Asymmetric Amine Synthesis

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Development of an Amine Dehydrogenase for Synthesis of Chiral Amines**

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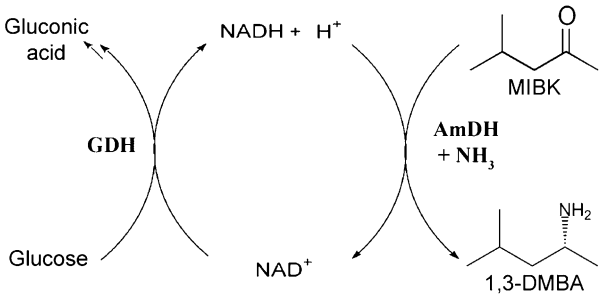
Biocatalysts are increasingly prevalent in the large-scale synthesis of enantiomerically pure compounds (EPCs), which are mainly used as active pharmaceutical ingredients (APIs). Enantiomerically pure forms can lead to lower dosages, improved efficacy, and even allow for extension of patents.[1] In 2000, 35% of APIs were chiral compounds, and this number was expected to increase to 70% by 2010.[2] However, many sought-after reactions lack a suitable enzymatic production route. A recent assessment by the ACS Green Chemistry Institute, Pharmaceutical Roundtable noted that the asymmetric synthesis of amines from prochiral ketones and free ammonia was one of the top aspirational reactions challenging the pharmaceutical industry.[3]

Our novel enzyme achieves exactly that aim: it creates amines with high selectivity. Previously characterized amine dehydrogenases were incapable of effecting the reductive amination of ketones and lacked stereospecificity.[4]

While chiral amines can be produced both chemically and enzymatically, the large-scale production of chiral amines is still challenging and heavily reliant on traditional methods of chemical synthesis.[1,5,6] Common methods include resolution though fractional crystallization[7] and the hydrogenation of C=N bonds, particularly in enamines.[8] Nonetheless, some chemoenzymatic routes, particularly with transaminases,[9] have shown promise in the dynamic kinetic resolution of racemic amines[10] and the direct asymmetric synthesis of amines with ω-transaminases (ω-TA), as used in the synthesis of sitagliptin.[11,12] This novel process has eliminated the use and removal of a less-selective rhodium catalyst, yet requires the use of a sacrificial amine source. The undesired ketone by-product must also be removed to shift the reaction equilibrium beyond about 50% conversion.

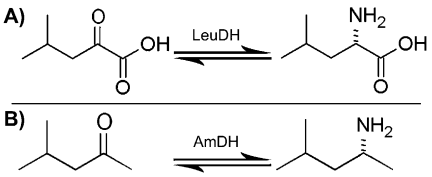
Asymmetric synthesis by amine dehydrogenases (AmDHs) would be the ideal route to produce chiral amines. When paired with a cofactor recycling system, such as glucose/glucose dehydrogenase (GDH) or formate/formate dehydrogenase (FDH), amine dehydrogenases allow for the direct production of chiral amines, with the consumption of only an inexpensive reducing agent, such as glucose or formate, and free ammonia (Scheme 1).[5b,13]



Scheme 1. Asymmetric synthesis reaction scheme with an amine dehydrogenase paired with a glucose dehydrogenase cofactor recycling system.

By using an existing amino acid dehydrogenase scaffold, we have successfully altered the substrate specificity through several rounds of protein engineering to create an amine dehydrogenase. Instead of the wild-type α-keto acid, the amine dehydrogenase now accepts the analogous ketone, methyl isobutyl ketone (MIBK), which corresponds to removal of the carboxyl moiety (Scheme 2). The wild-type leucine dehydrogenase exhibited no measurable activity toward the reductive amination of MIBK.

Leucine dehydrogenase from *Bacillus stereothermophilus* served as the initial protein scaffold. The only crystal structure currently available for leucine dehydrogenase (LeuDH) is an apo crystal structure from *Bacillus sphaericus* (PDB:



Scheme 2. A) Wild-type leucine dehydrogenase reaction. B) Novel amine dehydrogenase reaction.

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1LEH).[14] The determination of substrate–binding pocket interactions was instead based upon the holo crystal structures of an analogous protein, phenylalanine dehydrogenase (PheDH) from *Rhodococcus sp.* M4 (PDB: 1C1D and 1BW9).[15] LeuDH and PheDH have a nearly identical secondary structure, with a backbone RMSD of only 0.234 Å, thus allowing for reasonable estimation of the interactions of the substrate with the binding pocket. By using these structures in conjunction with knowledge of the reaction mechanism, amino acid residues surrounding the L-phenylalanine substrate binding pocket were identified (Figure 1). As previously discovered by Sekimoto et al., residues Lys80 and Asp115 are essential to the catalytic

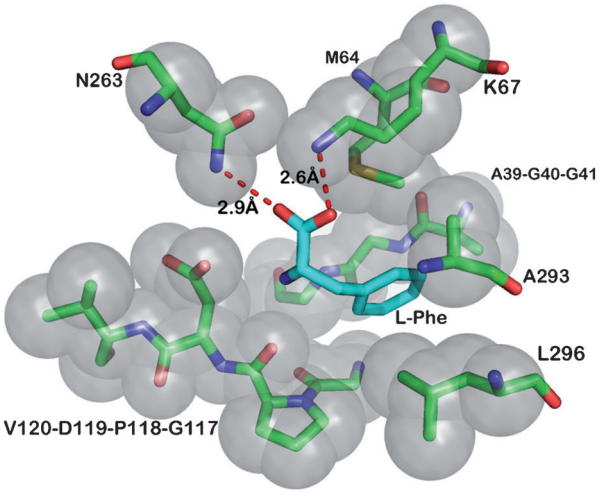

Figure 1. PheDH active site with bound L-phenylalanine and surrounding residues suitable for mutation (PDB: 1C1D).[15]

mechanism of the enzyme.[16] These residues were excluded from our mutation libraries so as to conserve activity. Conversely, residue Lys68 of LeuDH (Figure 1, PheDH: K67) directly interacts with the carbonyl moiety of the amino acid substrate through favorable charge interactions, and was chosen as the initial point of mutation. Library 1 included mutation to each of the remaining 19 amino acids. The resulting variants were cloned into pET28a vector and BL21(DE3) competent cells for subsequent expression and His-tag purification. Each purified variant was analyzed spectrophotometrically at 340 nm to correlate the NADH/NAD^+ cofactor conversion with the amination and deamination activity. This library yielded a single beneficial mutation, Lys68Met, that exhibited low, yet unprecedented activity for reductive amination (0.2 $mU\,mg^{-1}$). A higher activity of 3.4 $mU\,mg^{-1}$ was observed in the oxidative deamination of racemic 1,3-dimethylbutylamine (1,3-DMBA).

The remaining binding pocket residues were broken into groups on the basis of CASTing principles to create libraries 2, 3, 4, and 6 (Table 1).[17] Residues within each group were mutated simultaneously to capture any synergistic effects of neighboring residues. The mutational breadth at each position was constrained through degenerate codons to

Table 1: Grouping of active-site residues in LeuDH.

Library	PheDH	LeuDH with degenerate codons	Library size[a]
1	K67	K68[b]	20
2	M64	M65 NNK	3066
	K67	K68 NNK	
3	G117	A113 GBG	3450
	P118	E114 NNT	
	D119	D115 RAB	
	V120	V116 RTK	
4	A39	L40 DBW	2910
	G40	G41 GBC	
	G41	G42 DBW	
5	S190	A187 NNK	94
6	A293	V291 DBS	969
	L296	V294 DBS	
7	G117	A113 NNK	94
8	I293	I292 NNK	94
9	E300	E297 NNK	94
10	N263	N261 NNK	94
11	K67	K68 DDK	969
	N263	N261 DDK	

[a] Required for 95% confidence of entire library coverage. [b] Analyzed as individual mutants using purified protein.

avoid amino acids likely detrimental to substrate binding (such as clear steric hindrance or unfavorable charge interactions). Removing a subset of detrimental amino acids limits the library size, so as to improve screening efficiency, while maintaining an increased chance of altering the substrate specificity.

Single residue libraries (5, 7, 8, and 9) were broadly mutated to all 20 amino acids (codon: NNK), because of their small size and the insignificant decrease in the required screening effort, by applying a more restrictive degenerate codon. These residues were previously identified as influential in changing the substrate specificity of *Bacillus sphaericus* PheDH.[18]

Library 10 included mutation of binding pocket residue N261 (Figure 1, PheDH: N263). This position was not initially considered for mutation, despite its interaction with the carboxy moiety of the wild-type ligand, because of its involvement in binding the cofactor. However, as only the backbone amino group of the residue interacts with the cofactor, an exception was made for mutation at this position. Lastly, residues K68 and N261 were mutated simultaneously to create library 11 because of their proximity in the binding pocket and their comparable interactions with the carboxy moiety of the wild-type substrate.

Individual colonies from libraries 2–11 were selected and expressed in 96-well plates. A high-throughput absorbance-based assay was ultimately determined as the simplest and most accurate method. The initial, low level of amination activity is undetectable with the high-throughput assay because of the high background absorbance in the presence of cell lysate. Therefore, the enzymatic activity was measured in the more-active deamination direction, which gives a better signal to noise ratio and less false positives.

Absorbance measurements were taken at two wavelengths, 340 and 600 nm, to determine the substrate conversion and biomass loading, respectively. The increasing

absorbance at 340 nm was correlated to the reduction of the NAD^+ cofactor, and can be related stoichiometrically to substrate conversion. The absorbance at 600 nm accounted for differences in the biomass loading and was used, accordingly, to normalize the absorption at 340 nm. Successful variants were identified on the basis of their normalized 340 nm absorbance, relative to the control plate that lacks any substrate.[19] This ratiometric procedure eliminated false positives arising from the aggregation of cell debris and decreased experimental error.

The resulting hits were purified and analyzed spectrophotometrically for amination and deamination activity. The best variant was then used as the basis for subsequent rounds of mutation.

Early rounds of mutation (libraries 1–9) identified either the wild-type sequence or single variants which improved simultaneously the activity in both the amination and deamination directions, thus allowing for the straightforward selection of the top variant. Library 10 identified distinct mutations for the most-active amination and deamination variants. The most active amination and deamination mutations were with position Asn261 exchanged by Cys and Val. Library 11 further improved the amination activity by identifying synergistic mutations at positions 68 and 261, with these positions mutated from those in libraries 1 and 10. The final amine dehydrogenase contained four mutations, which are summarized in Table 2.

The most-active variants showed amination and deamination activity toward a number of ketones and amines, respectively (Table 3). The final variant (K68S/E114V/N261L/V291C) showed enhanced amination activity over the previous library 10 mutant toward all the ketones investigated, except methyl acetoacetate.

The enantioselectivity was initially estimated by measuring the deamination activity toward individual enantiomers of methylbenzylamine (MBA). MBA was used in place of 1,3-DMBA for selectivity experiments since it had a reasonable level of activity and individual enantiomers of MBA were commercially available. A preference towards (*R*)-MBA over the corresponding *S* enantiomer was evident from the deamination activities of 0.586 U mg^{-1} and 0.002 U mg^{-1}, respectively. These preliminary results were corroborated through direct measurement of the enantioselectivity of the MIBK amination in producing chiral 1,3-DMBA. Enzymatic conversion was allowed to continue for a total of 48 h with a GDH-cofactor recycling system, and gave a ketone substrate conversion of (92.5 ± 2.6) %. The resulting amine product was extracted from the aqueous reaction phase using toluene, subsequently derivatized using trifluoroacetic anhydride (TFAA), and analyzed by gas chromatography. The derivatization of 1,3-DMBA allowed adequate baseline separation of the peaks, with elution times of 47.5 and 48.9 min for the *S* and *R* enantiomers, respectively. The enantiomer peaks were identified through polarimetry since pure enantiomer standards were not available.[19] The resulting data gave an *ee* value of 99.8 % toward (*R*)-1,3-DMBA, which corresponds to the wild-type *S* selectivity (change of Cahn–Ingold–Prelog preference upon replacement of the carboxy group by a methyl group). The amine product was extracted from an 175 mL-scale reaction using methyl *tert*-butyl ether and concentrated through rotovap distillation. 29.5 mg of (*R*)-1,3-DMBA at 84.7 % conversion resulted, as confirmed by the ^{1}H NMR spectrum and chiral gas chromatography.[19]

In conclusion, we have successfully developed an amine dehydrogenase, by starting with an existing amino acid dehydrogenase as a template and subsequent active-site-targeted protein engineering. Eleven rounds of protein engineering completely inverted the enzyme's specificity and created amination activity of 0.69 U mg^{-1} with a corresponding k_{cat} value of 0.46 s^{-1}. In doing so, the native activity toward L-Leu was reduced to immeasurably low levels. The enantioselectivity of the wild-type enzyme was maintained,

Table 3: Substrate profiles of top amination and deamination variants.

Substrate[a]	K68M/E114V/N261V/V291C[b]		K68S/E114V/N261L/V291C[b]	
(*R*)-MBA	476.5	±1.4	586.3	±4.1
(*S*)-MBA	5.0	±0.0	1.6	±0.0
(*R*/*S*)-MBA[c]	484.0	±3.5	784.6	±13.4
cyclohexylamine	–		56.0	±0.0
cyclohexanone	18.8	±0.0	123.4	±3.5
ethyl pyruvate	19.8	±7.0	13.2	±5.8
methyl acetoacetate	4.5	±0.7	4.5	±0.6
ethyl-3-oxohexanoate	6.4	±4.9	14.0	±1.2
acetophenone	3.5	±0.7	58.8	±1.7

[a] Activity measured with 20 mM substrate. [b] Specific activity in mU mg^{-1} protein, with the error representing one standard deviation. [c] Activity measured with 40 mM racemic MBA, 20 mM of each enantiomer.

Table 2: Accumulated mutations and resulting improvements to the specific activity and K_M value of leucine dehydrogenase.[a]

Library	Mutations	Reductive amination specific activity[b]		K_M[c]		Oxidative deamination specific activity[b]		K_M[c]	
wild-type	–	n.m.[d]		n.m.		n.m.		n.m.	
1	K68M	0.0002		n.m.		0.0034		n.m.	
3	K68M, E114V	0.015		n.m.		0.65		48	
6	K68M, E114V, V291C	0.016	±0.004	70.2	±35	1.02	±0.41	77.1	±66
10[e]	**K68M, E114V, N261V, V291C**	0.089	±0.007	10.3	±3.6	**2.81**	**±0.44**	**30.9**	**±13**
	K68M, E114V, N261C, V291C	0.236	±0.04	21.6	±12	1.76	±0.20	14.8	±6.0
11	**K68S, E114V, N261I, V291C**	**0.690**	**±0.07**	**15.1**	**±5.1**	2.64	±0.28	57.5	±12.5

[a] Error values represent 95 % confidence intervals of nonlinear fit parameters. [b] Maximum specific activity (units/mg protein). [c] MIBK or 1,3-DMBA substrate (mM). [d] n.m. = not measurable (< 0.1 mU mg^{-1}). [e] Separate variants gave maximum amination and deamination activity.

and yielded (*R*)-1,3-DMBA with an *ee* value of 99.8% at 92.5% conversion. This amine dehydrogenase exhibited activity toward a number of different substrates. This is the first example of a cofactor-dependent amine dehydrogenase capable of selectively synthesizing chiral amines from a prochiral ketone and free ammonia.

Experimental Section

Leucine dehydrogenase (E.C. 1.4.1.9) from *Bacillus stereothermophilus* was donated by Assistant Professor Bert C. Lampson from East Tennessee State University, followed by isolation of the genomic DNA through application of method B described by Mehling et al.[20] Mutant libraries were created using overlap PCR (Molecular Cloning, 2000, Sambrook) and custom oligoprimers (MWG Operon). For initial high-throughput screening, mutant proteins were expressed in pET17b vector (Invitrogen) and BL21(DE3) competent cells (Invitrogen). Colonies were selected using a Genetix QPix2 colony picker. Well plates were assayed by using methods described in the Supporting Information. For purification, proteins were expressed in pET28a vector (Invitrogen) in BL21(DE3) with a C-terminal His-tag. All proteins were expressed in MagicMedia (Invitrogen) at 37 °C for 24 h.

His-tagged proteins were purified on Ni-NTA column materials (Thermo Scientific). Cell pellets (50 mL liquid culture) were resuspended in 50 mM phosphate buffer (6 mL) at pH 8 containing 20 mM imidazole and 300 mM NaCl. This was followed by sonication and centrifugation to remove cell debris. Clarified cell lysate was bound to the resin on ice for 1 h, then purified by column chromatography. Protein-bound resin was washed twice with 50 mM phosphate buffer (5 mL) at pH 8 containing 50 mM imidazole and 300 mM NaCl. Purified protein was eluted with the same buffer solution but using 250 mM imidazole.

Deamination reactions were performed in 250 mM Na_2CO_3 buffer at pH 10, 1 mM NAD^+, and various concentrations of amines. Amination reactions were performed in 0.5 M NH_4Cl/NH_4OH buffer at pH 9.6, 200 μM NADH, and various concentrations of ketones. The overall conversion of the amination was measured in 1.6 mL reaction volumes (1.5 mL of 0.5 M NH_4Cl/NH_4OH buffer at pH 9.6, 213 μM NADH, 10.67 mM MIBK substrate, 12.8 mM glucose, and 16 activity units of glucose dehydrogenase (85 U mg^{-1}) and 100 μL AmDH K68S/E114V/N261L/V291C at a concentration of 1.3 mg mL^{-1}). The reactions were incubated for 24 h, after which another 100 μL of enzyme was added for the remaining 24 h. Reactions were stopped by the addition of 10 N NaOH (250 μL), and then extracted with toluene and derivatized with TFAA.

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