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DOI
10.1021/acscatal.3c04995

Publication date
2024

Document Version
Final published version

Published in
ACS Catalysis

ACS Catalysis

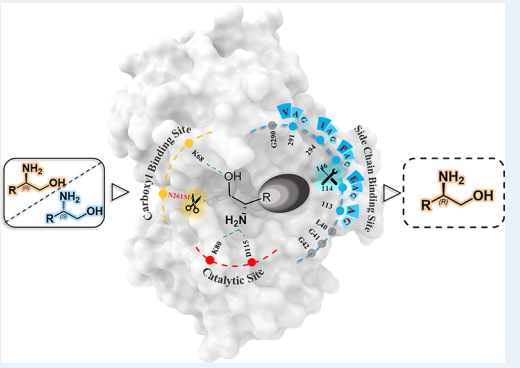
pubs.acs.org/acscatalysis
Research Article

Substrate-Specific Evolution of Amine Dehydrogenases for Accessing Structurally Diverse Enantiopure (*R*)-*β*-Amino Alcohols

Xinjian Yin, Wenzhong Gong, Yujing Zeng, Hulin Qiu, Lan Liu, Frank Hollmann,* and Bishuang Chen*

Cite This: *ACS Catal.* 2024, 14, 837−845

ABSTRACT: The biocatalytic oxidative deamination of *β*-amino alcohols holds significant practical potential in kinetic resolution and/or deracemization process to access (*R*)-*β*-amino alcohols. This study exemplifies a notable instance of acquisition and utilization of this valuable oxidative deamination activity. Initially, the mutation N261M (M0) was identified to endow a native valine dehydrogenase with oxidative deamination activity toward a few (*S*)-*β*-amino alcohols. Subsequently, a phylogenetic analysis-guided, double-code saturation mutagenesis strategy was proposed to engineer M0's side-chain binding site. This strategy facilitated the substrate-specific evolution of M0, resulting in the creation of a panel of mutants (M1−M4) with noteworthy oxidative deamination activity toward structurally diverse (*S*)-*β*-amino alcohols. Using these engineered amine dehydrogenases, termed as *β*-amino alcohol dehydrogenases (*β*-AADHs), the complete kinetic resolution and even deracemization of a range of *β*-amino alcohols have been achieved. This work reports distinct biocatalysts and a synthetic strategy for the synthesis of enantiopure (*R*)-*β*-amino alcohols and offers an innovative approach for substrate-specificity engineering of enzymes.



KEYWORDS: *biocatalysis, amine dehydrogenases, oxidative deamination, enantiopure β-amino alcohols, protein engineering*

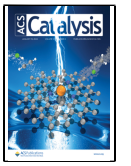
INTRODUCTION

Chiral *β*-amino alcohols are important building blocks for the synthesis of chiral auxiliaries, ligands in asymmetric synthesis, and crucial intermediates in pharmaceutical synthesis (Scheme S1).[1−6] For example, (*R*)-2-amino-3-methyl-1-butanol is a pivotal chiral intermediate in producing (*R*)-Roscovitine,[7,8] a second-generation orally available CDK inhibitor; (*R*)-2-amino-3-phenylpropanol represents a central chiral moiety in producing Solriamfetol,[9] a wakefulness-promoting medication; (*R*)-2-amino-4-methylpentan-1-ol serves as a foundational precursor for synthesizing a potent and selective antagonist of the Fractalkine receptor (CX3CR1).[10] The extensive applications of chiral *β*-amino alcohols underscore the significant importance of developing efficient synthetic methodologies for their production.

Besides some chemical synthetic routes,[11−15] biocatalytic strategies have also been receiving considerable attention[16−23] mostly due to their high stereoselectivity and the mild reaction conditions. Particularly, amine dehydrogenase-catalyzed asymmetric reductive amination[24−32] and kinetic resolution[33−36] are two attractive processes to access chiral amines. Among them, the asymmetric reductive amination of *α*-hydroxy ketones by engineered amine dehydrogenase to yield *β*-amino alcohols has been extensively explored, achieving remarkable conversion rates and enantiomeric excess values of >99%.[37−39] Almost all known amine dehydrogenases exhibiting activity toward *β*-amino alcohols are mutants of natural L-amino acid dehydrogenases, exhibiting a strict (*S*)-stereoselectivity. Hence, (*R*)-enantiomers are so far inaccessible via stereoselective reductive amination of *α*-hydroxy ketone starting materials. Evolving some natural amine dehydrogenases is a potential option to obtain (*R*)-selective amine dehydrogenases toward *β*-amino alcohols, such as *Msme*AmDH, *Mvac*AmDH, *Cfus*AmDH, *Micro*AmDH, and *Apau*AmDH (possessing opposite stereoselectivity preference compared to engineered amine dehydrogenases).[40] However, these enzymes are practically inactive on *α*-hydroxy ketones, and they suffer from poor stereoselectivity. Hence, engineering these enzymes did not appear to be an attractive route to us.

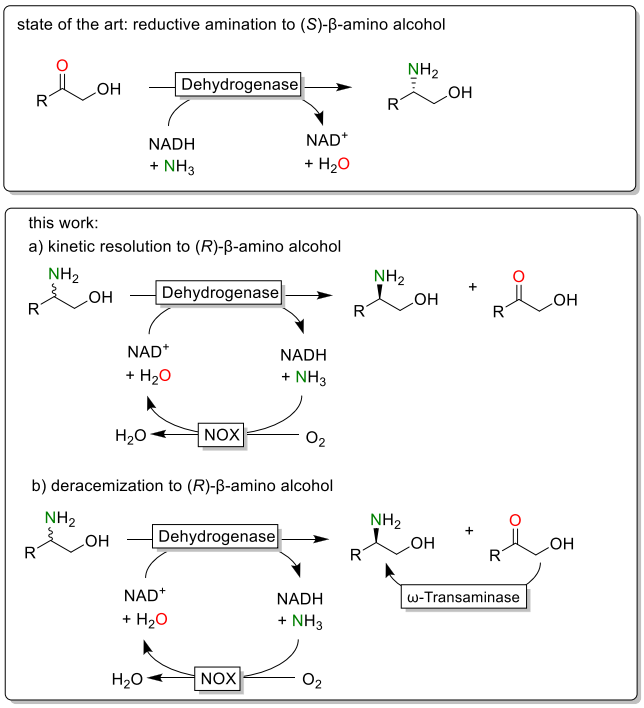
In contrast, the kinetic resolution and/or deracemization of readily available racemic *β*-amino alcohols (obtainable, e.g., through a simple one-step reduction of amino acids)[41,42] using amine dehydrogenases presents a promising alternative for the synthesis of (*R*)-*β*-amino alcohols (Scheme 1). Despite this potential, it should be noted that successful implementation

Received: October 18, 2023
Revised: December 16, 2023
Accepted: December 20, 2023
Published: January 3, 2024



https://doi.org/10.1021/acscatal.3c04995
ACS Catal. 2024, 14, 837−845

Scheme 1. Kinetic Resolution and Deracemization Process for the Synthesis of (*R*)-*β*-Amino Alcohols



faces considerable challenges. To the best of our knowledge, no successful case has been reported yet, primarily due to two significant hurdles encountered in the utilization of amine dehydrogenase-catalyzed oxidative deamination.[43,44] First, currently available (wild-type and engineered ones) amine dehydrogenases have been primarily developed for their reductive amination activity, resulting in relatively low catalytic efficiency for the oxidative deamination reaction,[31,37,39,45,46] especially concerning *β*-amino alcohols (<10 U/g).[36] Additionally, the strict substrate specificity inherited from amino acid dehydrogenases also extends to the derived amine dehydrogenases, creating a constraint on the range of substrates that can be converted.[47,48] Despite extensive research to expand the substrate spectra of amine dehydrogenases, in most cases, this remains a rather intricate issue.[24−27,30,49,50] This is because almost all mutants are screened based on one or a limited set of substrates, and their enhanced activity often lacks generality. Consequently, when faced with novel substrates, the screening process for suitable mutants remains time-consuming and labor-intensive, even for enzymes that have been extensively studied.

In this work, we report an efficacious solution to develop engineered amine dehydrogenases exhibiting remarkable oxidative deamination activity toward structurally diverse *β*-amino alcohols. These engineered amine dehydrogenases, termed as *β*-amino alcohol dehydrogenases (*β*-AADHs), have demonstrated their utility in kinetic resolution or deracemization processes, yielding diverse enantiopure (*R*)-*β*-amino alcohols with good ee values and conversion.

RESULTS AND DISCUSSION

Access to the Oxidative Deamination Activity of *β*-AADHs. As starting point for our investigation, we chose a valine dehydrogenase previously identified from a hot spring metagenomic library (HsValDH3).[51] Due to its considerable stability, HsValDH3 is an attractive starting point for evolution and future application. wt-HsValDH3 does not exhibit sizable activity toward *β*-amino alcohols, which is why we first aimed at addressing this issue. Since a crystal structure of HsValDH3 is not available yet, we employed AlfaFold2[52] to construct a three-dimensional model of HsValDH3. Subsequently, molecular docking with its native substrate, L-valine, was conducted to scrutinize the binding interactions (Figure 1A). According to the model, L-valine occupies a specific binding site nestled within the crevice formed between the substrate-binding domain and the coenzyme-binding domain of HsValDH3 (Figure 1A).

Utilizing insights from the resolved crystal structure and catalytic mechanism of amino acid dehydrogenases,[53,54] we partitioned the substrate-binding pocket of HsValDH3 into three discrete regions (Figure 1B): the catalytic site, the carboxyl-binding site, and the side-chain binding site. The catalytic site encompasses residues Lys80 and Asp115, both of which are pivotal for the catalytic function of amino acid dehydrogenases (Figure 1C).[53,54] The carboxyl-binding site is constituted by residues Asn261 and Lys68, governing the specificity for *α*-keto acids by forming hydrogen bonds and electrostatic interactions with the carboxyl group of L-valine (Figure 1C). Therefore, we targeted these two amino acid residues using a combinatorial saturation mutagenesis strategy, K68X/N261X (NNK degenerate codons). It is worth emphasizing here that, in contrast to previous studies,[28,31,37,39,45,46] we aimed at oxidative deamination, for which so far no efficient high-throughput screening method was available. Fortunately, the deamination product (i.e., the *β*-keto alcohol) proved to be susceptible to spontaneous oxidation by tetrazolium red (TTC, Figure 1D), which enabled us to establish a semiquantitative colorimetric activity assay based on the formation of the intensely red-colored triphenylformazan (TPF) (Figure S1). This way, we identified three mutants (N261M, N261L, and N261F), exhibiting oxidative deamination activity toward 2-aminobutanol. Interestingly, N261M (M0) proved to be a better mutant for oxidative reaction than the extensively documented K68S/N261L (2M), while 2M shows superiority in the reductive direction (Figure 1E). This observation reinforces the necessity of our dedicated effort in conducting oxidative deamination-specific screening.

To gain further insight into the molecular basis of these differences, we performed a molecular docking analysis of (*S*)-2-aminobutanol and 1-hydroxy-2-butanone for M0 and 2M, respectively (Figure 1F). In the case of M0, hydrogen-bonding interactions between the *α*-hydroxyl group of the amino alcohol and Lys68 were observed, which positioned the starting material in a near-attack conformation to the cofactor (NAD^+). No such interaction was observed in 2M. Rather, a new hydrogen bond network between the amino group of (*S*)-2-aminobutanol and K80 and D115 was observed, which may impede their involvement in the catalytic cycle and therefore may account for the drastically reduced catalytic activity in the oxidation of (*S*)-2-aminobutanol. Indeed, the kinetic parameters revealed that 2M exhibited a significantly lower k_{cat} value for (*S*)-2-aminobutanol compared to M0 (1.18 vs 5.23 s^{-1}) (Table 1). However, when 2M interacted with 1-hydroxy-2-butanone, the ketone group maintained a favorable catalytic distance with the catalytic residues, measuring 2.6 Å to K80 and 3.6 Å to D115. Furthermore, this binding arrangement resulted in a higher binding energy compared to M0 (−23.43 vs −21.36 kcal mol^{-1}) in line with the increased substrate affinity (lower K_m value) and catalytic efficiency (higher k_{cat} K_m^{-1}) for the *α*-hydroxy ketone (Table 1).

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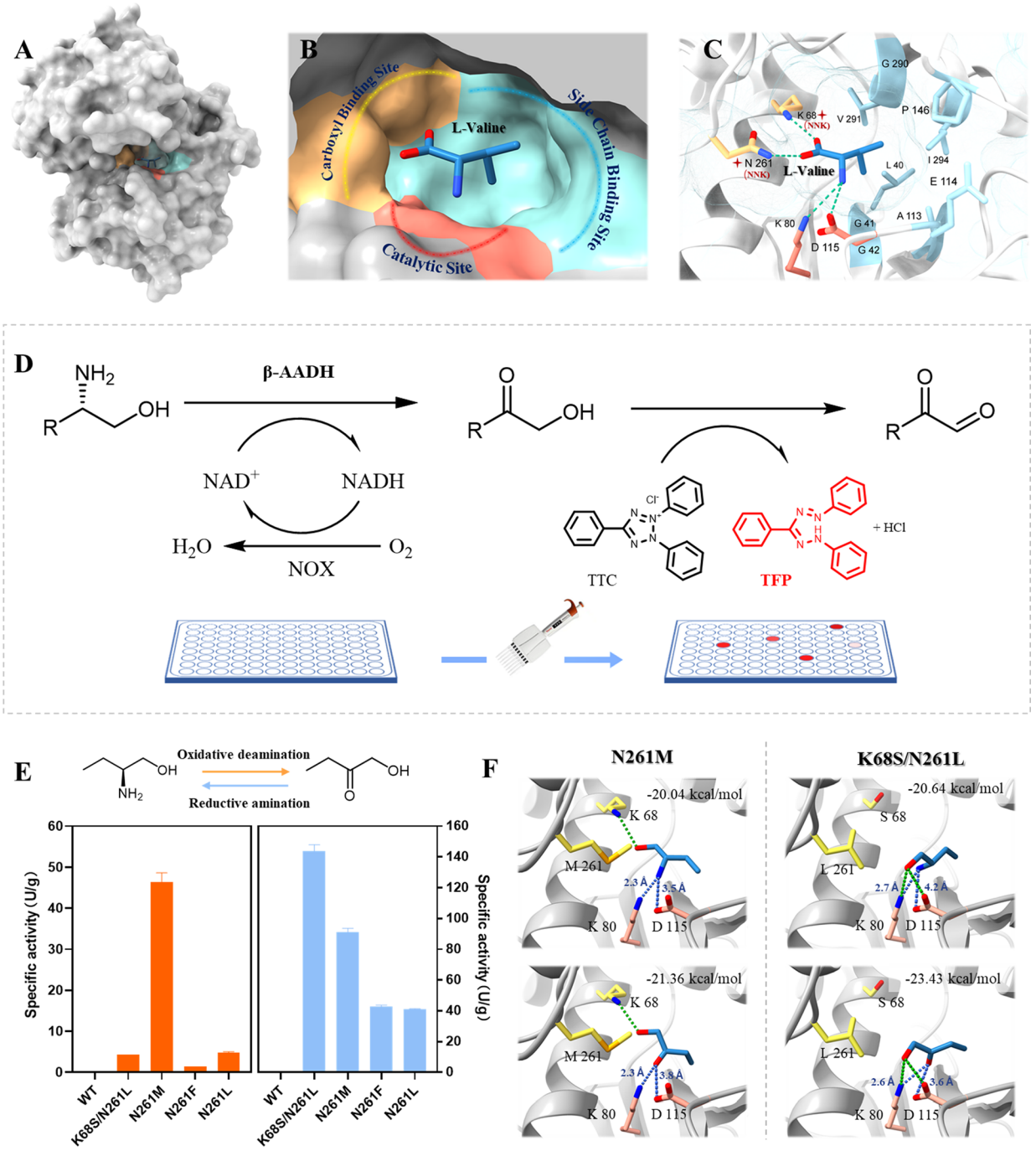
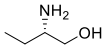
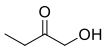


Figure 1. Access to the oxidative deamination activity of β-AADHs. (A) Overview of the three-dimensional model of HsValDH3 docked with L-valine. L-Valine is shown as a stick model. (B) Close-up view of the active pocket of HsValDH3. The substrate-binding pocket is shown as a surface model, with the catalytic site, carboxyl-binding site, and side-chain binding site structure represented in red, yellow, and cyan, respectively. (C) Residues were within the active pocket of HsValDH3. These residues constitute the catalytic site, carboxyl-binding site, and side-chain binding site, represented in red, yellow, and cyan, respectively. Hydrogen bonds that contribute to the binding of L-valine are represented as green-colored dotted lines. (D) Principle of the colorimetric high-throughput screening method for β-AADH. In the enzymatic system, β-amino alcohols undergo conversion into the corresponding α-hydroxy ketones catalyzed by β-AADH, with NAD^+ regeneration powered by the NADH oxidase (NOX). (E) Specific activities of the identified mutants toward (*S*)-2-aminobutanol and 1-hydroxy-2-butanone. The specific activity was determined using purified proteins. All enzymatic assays were performed in triplicate, at least, and the averaged values are reported. (F) Docking poses of (*S*)-2-aminobutanol and 1-hydroxy-2-butanone with N261M (M0) and K68S/N261L (2M). Hydrogen-bonding interactions are shown and highlighted as green dotted lines, and distance between substrates' active group and catalytic residues is shown and highlighted as blue dotted lines.

Substrate-Specific Evolution of M0 To Expand the Substrate Scope. The N261M mutation successfully conferred oxidative deamination activity to HsValDH3, resulting in the "first-generation" β-AADH (M0). But M0 converted only a rather narrow range of short-chain (*S*)-β-amino alcohols (Figure S2). This outcome aligns with our expectations as natural amino acid dehydrogenases exhibit stringent substrate specificity, and

https://doi.org/10.1021/acscatal.3c04995
ACS Catal. 2024, 14, 837−845

Table 1. Kinetic Parameters of the Mutants toward (*S*)-2-Aminobutanol and 1-Hydroxy-2-butanone[a]

Substrate	Enzyme	V_{max} (U/mg)	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
	N261M	1.01 ± 0.15	0.447 ± 0.032	5.23 ± 0.78	11.70
	K68S/N261L	0.23 ± 0.01	1.126 ± 0.021	1.18 ± 0.04	1.05
	N261M	0.39 ± 0.03	0.084 ± 0.003	2.02 ± 0.1	24.05
	K68S/N261L	0.26 ± 0.01	0.044 ± 0.002	1.38 ± 0.05	31.36

[a]Kinetic data were obtained using purified proteins. All enzymatic assays were performed at least in triplicate.

the resultant amine dehydrogenases typically retain similar specificity.

Introducing the N261M (HsValDH3 numbering) mutation to other amino acid dehydrogenases with larger binding pockets, such as phenylalanine dehydrogenases, offers a viable way to diversify the substrate spectrum (Figure S3). However, this approach suffers some limitations: it proves ineffective in generating active mutants for specific substrates, such as (*S*)-2-amino-2-phenylethan-1-ol, and is hardly capable of yielding mutants with enhanced catalytic activity for short-chain aliphatic substrates. Hence, we propose a phylogeny-guided, double-code saturation mutagenesis to reconfigure the side-chain binding site within M0's substrate-binding pocket. The active site pocket accommodating the alkyl substituent consists of nine residues: L40, G41, G42, A113, E114, P146, G290, V291, and I294 (Figure 1C). A multiple sequence alignment with some well-characterized amino acid dehydrogenases, particularly leucine dehydrogenase and phenylalanine dehydrogenase (Figure S4),[54−60] revealed that residues 40, 41, 42, and 290 are conserved, while residues 113, 114, 146, 291, and 294 showed notable variations (Figure 2A). To accommodate sterically more demanding alkyl substituents, targeting smaller amino acid residues appeared reasonable. Therefore, we devised a five-site, double-coded saturation mutagenesis library (Figure 2B) randomly introducing two small amino acids (alanine and glycine) to residues 113, 114, 146, 291, and 294 of M0. Notably, this strategy reduced the screening efforts significantly, as with

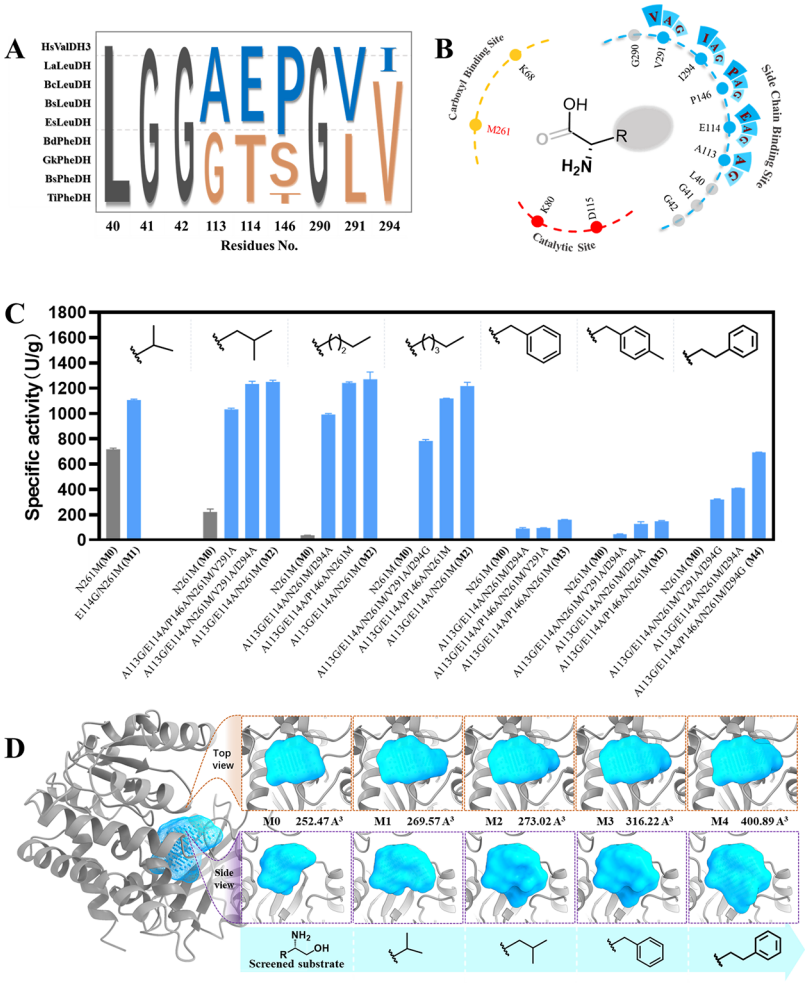


Figure 2. Evolution of the substrate scope of *β*-AADHs. (A) Phylogenetic analysis of the residues in the side-chain binding site. *La*LeuDH from *Labrenzia aggregate* (WP_006932274.1); *Bc*LeuDH from *Bacillus cereus* (WP_000171355.1); *Bs*LeuDH from *Bacillus sphaericus* (GEC82162.1); *Es*LeuDH from *Exiguobacterium sibiricum* (WP_012369820.1); *Bb*PheDH from *Bacillus badius* (BAA08816.1); *Gk*PheDHe from *Geobacillus kaustophilus* (KJE28589.1); TiPheDH from *Thermoactinomyces intermedius* (BAA00524.1); and *Bs*PheDH from *Bacillus sphaericus* (AAA22646.1). (B) Representation of the five-site double-code saturation mutagenesis library. (C) Screening results. The specific activity of the three most active mutants for each substrate was determined using purified proteins. All enzymatic assays were performed in triplicate, at least, and the averaged values are reported. (D) Active pocket structure analysis of the *β*-AADHs. The pocket volumes of the *β*-AADHs were measured using POVME 3.0.

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ACS Catal. 2024, 14, 837−845

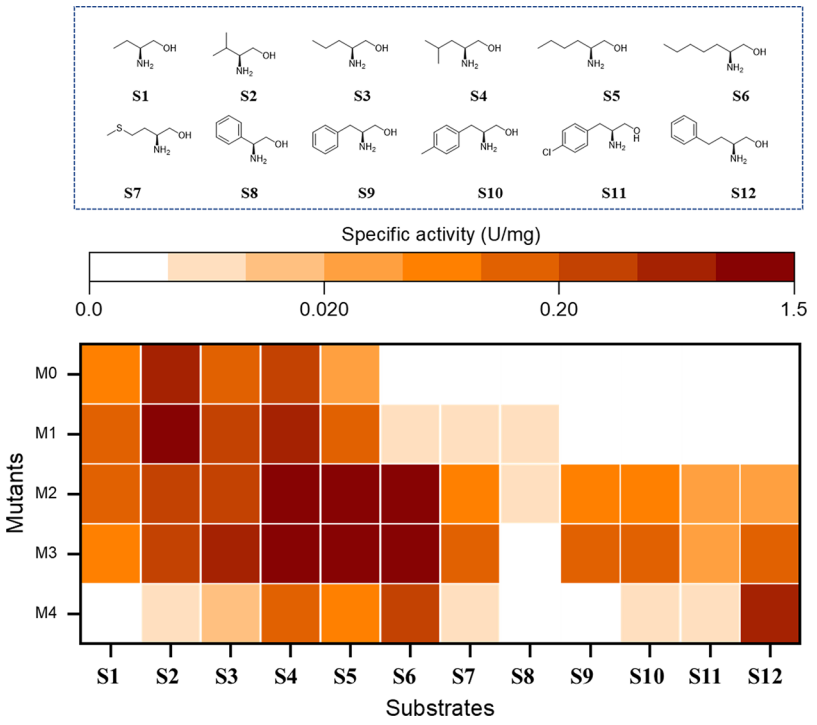

Figure 3. Substrate spectrum evaluation of the developed β-AADHs.

only 485 transformants, a 95% coverage was obtained.[61] Compared to conventional substrate-specificity engineering strategies used for amino acid dehydrogenases or amine dehydrogenases, such as rational site-directed mutagenesis[27,50,62,63] and iterative saturation mutagenesis (ISM),[29,31,64] our approach exhibits several significant benefits: first, the lower screening effort enables screening for more substrates in shorter times. Second, it allows the capture of potential synergistic effects arising from the combination of mutations at the five selected sites, an outcome that is nearly impossible to achieve by using traditional engineering strategies.

As shown in Figure 2C, for all 7 model substrates screened, several mutants with significantly increased catalytic activity were observed. The best-performing mutants for L-valinol, (*S*)-2-amino-4-methylpentane-1-ol, and (*S*)-2-aminohexane-1-ol exhibited a specific activity improvement of 1.5- to 27.6-fold compared to that of M0. Furthermore, for the four substrates initially showing no activity with M0, the generated mutants acquired substantial catalytic activity (0.15−1.22 U/mg).

Particularly mutants M1−M4 (M1: E114G/N261M; M2: A113G/E114A/N261M; M3: A113G/E114A/P146A/N261M; M4: A113G/E114A/P146A/N261M/I294G) excelled in their catalytic performance.

Next, we examined the substrate-binding pocket structure of the best mutants using POVME 3.0.[65] A compelling correlation between the active pocket size and substrate specificity (Figure 2D) was observed. As the side chains of the substrates used for screening increased, the pocket size of the best mutants obtained also gradually increased, indicating a notable positive correlation. It serves to validate the effectiveness and efficacy of our phylogenetic analysis-guided double-code saturation mutagenesis strategy in obtaining individual optimal mutants for each substrate.

Applications of Developed β-AADHs. To assess the applicability of the developed β-AADHs, we conducted a systematic evaluation of their substrate spectra. As illustrated in Figure 3, the "second-generation" β-AADHs (M1−M4) exhibited a remarkably expanded substrate scope compared to M0. Catalytic activity was observed for essentially all substrates tested.

Next, we explored the synthetic usefulness of newly generated β-AADHs. Six representative, racemic β-amino alcohol substrates (S1, S2, S3, S4, S9, and S12) were tested first in a kinetic resolution-type reaction using the previously identified optimal β-AADH mutant (Figure 3). For cofactor regeneration, the recombinant NADH oxidase (NOX) from *Streptococcus equi* was used.[66] As shown in Figure 4A, the majority of these model compounds were smoothly oxidized in high enantioselectivity, resulting in 50% conversion (i.e., full theoretical) and essentially optically pure (*R*)-β-amino alcohols. In two cases, even after prolonged reaction times, the conversions did not exceed 49%, and consequently, the optical purities of the remaining amino alcohols were disappointingly low (92−96% ee). The origin of this slower reaction rate is not fully understood and will be investigated in future studies. Possibly, low affinity toward the starting material (high K_m values), product inhibition, and/or enzyme inactivation may account for this.

Inspired by previous work regarding deracemization of racemic amines without α-functionalization,[33] we also evaluated the deracemization of the racemic β-amino alcohols by extending the reaction shown in Figure 4A with an enantioselective transamination of the undesired keto alcohol byproduct (Figure 4B). For this, we chose the (*R*)-selective ω-transaminase from *Bacillus megaterium* (*Bm*TA) (Table S1).[67] The stereoselectivity of available (*R*)-selective ω-transaminases is generally low.[19,68,69] This implies that the amino alcohols produced by these transaminases will not be enantiomerically pure. However, the proposed three-enzyme cascade deracemization system effectively addresses this issue as undesired (*S*)-β-amino alcohols are transferred back to α-hydroxy ketones by the engineered β-AADHs. Consequently, only the (*R*)-product is preserved in the system, theoretically giving excellent optical

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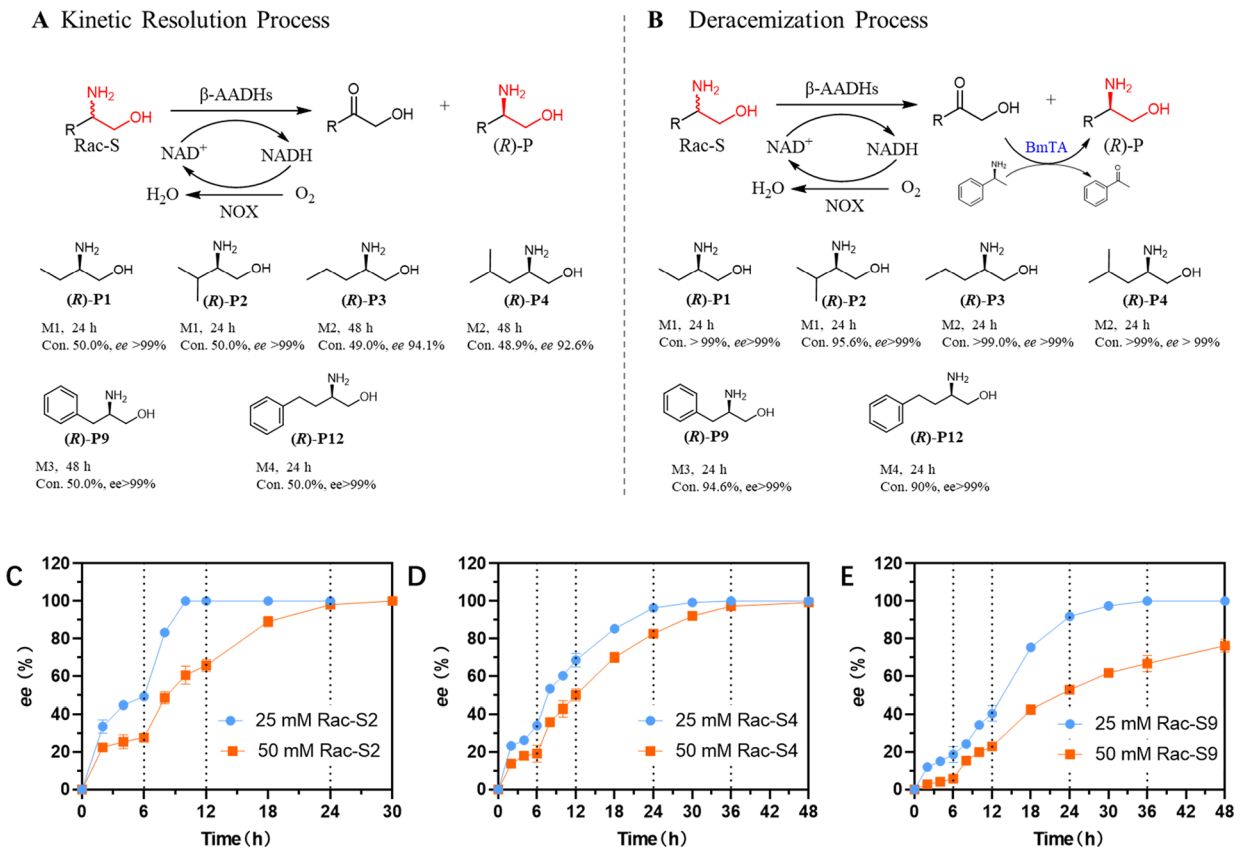

Figure 4. Kinetic resolution and deracemization process for the synthesis of (*R*)-*β*-amino alcohols. (A) Kinetic resolution of racemic *β*-amino alcohols. Reaction conditions: *β*-AADHs (2 mg/mL purified protein), NOX (2 mg/mL purified protein), NAD^+ (0.5 mM), racemic *β*-amino alcohols (10 mM), pH 9.0 Tris−HCl (0.1 M), 2 mL of total volume. The reaction was performed at 37 °C and 200 rpm for 24 or 48 h. Conversion for kinetic resolution process was defined as the percentage ratio of consumed (*S*)-*β*-amino alcohols to the initial racemic *β*-amino alcohol substrate. (B) Deracemization of racemic *β*-amino alcohols (10 mM). Reaction conditions: *β*-AADHs (2 mg/mL purified protein), NOX (2 mg/mL purified protein), *Bm*TA (2 mg/mL for S1, S2, S3, S4; 10 mg/mL for S9 and S12, purified protein), NAD^+ (0.5 mM), PLP (0.5 mM), racemic *β*-amino alcohols (10 mM), (*S*)-phenethylamine (50 mM), pH 9.0 Tris−HCl (0.1 M), 2 mL of total volume. The reaction was performed at 37 °C, 200 rpm for 24 or 48 h. Conversion for the deracemization process was defined as the percentage ratio of the (*R*)-*β*-amino alcohol product to the initial racemic *β*-amino alcohol substrate. (C−E) Deracemization of racemic S2, S4, and S9 (25 or 50 mM). Reaction conditions: NAD^+ (0.5 mM), PLP (0.5 mM), racemic *β*-amino alcohols (25 or 50 mM), and (*S*)-phenethylamine (100 mM), pH 9.0 Tris−HCl (0.1 M), 2 mL of total volume. The enzymes [1 mg/mL *β*-AADHs (M1 for S2, M2 for S4, and M3 for S9), 2 mg/mL NOX, and 2 mg/mL *Bm*TA] were added into the reaction system at specific time points: 0, 6, 12, 24, and 36 h. The reaction was performed at 37 °C, 200 rpm.

purity for various *β*-amino alcohol products. Gratifyingly, all six racemic *β*-amino alcohols were efficiently deracemized, resulting in the production of (*R*)-*β*-amino alcohols with ee values >99% and conversions ranging from 90 to 99%. Even for substrates S3 and S4, which exhibited imperfect ee values in the kinetic resolution process, the deracemization process yielded satisfactory results.

Motivated by these results, we proceeded to explore the deracemization process with higher substrate loadings (25−50 mM) (Figure 4C−E). To surmount enzyme deactivation issues observed in prior batch reactions (Figure S5), particularly concerning NOX and *Bm*TA, we added these enzymes at intervals (0, 6, 12, 24, and 36 h). Applying this strategy, complete deracemization of 25 mM racemic S2, S4, and S9 substrates was achieved within 10, 30, and 36 h, respectively, with optical purities surpassing 99% ee. Upon further increasing the substrate concentration to 50 mM, S2 and S4 achieved complete deracemization within 30 and 48 h (ee > 99%), respectively, while the final ee value for S9 stood at 76%.

Preparative-scale deracemization reactions were performed on a 50 mL scale with 50 mM racemic S2 and S4. These reactions mirror the process observed in the 2 mL-scale reactions (Figure S6). The deracemization of racemic S2 and S4 reached completion (>99% conversion) within 34 and 48 h (ee > 99%), respectively, yielding a final concentration of (*R*)-R2 and (*R*)-R4.

Compared with extensively explored and optimized amine dehydrogenase-catalyzed reductive amination processes for (*S*)-*β*-amino alcohol production,[37−39] the productivity observed here (2.1 mM h^{-1}) somewhat falls back (up to 100 mM h^{-1}) albeit also using approximately 20−100 times less of the biocatalyst. Hence, we are convinced that further reaction engineering will yield reaction schemes at least comparable to those of the above-mentioned (*S*)-amino alcohol syntheses.

CONCLUSIONS

In this contribution, we have expanded the biocatalytic toolbox for chiral *β*-amino alcohol synthesis with a newly designed amine dehydrogenase from a wild-type valine dehydrogenase. A mutation N261M in the carboxyl-binding site was identified to endow HsValDH3 with oxidative deamination activity, creating the "first-generation" *β*-AADH (M0). Guided by phylogenetic

https://doi.org/10.1021/acscatal.3c04995
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analysis, double-code saturation mutagenesis in M0's substrate-binding pocket led to the "second-generation" β-AADHs (M1−M4) with broadened substrate spectra.

Also, the newly developed β-AADHs themselves exhibit a significant potential for the preparative kinetic resolution and deracemization of readily available racemic β-amino alcohols.

Further work in our laboratory will focus on the further optimization of the methodology, generating even more active and selective β-AADHs and embedding the deracemization in more complex cascades to convert simple starting materials into diverse value-added products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.3c04995.

Detailed description of experimental procedures; analytic conditions; primer sequences; enzyme sequences; GC conditions; HPLC conditions; colorimetric high-throughput screening results; specific activities of amino acid dehydrogenases; Michaelis−Menten diagrams; pre-column derivatization HPLC analysis; GC analysis; sequence information; and supporting HPLC, NMR, and MS spectra (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (Nos. 22108317 and 42376097); Guangdong Basic and Applied Basic Research Foundation (2019A1515111051, 2021A1515010829, and 2023A1515030226); and China Postdoctoral Science Foundation (2020M680134).

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