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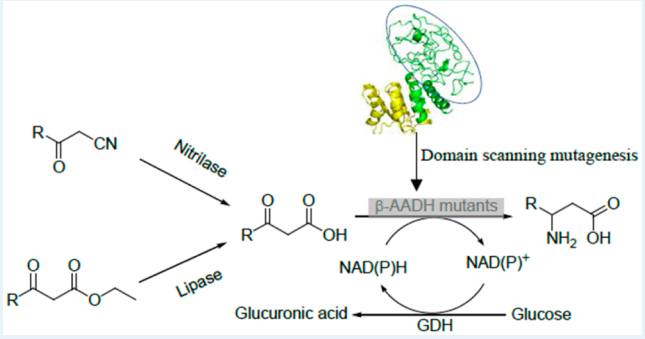
Development of β-Amino Acid Dehydrogenase for the Synthesis of β-Amino Acids via Reductive Amination of β-Keto Acids

Dalong Zhang, Xi Chen, Rui Zhang, Peiyuan Yao, Qiaqing Wu, and Dunming Zhu*

National Engineering Laboratory for Industrial Enzymes and Tianjin Engineering Center for Biocatalytic Technology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 Xi Qi Dao, Tianjin Airport Economic Area, Tianjin 300308, China

Supporting Information

ABSTRACT: An L-*erythro*-3,5-diaminohexanoate dehydrogenase from *Candidatus* Cloacamonas acidaminovorans (3,5-DAHDHcca) was engineered to β-amino acid dehydrogenase (β-AADH) by screening a domain scanning mutagenesis library. The best β-AADH mutant displayed about 200-fold activity toward (*R*)-β-homomethionine compared with the wild-type enzyme. (*R*)-β-Homomethionine, (*R*)-β-phenylalanine, and (*S*)-β-aminobutyric acid were obtained via β-AADH-catalyzed reductive amination of the corresponding β-keto acids, which were generated from β-keto-nitriles or β-keto-esters with nitrilase or lipase. This is the first example of the direct reductive amination of β-keto acids catalyzed by β-AADH to give β-amino acids, opening a new venue for the synthesis of chiral β-amino acids.

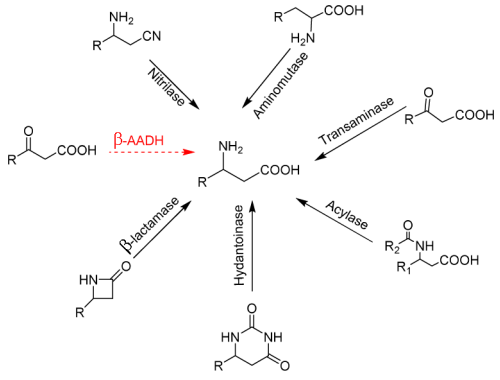


KEYWORDS: *asymmetric catalysis, β-amino acid dehydrogenase, β-amino acid, biocatalysis, reductive amination, enzyme engineering*

Chiral amines are the key building blocks for many chiral pharmaceuticals, agrochemicals, and other fine chemicals.[1] Among them, β-amino acids not only show unique biological and pharmacological properties in free form, but also are key building blocks for the synthesis of a variety of bioactive molecules.[2] Because of their significance, the synthesis of chiral β-amino acids and derivatives has gained increasing attention, and considerable progress has been achieved in recent years.[3] Chemical approaches include kinetic resolution of chemically synthesized racemic β-amino acids, homologation of chiral α-amino acids, and asymmetric synthesis.[3a,4] However, the inherent drawback of kinetic resolution, acquisition of chiral precursor, high loading of expensive catalysts or chiral auxiliaries, and uncontrollability of reaction condition have limited their application at an industrial scale.[3a]

As an alternative, environmentally friendly and enantioselective biocatalysis shows captivating potentiality in asymmetric synthesis of chiral β-amino acids (Scheme 1).[5] Aminomutases, which possess the capability to migrate amino groups from C2 to C3 of amino acids, catalyze the conversion of α-amino acids to β-isomers. Although this seems to be an ideal procedure for preparation of chiral β-amino acids from their α-counterparts,[6] low activity, strict substrate specificity, and the difficult separation of product β-amino acids from substrate α-amino acids prevent their broad applications, as observed for lysine 2,3-aminomutase, which catalyzes the interconversion of L-α-lysine and L-β-lysine,[7] and phenylalanine aminomutase, which is utilized only to synthesize β-styryl- and β-aryl-β-alanine

Scheme 1. Enzymatic Routes to Enantiopure β-Amino Acids



derivatives.[8] Hydrolytic enzymes such as hydantoinases and nitrilases provide a mild method to hydrolyze cyclic amides or β-amino nitriles to give β-amino acids, respectively.[9] To obtain a 100% yield, extra racemase or an efficient chemical racemization must be adopted for racemic substrates, or homologous chiral substrates are required for these enzymes. Optically pure α and β-amino acids can also be synthesized by reductive amination of the corresponding keto acids with β-transaminases,[10] and high conversion can be obtained if the

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coproduct keto acid is removed.[11] Each approach has its own advantages and limitations. The development of an efficient, practical, and simple process for large-scale synthesis of enantiopure β-amino acids remains a challenging task.[3a]

As such, the ACS Green Chemistry Institute, Pharmaceutical Roundtable noted that the asymmetric synthesis of amines from prochiral ketones and ammonia was one of the top aspirational reactions in the pharmaceutical industry.[12] The direct reductive amination of carbonyl compounds by amino acid dehydrogenases to produce chiral amines would be very attractive in terms of atomic economy and impact on the environment. It is well-known that α-amino acid dehydrogenases catalyze the reductive amination of α-keto acids to afford α-amino acids.[13] However, no enzyme has been reported for the reductive amination of β-keto acids, except L-*erythro*-3,5-diaminohexanoate dehydrogenase (3,5-DAHDH), which was identified to catalyze the oxidative deamination of (3*S*,5*S*)-diaminohexanoate ((3*S*,5*S*)-DAH) to 3-keto-5-aminohexanoate in the fermentation pathway of lysine.[14] All reported 3,5-DAHDHs have strict substrate specificity toward (3*S*,5*S*)-DAH and show hardly any activity toward other β-amino acids, even the other three stereoisomers of 3,5-DAH.[15] In the present study, a 3,5-DAHDH (NCBI reference sequence: YP_001741409.1) from *Candidatus* Cloacamonas acidaminovorans (3,5-DAHDHcca)[16] was expressed in *Escherichia coli* BL21(DE3) host cells as a soluble protein (Supporting Information Figure S1) and engineered to β-amino acid dehydrogenase for the reductive amination of β-keto acids.

Currently, the structures of 3,5-DAHDHs has not been reported, and their sequences showed low homogeneity of <30% with the proteins with crystal structures available in the PDB. It is difficult to obtain reliable structural modeling by a single structure template. As such, the 3D-model of 3,5-DAHDHcca was built with online server I-TASSER on the basis of multiple-threading alignments and iterative template fragment assembly simulations.[17] The C-terminus of the model structure with the highest C score in the structural modeling formed a regular secondary structure, which could structurally superimpose approximately with the C-terminus of leucine hydrogenase (1LEH, a chain),[18] but the fold constitution of the N-terminus could not be predicted with available protein structures.

Generally, NAD(P)H-dependent dehydrogenases are composed of two functional domains: a NAD(P)H-binding domain and a substrate-binding and catalytic domain.[19] 3,5-DAHDHcca showed a multidomain feature by functional assay with the Conserved Domain Database. The C-terminus of 3,5-DAHDHcca (approximately residues 180−300) possesses a typical Rossmann fold,[20] which contains a conserved sequence motif GX1-2GXXG, and residues Gly199 and Gly202 are located on the ligand-binding loop connecting a β-strand and an α-helix (Figure 1 A,B). The residues 180−300 were regarded as the NAD(P)H-binding domain of 3,5-DAHDHcca. The N-terminus and residual C-terminal portion, residues 295−352, were inferred to execute substrate binding and catalytic function.

NAD(P)H-binding and substrate-binding/catalytic portions are usually located in the core of dehydrogenases.[20d] Structurally, the first 40 amino acid residues of the N-terminus and residues 325−352 on the C-terminal α-helix of 3,5-DAHDHcca, as shown in the model structure, were topologically far away from the catalytic center; thus, they were considered to exert little impact on catalysis and substrate

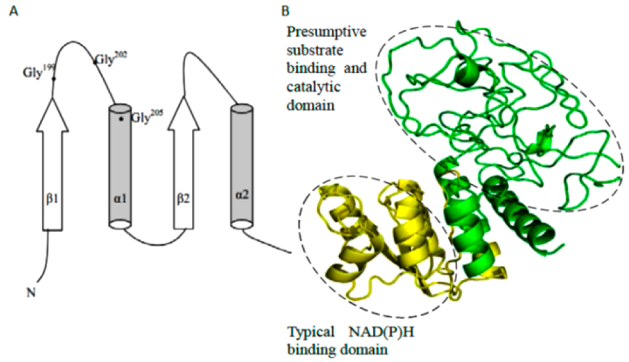

Figure 1. Simulated 3,5-DAHDHcca structure based on multiple-threading alignments with I-TASSER. (A) Diagrammatic presentation of secondary structure and sequence motifs (GX1-2GXXG) of the NAD(P)-binding Rossmann fold; the loop connecting β-strand 1 (β1) and α-helix 1 (α1) is referred to as the ligand-binding loop (cylinders represent α-helices and arrows are β-strands). (B) The 3,5-DAHDHcca modeling structure is composed of two domains: a NAD(P)H-binding domain with a Rossmann fold motif and a substrate-binding and catalytic domain with unrecognizable folds.

binding and were excluded from our experimental survey. The residues 40−180 and 295−325 were presumptively included in the substrate binding domain, and a point-by-point scanning mutation strategy was implemented with an aim to enlarge the substrate profile (Chart S1 in the Supporting Information). Eighty-five libraries were constructed, and each contained double-site mutations with an interval of three residues. This strategy was chosen because the side chains of residues *n* and *n* + 4 might be spatially close in the α-helical secondary structure and might have a mutual effect on the enzyme activity. The mutational breadth at each position was constrained with degenerate codons NDT encoding 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly), which were constituted of polar, nonpolar, charged, noncharged, aromatic, and nonaromatic side chains, to reduce the required screening efforts.

The resulting mutant libraries were screened with a mixture of substrates, including (*S*)-β-aminobutyric acid ((*S*)-β-AB), β-alanine (β-Ala), (*R*)-β-homoserine ((*R*)-β-homoSer), (*R*)-β-homomethionine ((*R*)-β-homoMet), (*S*)-β-homoglutamine ((*S*)-β-homoGln), (*R*)-β-phenylalanine ((*R*)-β-Phe), and (*S*)-β-homolysine ((*S*)-β-homoLys) using cleared cell lysates by measuring the absorbance of the produced NADPH at 340 nm. Wild-type 3,5-DAHDHcca barely exhibited any activity toward these substrates. Mutants E310G/A314Y and E310S/A314N were identified to show obvious oxidative deamination activity toward such mixed substrates. The purified mutant enzymes E310G/A314Y and E310S/A314N displayed the activity of 0.17 and 0.13 U mg^{-1} toward (*R*)-β-homoMet, which were ~200-fold that of the wild-type enzyme (Table 1). Although these mutant enzymes were active toward the β-amino acids in Table 1, they did not exhibit detectable activity toward other analogous substrates, such as β-alanine, (*S*)-β-homoglutamine, and (*R*)-β-homothreonine.

It was interesting that both mutant enzymes with activity toward these β-amino acids had mutations at Glu310 and Ala314. As such, these two amino acid residues may play an important role in determining the substrate specificity of 3,5-DAHDHcca. To explore their effects on the enzyme activity, the substitutions of residues Glu310 and Ala314 of 3,5-DAHDHcca to another 19 amino acids were performed. As

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Table 1. Oxidative Deamination Activity (mU mg^{-1}) of the Purified β-AADHs toward β-Amino Acids

	WT	E310G	E310S	E310G/A314Y	E310S/A314N
(3*S*,5*S*)-DAH	$(3.0 \pm 0.2) \times 10^{3}$	$(5.2 \pm 0.4) \times 10^{1}$	9.5 ± 0.9	$(5.1 \pm 0.7) \times 10^{1}$	$(1.8 \pm 0.1) \times 10^{1}$
(*R*)-β-homoMet	$(6.9 \pm 0.7) \times 10^{-1}$	$(6.3 \pm 0.9) \times 10^{1}$	$(5.8 \pm 0.5) \times 10^{1}$	$(1.7 \pm 0.1) \times 10^{2}$	$(1.3 \pm 0.1) \times 10^{2}$
(*R*)-β-Phe	n.m.	4.9 ± 0.1	$(3.1 \pm 0.3) \times 10^{-1}$	$(6.3 \pm 0.8) \times 10^{-1}$	$(5.1 \pm 0.4) \times 10^{-1}$
(*S*)-β-homoTyr	n.m.	$(6.9 \pm 0.2) \times 10^{-1}$	$(1.8 \pm 0.0) \times 10^{-1}$	$(1.3 \pm 0.2) \times 10^{-1}$	$(2.1 \pm 0.3) \times 10^{-1}$
(*S*)-β-AB	n.m.	$(5.4 \pm 1.5) \times 10^{-1}$	$(3.1 \pm 0.6) \times 10^{-1}$	$(5.3 \pm 0.8) \times 10^{-1}$	$(5.8 \pm 0.2) \times 10^{-1}$
(*S*)-β-homoLys	$(5.5 \pm 0.4) \times 10^{-1}$	$(7.1 \pm 0.1) \times 10^{-1}$	$(6.3 \pm 0.2) \times 10^{-1}$	1.8 ± 0.2	1.5 ± 0.3
(*R*)-β-homoSer	n.m.	$(7.9 \pm 0.1) \times 10^{-1}$	$(3.2 \pm 0.5) \times 10^{-1}$	$(6.3 \pm 0.7) \times 10^{-1}$	$(6.1 \pm 0.6) \times 10^{-1}$

n.m. = not measurable.

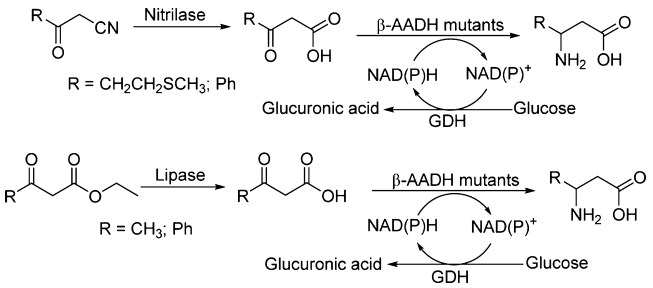
shown in Figure S2 of the Supporting Information, mutants E310G and E310S evidently manifested oxidative deamination activity toward (*R*)-β-homoMet. For the native substrate (3*S*,5*S*)-DAH, all the mutants of E310 showed much lower activity than the wild-type enzyme. On the other hand, the substitutions of Ala314 did not significantly impact the enzyme activity toward (*R*)-β-homoMet and (3*S*,5*S*)-DAH. Therefore, the replacement of residue Glu310 obviously affected the substrate profile, whereas the mutation of site Ala314 did not exert significant influence on the enzyme activity.

The wild-type 3,5-DAHDHcca showed little activity toward (*R*)-β-homoMet and (*S*)-β-homoLys, whereas mutants E310G/A314Y and E310S/A314N displayed significantly higher activity than wild-type enzyme and the single mutants E310G and E310S. The substitutions of residues Glu310 and Ala314 preferably enlarged the substrate profile. Both single and double mutations increased the activity not only toward β-amino acids with aliphatic side chains ((*S*)-β-AB, (*R*)-β-homoSer, (*R*)-β-homoMet, (*S*)-β-homoLys) but also toward those with aromatic side chains ((*R*)-β-Phe and (*S*)-β-homotyrosine ((*S*)-β-homoTyr)).

The kinetic parameters (k_{cat}, K_m) of mutants E310G, A314Y, E310G/A314Y, E310S, A314N, and E310S/A314N against (*R*)-β-homoMet, (*S*)-β-AB, and (*R*)-β-Phe were measured (Table S4 in the Supporting Information). The results showed that the mutation of A314 exerted no effect on the K_m and k_{cat} against the native substrate. For the mutation of E310 and the double mutations, the k_{cat} decreased in all the cases, and the K_m of E310G and E310G/A314Y decreased to half of the wild-type enzyme, but those of E310S and E310S/A314N increased more than 10 times. For (*R*)-β-homoMet, the wild-type and all mutant enzymes had similar K_m's but different k_{cat}'s, indicating that the higher activity of mutants E310G, E310G/A314Y, E310S, and E310S/A314N was due to their high k_{cat}. For β-amino acids (*S*)-β-AB and (*R*)-β-Phe, the activity of the mutants of A314 and wild-type enzyme was too low to measure the kinetic data. Introduction of a mutation at A314 into the mutants of E310 had some effect on their K_m and k_{cat} against these two substrates. Because the differences of K_m for the wild-type and mutant enzymes were not dramatic, the activity change was mainly a result of the k_{cat}, and residue 310 played a much more important role in determining the enzyme activity.

These mutants were also examined for the reductive amination of β-keto acids. Because β-keto acids were not readily available and not very stable in aqueous solution,[21] stable β-ketoesters ethyl benzoylacetate and ethyl 3-ketobutanoate were hydrolyzed using lipase Novozyme435 to benzoylacetic acid and 3-ketobutyric acid, or similarly, stable β-ketonitriles 5-(methylthio)-3-oxopentanenitrile and benzoylacetonitrile were hydrolyzed to 5-(methylthio)-3-oxopentanoic acid and benzoylacetic acid with nitrilase NIT6402 (Scheme 2). The

Scheme 2. Synthesis of β-Amino Acids from β-Keto Acids Catalyzed by β-AADH Mutants with the Cofactor Regeneration System of GDH and D-Glucose



reductive aminations of 5-(methylthio)-3-oxopentanoic acid, benzoylacetic acid and 3-ketobutanoic acid were carried out using mutant E310G/A314Y or E310G as the biocatalyst and D-glucose dehydrogenase (GDH) from *Bacillus subtilis* for NADPH regeneration (Scheme 2). The reaction products were derivatized with 2,4-dinitrofluorobenzene (DNFB), and the derivatives were analyzed with HPLC. Their identities were confirmed by LC−MS (Supporting Information Figures S3−8).

Compared with the control experiments (without β-AADH), (*R*)-β-homoMet, (*R*)-β-Phe, or (*S*)-β-AB was generated with β-AADH mutants as the catalyst. The products were also isolated in the preparative reactions with yields of 12−22% and confirmed by ^{1}H NMR (Figures S12−S14 in the Supporting Information). The absolute configurations of the products were determined by HPLC analysis after being derivatized with chiral reagent *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Figures S9−S10 in the Supporting Information), except β-homoMet, whose configuration was determined by comparing its optical rotation with that of the standard sample. It has been reported that (3*S*)-amino-3-phenylpropionic acid was produced from the ketocarboxylic acid ester with 20% yield by coupling a transaminase with a lipase using 3-aminobutyric acid as amino donor.[10a] These results demonstrated the reductive amination activity of the mutant enzymes toward the β-keto acids other than its native substrate.

In conclusion, an L-*erythro*-3,5-diaminohexanoate dehydrogenase from *Candidatus* Cloacamonas acidaminovorans was identified to have high activity toward (3*S*,5*S*)-DAH, but no or little activity was observed for other β-amino acids. By screening a domain scanning mutagenesis library, several mutant enzymes with mutations at E310 and A314 were obtained to display enhanced activity toward the β-amino acids other than the native substrate (3*S*,5*S*)-DAH. To overcome the instability of β-keto acids in reductive amination, 5-(methylthio)-3-oxopentanoic acid and benzoylacetic acid were generated via the hydrolysis of the corresponding β-keto nitriles by nitrilase NIT6402. The resulting β-keto acids were

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converted to (*R*)-*β*-homoMet and (*R*)-*β*-Phe via the reductive amination catalyzed by mutant E310G/A314Y or E310G. Similarly, (*S*)-*β*-AB and (*R*)-*β*-Phe were also synthesized from the corresponding *β*-keto esters by combining the hydrolysis of the *β*-keto esters with lipase Novozyme435 and the reductive amination with mutant E310G or E310G/A314Y. This is the first example that *β*-AADH catalyzed the reductive amination of aromatic and aliphatic *β*-keto acids to generate the corresponding *β*-amino acids, offering a new approach for the synthesis of valuable *β*-amino acids. The structural and molecular dynamics analysis would shed insights into the substrate-binding mechanism and how these mutations affected the substrate specificity and provide guidance for the development of new *β*-AADHs for the synthetic applications. These studies are currently performed in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/cs5017358.

Experimental procedures; primers, SDS-PAGE of DHADHcca and mutants; activity of mutant enzymes; HPLC, LC−MS, and NMR analysis

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: zhu_dm@tib.cas.cn.

Notes

The authors declare no competing financial interests.

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