturation mutagenesis at other 20 selected residues, as a new amine dehydrogenase for the highly enantioselective reductive amination of phenylacetone **1** and 4-phenyl-2-butanone **3** to give (*R*)-amphetamine **2** and (*R*)-1-methyl-3-phenylpropylamine **4** in >98% *ee*, respectively. TM_pheDH showed a k_{cat} of 0.70 and 0.72 s^{-1} and k_{cat}/K_m of 0.18 and 0.50 s^{-1} mM^{-1} for the conversion of **1** to (*R*)-**2** and of **3** to (*R*)-**4**, respectively. Coupling of TM_pheDH and glucose dehydrogenase allowed 95% conversion of 15 mM **3** to (*R*)-**4** with NAD^+ recycling. Molecular docking provided with some insight into the role of key mutations, which could be very useful for further engineering amine dehydrogenase with higher activity and/or different substrate specificity. The engineered TM-pheDH with different substrate specificity is an important addition to the family of amine dehydrogenases with a very limited number thus far, contributing to the expending of synthetic scope of amine dehydrogenases-catalyzed asymmetric reductive amination of ketone in green and sustainable pharmaceutical manufacturing.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/cs501906r.

Chemicals, strains and biochemicals, analytic method; procedures for directed evolution, cell growth, enzyme purification, biotransformation, enzyme simulation and modeling, kinetics; HPLC chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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