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The Evolution of an Amine Dehydrogenase Biocatalyst for the Asymmetric Production of Chiral Amines

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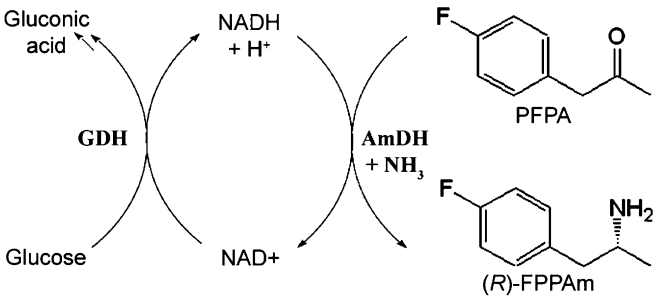
Abstract: The reductive amination of ketones to produce chiral amines is an important transformation in the production of pharmaceutical intermediates. Therefore, industrially applicable enzymatic methods that enable the selective synthesis of chiral amines could be very useful. Using a phenylalanine dehydrogenase scaffold devoid of amine dehydrogenase activity, a robust amine dehydrogenase has been evolved with a single two-site library allowing for the direct production of (*R*)-1-(4-fluorophenyl)-propyl-2-amine from *para*-fluorophenylacetone with a k_{cat} value of 6.85 s^{-1} and a K_M value of 7.75 mM for the ketone substrate. This is the first example of a highly active amine dehydrogenase capable of accepting aliphatic and benzylic ketone substrates. The stereoselectivity of the evolved amine dehydrogenase was very high (>99.8% *ee*) showing that high selectivity of the wild-type phenylalanine dehydrogenase was conserved in the evolution process. When paired with glucose/glucose dehydrogenase, NADH cofactor can be effficiently regenerated and the reaction driven to over 93% conversion. The broad specificity, high selectivity, and near complete conversion render this amine dehydrogenase an attractive target for further evolution toward pharmaceutical compounds and subsequent application.

Keywords: amine dehydrogenase; asymmetric catalysis; biocatalysis; chiral amines; directed evolution

Introduction

One of the shortcomings of biocatalysis is the limited number of reactions which have identified enzymatic routes. As the field of protein engineering grows, the characterization of novel biocatalysts expands the repertoire of accessible reactions.[1–4] A prominent example of this novel application is the recent evolution of an ω-transaminase to produce sitagliptin.[5,6] Biocatalysis is emerging as an essential tool in the asymmetric synthesis of chiral intermediates, which play a particularly important role as building blocks for the pharmaceutical industry. Therapeutic compounds often act as structurally optimized inhibitors of biological processes, and since the human body functions using chiral chemistry, these compounds almost always contain chiral centers. Enantiomerically pure forms of active pharmaceutical ingredients (APIs) can lead to lower dosages, increased efficacy, and elimination of detrimental side-effects.[7] In 2006, 80% of small-molecule drugs approved by the FDA were chiral and 75% were single enantiomers.[8] The production of chiral amines is of particular importance due to their highly active and diverse influence on biological functions.[3–5,7,9] Amine-based pharmaceuticals are used in a wide range of functions including; stimulants[10], decongestants[11], vasoconstrictors[12], and antidepressants.[13]

The production of chiral amines remains difficult through traditional chemical catalysis,[3,14] and the direct amination of ketones with free ammonia to produce chiral amines has been identified as one of the most aspirational reactions challenging the pharmaceutical industry by the American Chemical Society's Green Chemistry Institute, Pharmaceutical Roundtable.[15] Recently, we have demonstrated this reaction by an amine dehydrogenase (AmDH) that was obtained through the modification of a leucine dehydrogenase (LeuDH) scaffold to accept ketone substrates[1]. This biocatalytic route of production



Scheme 1. The asymmetric amination of *para*-fluorophenylacetone to (*R*)-1-(4-fluorophenyl)-propyl-2-amine by a PheDH-based amine dehydrogenase.

offers several advantages; elimination of heavy metals, high selectivity (>99.8% *ee*) and conversion (>92%).

This work herein reports the development of a second amine dehydrogenase catalyst based on the scaffold of phenylalanine dehydrogenase (PheDH, EC 1.4.1.20).[16] This PheDH-based AmDH (PheDH-AmDH) is capable of aminating *para*-fluorophenylacetone to (*R*)-1-(4-fluorophenyl)-propyl-2-amine (Scheme 1), and offers several advantages over the LeuDH-based AmDH (LeuDH-AmDH) including markedly increased specific activity and broader substrate scope which includes amination of aromatic ketones.

Results and Discussion

Transposition of LeuDH-AmDH Mutations

The original AmDH scaffold, LeuDH from *Bacillus stearothermophilus,* shares a reasonable sequence similarity to PheDH from *Bacillus badius* (48% identity, 66% similarity). These proteins are even more closely related in their folded structure, sharing a backbone RMSD of only 1.69 Å.[17] The initial strategy to develop PheDH-AmDH activity was to evaluate each of the influential mutations identified in LeuDH-AmDH (K67M, E113V, and N261V)[1], individually and in combination, on the PheDH scaffold to directly create AmDH activity (Table 1). The analogous substitutions in PheDH were identified by sequence alignment. The PheDH double variant K77M/N276V was the best combination of the known LeuDH mutations. Both of these residues interact with the wild-type substrate at the carboxyl moiety, and their synergistic effects are reflected in the large increase in AmDH activity of the double variant on methyl isobutyl ketone (MIBK) compared to the respective single variants (Figure 1).

The K77M/N276V double variant was further characterized for amination of *p*-fluorophenylacetone

Table 1. AmDH activity on 1,3-dimethyl butyl amine (1,3-DMBA) and methyl isobutyl ketone (MIBK) by PheDH variants derived from analogous LeuDH-AmDH mutations.

LeuDH Variant	PheDH Variant	Deamination Activity[a] $k_{cat,app}$ [s^{-1}]	Amination Activity[b] $k_{cat,app}$ [s^{-1}]
wild-type	wild-type	n.m.[c]	n.m.
K67M	K77M	0.0033 ± 0.0000	0.0009 ± 0.0000
E113V	T123V	0.0013 ± 0.0000	n.m.
N261V	N276V	0.0016 ± 0.0000	n.m.
K67M	K77M	0.0010 ± 0.0000	n.m.
E113V	T123V		
K67M	**K77M**	**0.0722 ± 0.0040**	**0.0037 ± 0.0000**
N261V	**N276V**		
K67M	K77M	0.0011 ± 0.0000	n.m.
E113V	T123V		
N261V	N276V		

[a] Deamination: 0.1 M glycine buffer pH 10.0, 1 mM NAD^+, 20 mM 1,3-DMBA.
[b] Amination: 225 mM NH_4Cl/NH_4OH buffer pH 9.6, 200 μM NADH, 40 mM MIBK.
[c] n.m. = not measurable.

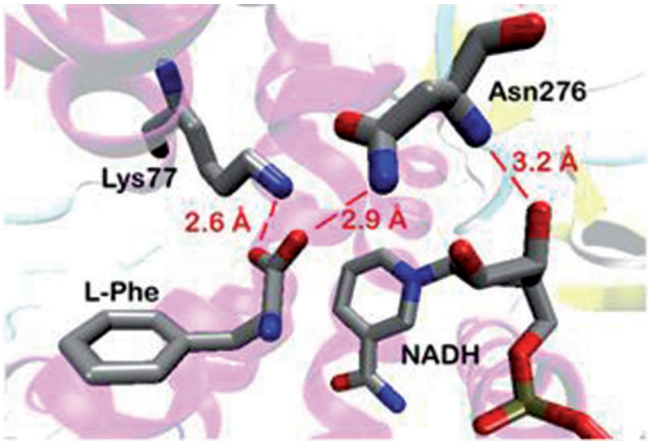


Figure 1. Interaction of proximate residues Lys77 and Asn276 observed in the crystal structure of PheDH from *Rhodococcus sp.* M4, PDB: 1C1D.

(PFPA). PFPA was selected as the test substrate since phenylacetone, the simplest ketone analog of phenyl pyruvate, the natural substance of PheDH, is regulated as a Schedule 2 controlled substance and thus is not readily available.[18] Amination activity was significantly higher toward PFPA, compared to MIBK, with a k_{cat} value of 0.128 s^{-1} and a K_M value of 4.61 mM, and resulted in the formation of 1-(4-fluorophenyl)-propyl-2-amine (FPPAm). This elevated activity allowed for stringent screening of subsequent mutational libraries, and variants with lower activity were not considered for further evaluation.

Since the K77M/N276V variant showed good activity for PFPA, a library was designed to evaluate if

Table 2. Characterization of his-tag-purified PheDH library 1 hits. Kinetic parameters k_{cat} and K_M determined by non-linear fit for the amination of PFPA.

K77 Residue	N276 Residue	Non-Linear Michaelis–Menten $k_{cat,app}$ [s^{-1}][a]		K_M [mM]	
Ser	**Leu**	**2.80**	**±0.54**	**4.4**	**±1.6**
Trp	**Glu**	**2.63**	**±0.16**	**5.3**	**±0.6**
Met	Met	1.82	±0.19	7.9	±1.3
Ser	Ser	1.31	±0.36	14.5	±5.2
Cys	Leu	1.29	±0.25	3.5	±1.4
Met	Leu	1.28	±0.10	4.2	±0.6
Ser	Val	1.06	±0.19	4.4	±1.5
Met	Cys	0.90	±0.08	1.5	±0.4
Met	Leu	0.86	±0.18	4.6	±1.8
Ser	Phe	0.79	±0.18	22.2	±0.4
Met	Ser	0.66	±0.07	4.9	±1.0
Val	Leu	0.56	±0.06	3.3	±0.8
Cys	Ile	0.55	±0.06	7.7	±1.4
Gly	Ile	0.34	±0.07	5.9	±2.0
Ser	Gly	0.28	±0.07	6.8	±2.7
Ser	Met	0.10	±0.04	28.6	±13.8
Gly	Cys	0.029	±0.00	–	
Ser	Glu	0.024	±0.01	9.0	±7.6
Trp	Ile	0.019	±0.01	–	
Trp	Gly	0.017	±0.02	–	
Gly	Val	0.017	±0.01	–	

[a] Apparent k_{cat} values in 225 mM NH_4Cl/NH_4OH pH 9.6, 200 μM NADH.

other variants at these two positions could yield further synergistic improvements. Since previous libraries had identified LeuDH K67M as a beneficial mutation, it was essential that the degenerate codon at that position include methionine to ensure the identification of the best variant. With a two-site library, codon selection must also be efficient by limiting codon redundancy to maintain a manageable screening effort. A two-site NNK library at these residues would encode for all 20 amino acids at each position. This large number of amino acid substitutions and codon redundancy results in a significant screening effort of 3066 colonies to achieve statistical coverage. The more restricted and efficient codon DDK greatly reduces the screening effort to 969 colonies by limiting the redundancy while still including a structurally-diverse selection of 15 amino acids.

The high-throughput screening of this diverse two-site PheDH K77DDK N276DDK library identified 36 highly-active colonies, containing 21 unique variants for subsequent characterization. These variants were purified and individually characterized for kinetic parameters in the amination of PFPA to more accurately determine which combination of mutations performed best (Table 2). All unique variants within the 21 pairs of mutations were active amine dehydrogenases, eight of which provided apparent k_{cat} values greater than 1 s^{-1} in the amination of PFPA. The K77S/N276L variant was selected as the top candidate for further characterization, since it had the highest apparent k_{cat} value and a lower PFPA K_M value than the next best K77W/N276E variant.

Characterization of PheDH-AmDH

The K77S/N276L variant was characterized in detail for a number of properties including; pertinent kinetic parameters, substrate specificity, thermostability, overall conversion, and enantioselectivity.

Kinetic parameters, $k_{cat,app}$ and K_M, were determined for the enzyme and each of the amination and deamination substrates. The original assay conditions of 225 mM NH_4Cl/NH_4OH buffer did not saturate the enzyme with respect to NH_3. The k_{cat} value for this AmDH enzyme with all three substrates saturated in 2 M NH_4Cl/NH_4OH buffer at pH 9.6 was 6.85 ± 0.59 s^{-1} at 25 °C. This k_{cat} value was nearly 15-fold greater than the maximum observed k_{cat} of 0.46 s^{-1} for the previously developed LeuDH-AmDH.[1] The increase in buffer concentration did have an effect upon the K_M value of PFPA, which increased to 7.75 ± 1.67 mM under saturated reaction conditions of 2 M NH_4Cl/NH_4OH buffer (pH 9.6) with 200 μM NADH. The K_M value for NADH was 23.9 ± 0.6 μM and was similar to the K_M value of native PheDH for NADH.

The significant increase in the K_M for NH_3 ($K_{M,\ NH_3}$ = 557 ± 107 mM) seen in the amination of PFPA, as compared to the amination of phenyl pyruvate by the wild-type PheDH ($K_{M,\ NH_3}$ = 37.6 ± 5.2 mM) can be at least partially attributed to decreased activation of the carbonyl moiety of PFPA, with its neighboring methyl group, compared to the carbonyl moiety in phenyl pyruvate, which is activated by the adjacent carboxyl group. The smaller δ+ charge of the α-carbon decreases its electrophilicity, requiring a higher concentration of the ammonia nucleophile.

The deamination of (*R/S*)-FPPAm exhibited slightly lower activity with a k_{cat} value of 1.92 ± 0.10 s^{-1}. The K_M values of the amine substrate and NAD^+ were similar to those for the amination reaction being 3.42 ± 0.42 mM and 61.6 ± 12.1 μM, respectively.

The substrate scope of the K77S/N276L PheDH-AmDH double variant was evaluated by determining the amination and deamination activities toward a number of different ketones and amines. The diversity of these ketones varied in structure from small aliphatic ketones such as 3-methyl-2-butanone, to larger aromatic ketones with additional functionality, such as phenoxy-2-propanone.

The enzyme showed elevated activity toward methyl ketones versus ethyl ketones or cyclic ketones (Table 3). The top five most active ketones; PFPA,

Table 3. Activity of K77S/N276L PheDH-AmDH towards various substrates.[a]

Substrate	Activity [mU mg^{-1}]	
(*R*)-MBA[b]	1.9	±1.8
(*S*)-MBA[b]	n.m.	
(*R/S*)-MBA[b,c]	0.5	±0.2
MIBK	77.0	±1.3
1,3-DMBA[b]	166.3	±0.0
acetophenone	n.m.	
phenoxy-2-propanone	540.8	±6.9
2-hexanone	155.7	±1.4
3-hexanone	1.6	±0.5
3-pentanone	1.3	±0.0
cyclopentanone	0.9	±0.5
cyclohexanone	27.5	±1.4
ethyl pyruvate	18.4	±5.9
benzaldehyde	31.4	±8.7
2-methylcyclohexanone	19.3	±1.1
3-methylcyclohexanone	41.1	±2.1
3-methyl-2-butanone	72.7	±1.4

[a] Amination in 225 mM NH_4Cl/NH_4OH, pH 9.6, 200 μM NADH, 20 mM substrate at 25 °C.
[b] Deamination in 0.1 M glycine buffer, pH 10.0, 1 mM NAD^+, 20 mM substrate at 25 °C.
[c] 40 mM, 20 mM of each enantiomer. n.m. = not measurable, < 0.1 mU mg^{-1}.

phenoxy-2-propanone, 2-hexanone, methyl isobutyl ketone and 3-methyl-2-butanone are all methyl ketones. This specificity is also observed in the large differences in activity toward 2-hexanone (155.7 mU/mg) versus 3-hexanone (1.6 mU/mg): despite their structural similarities, the methyl ketone 2-hexanone exhibited approximately 100-fold higher activity. Similarly, the enzyme requires at least a methylene linkage between the carbonyl carbon and the substrate's phenyl ring. This is reflected by the lack of activity toward acetophenone, as well as the low deamination activity of methylbenzylamine (MBA).

Conversion with Cofactor Recycle System

Conversion of the PFPA to FPPAm was demonstrated with K77S/N276L PheDH-AmDH using a glucose/glucose dehydrogenase (GDH) system to regenerate NADH at the expense of glucose. Over two days, the AmDH converted 93.8% of the substrate to the FPPAm product with 73.9% isolated yield. The product amine was confirmed by ^{1}H NMR.

Enantioselectivity

Equally important to conversion was maintaining the near perfect enantioselectivity of the wild-type enzyme while altering its substrate specificity. Preservation of wild-type PheDH's selectivity, which converts phenyl pyruvate to (*S*)-phenylalanine would result in asymmetric production of (*R*)-FPPAm from PFPA due to a change in Cahn–Ingold–Prelog priority. The product amine resulting from enzymatic conversion of PFPA was derivatized with trifluoroacetic anhydride and analyzed by chiral GC to show a single peak corresponding to one of the peaks observed in chiral GC analysis of racemic FPPAm. No peak was observed at the retention time for the opposite enantiomer indicating that the enzymatic conversion was highly selective (> 99.8% *ee*). The absolute configuration of the enzymatic product was assigned as (*R*)-FPPAm by optical rotation. These results showed that AmDH was highly enantioselective in the conversion of PFPA to (*R*)-FPPAm and preserved the high enantioselectivity of the wild-type PheDH.

Thermostability

The thermostability of K77S/N276L PheDH-AmDH was compared to that of its wild-type scaffold using two different criteria, melting temperature as measured by circular dichroism (CD) and a profile of temperature *versus* activity. The results of these evaluations indicated that the thermostability of the mutant enzyme was also affected, in addition to the changes to its substrate specificity. Both the wild-type PheDH and the AmDH exhibited similar characteristics in the loss of secondary structure as indicated through CD (Figure 2). At temperatures above 30 °C, there was an earlier onset of the pronounced loss of structure in the double variant compared to the wild-type; however, the slope of the folded fraction over temperature remained constant, indicating equivalent

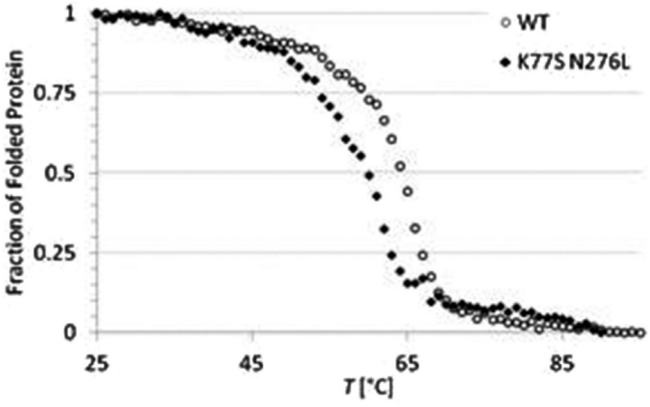


Figure 2. Fraction of folded PheDH-AmDH as measured by circular dichroism spectroscopy of wild-type PheDH and top PheDH-derived AmDH variant, K77S/N276L, with normalized ellipticity at 222 nm representing the fraction of folded protein (protein concentrations 100 μg mL^{-1} in 50 mM sodium phosphate buffer pH 8.0).

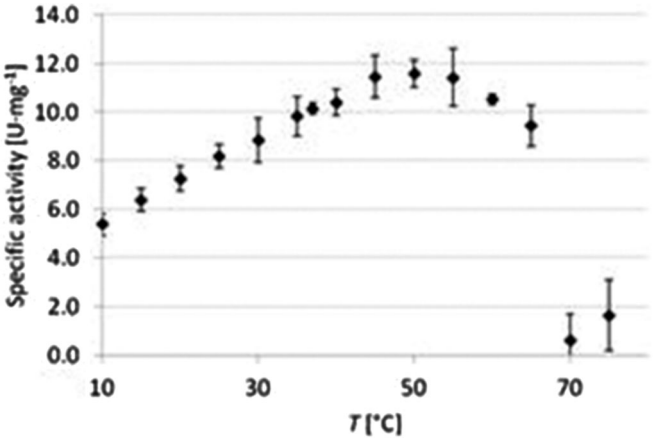


Figure 3. Profile of activity versus temperature for PheDH K77S/N276L double variant (AmDH). Amination activity measured in 2 M NH_4Cl/NH_4OH buffer pH 9.6, 200 μM NADH, and 20 mM PFPA for 2 min.

enthalpy of melting. The K77S/N276L variant resulted in a melting point of 59.9 °C, 4.4 °C less than the wild-type. The specific cause for this change is unknown, but could be due to either disruption of the packing of the wild-type enzyme's hydrophobic core or a net decrease in hydrophobicity by the amino acid substitutions resulting in a lower folding entropy.[19]

The thermostability of the double variant K77S/N276L was additionally investigated by an activity *versus* temperature profile (Figure 3). This profile shows the enzyme's near-linear increase in the lower temperature range (10–45 °C), corresponding to a low apparent Arrhenius activation energy of 241 J mol^{-1}.[20] At 50 °C, close to the temperature of maximum activity, the maximum specific activity was 11.6 U mg^{-1}. Above 50 °C, the enzyme began to rapidly lose activity as a result of denaturation. This data correlated well with the CD data where a dramatic loss of secondary structure was also observed above 50 °C.

Conclusions

Previous knowledge gained in the evolution of an amine dehydrogenase from LeuDH,[1] particularly the influence of binding pocket residues Lys77 and Asn276, allowed for the rapid evolution of an AmDH from PheDH from *Bacillus badius*. With a reasonably active starting variant K77M/N276V, a stringent high-throughput screening of a single two-site library across the sites 77 and 276 identified a variant, K77S/N276L, with high reductive amination activity with a k_{cat} value of 6.85 s^{-1} at 25 °C. The evolved AmDH demonstrated extremely high enantioselectivity [> 99.8% *ee* in the conversion of PFPA to (*R*)-FPPAm], which is a critical feature for the synthesis of chiral amines. The reaction can be simply and inexpensively driven to high conversions in excess of 90% when paired with a cofactor recycle system. With a temperature of maximum activity of 50 °C, at which the observed specific activity rises to 11.6 U mg^{-1}, the K77S/N276K AmDH variant is sufficiently stable for further evolution,[21] despite a 4.4 °C decrease in thermostability in comparison with wild-type PheDH. The novel AmDH exhibits amination activity towards a range of ketone substrates, with preference for methyl ketones, and beta-positioned ketones in the case of aromatic substrates. In summary, K77S/N276K AmDH is a good starting point for further evolution to increase activity toward specific API targets or fine chemicals.

Experimental Section

General

Substrates were obtained from suppliers and used without further purification. NADH was obtained from Amresco (Solon, OH). NAD^+, NH_4OH, PFPA, (*R*)-MBA, (*R/S*)-MBA, MIBK, 1,3-DMBA, phenoxy-2-propanone, 2-hexanone, ethyl pyruvate, 2-methylcyclohexanone, 3-methylcyclohexanone, and 3-methyl-2-butanone were obtained from Sigma (St. Louis, MO). (*S*)-MBA, acetophenone, cyclopentanone, and benzaldehyde were obtained from Fluka (St. Louis, MO). NH_4Cl was obtained from BDH (London, UK). (*R/S*)-FPPAm and 3-pentanone were obtained from Alfa Aesar (Ward Hill, MA). 3-Hexanone was obtained from Riedel-de Haen (Seelze, Germany). Cyclohexanone was obtained from Mallinckrodt (Phillipsburg, NJ). ^1H NMR spectra were obtained in $CDCl_3$ at 400 MHz on a Varian Mercury VX400 spectrometer. Gas chromatography was performed on a Shimadzu GC-2010 gas chromatographer equipped with a Restec Rt-BDEXcst column.

Gene Cloning and Expression

Genomic DNA from *Bacillus badius* Bachelor (ATCC# 14574) was purchased from the American Type Culture Collection. The gene was amplified using a standard PCR protocol[22] with the forward primer, 5′-GGAATTC**CATA**TGAGCT TAGTAGAAAAAACATCCATCA-3′ and reverse primer, 5′-CCGCT**CGAGTA**TTAGTTGCGAATATCCCATTTTG-3′. These primers simultaneously inserted the restriction sites *Nde*I and *Xho*I prior to and following the gene, respectively. The locations of the restriction sites are indicated by bold type with each primer. This made for simple digestion and ligation into either pET17b or pET28a plasmids.

Wild-type PheDH from *Bacillus badius* and variants were expressed in a pET28a, BL21 (DE3) system at 37 °C in MagicMedia™ media for 24 h, and subsequently his-tag purified using standard Ni-NTA affinity chromatography.

Activity Measurements

Activity of purified proteins was measured using a spectrophotometric assay at 340 nm, corresponding to the cofactor

NADH ($\varepsilon_{340} = 6220\,M^{-1}cm^{-1}$).[23] For reductive amination, reactions were performed in 225 mM NH_4Cl/NH_4OH buffer at pH 9.6, with 200 μM NADH and 20 mM of the ketone substrate, unless otherwise specified. For oxidative deamination, reactions were performed in 0.1 M $NaHCO_3/Na_2CO_3$ buffer at pH 10.0, with 1 mM NAD^+ with 10 mM of the amine substrate of interest. All reactions were performed at 25 °C unless otherwise specified.

Mutagenesis

After the first 10 rounds of mutagenesis, three mutations were previously identified to be most influential in creating reductive amination activity in a LeuDH-based scaffold; K67M, E113V, and N261V.[1] Analogous mutations to these were identified through sequence alignment and applied to a similar amino acid dehydrogenase scaffold, phenylalanine dehydrogenase (PheDH) from *Bacillus badius* (48% identity, 66% similarity). Each of the point mutations, (K77M, T123V, N276V) as well as combinations of these mutations, was evaluated for amination activity.

Mutant libraries were generated using overlap extension PCR. After identification of mutational sites, primers were designed according to the guidelines of the QuikChange® Site Directed Mutagenesis Protocol.[24] These primers were then used in the overlap extension protocol described in Molecular Cloning: A Laboratory Manual.[22] After mutation, the resulting mutated gene was digested using *Nde*I and *Xho*I, and ligated into pET17b. The resulting plasmids were transformed into BL21 (DE3) *E. coli* competent cells.

Library Screening

After mutagenesis, colonies were robotically picked using a Genetix QPix2 colony picker and expressed in microtiter plates containing 250 μL of MagicMedia™ media for 18 h at 37 °C. After expression, the plates were pelleted at 1000 rpm for 30 min and frozen until screening at −80 °C. As the expression plates thawed, the cell pellets were gently vortexed with 30 μL of B-PER to uniformly resuspend and lyse the cells. The crude cell lysate was split in 10 μL aliquots into two plates; a reaction and background plate. A reaction mixture (200 μL) containing 10 mM PFPA and 200 μM NADH in 225 mM NH_4Cl/NH_4OH buffer was added to each well. The same mixture lacking PFPA was added to the background plate. Absorbance measurements at 340 nm began immediately and continued periodically over the course of 1.5 h.

Active variants were identified by the rate of change in absorbance at 340 nm, corresponding to the fastest activity. The wells exhibiting the fastest rate change over that of the background plate were selected for further characterization. Some variants consumed the cofactor too quickly (< 5 min) to be observed over multiple time points, and gave low absorbance at 340 nm in the initial time point. These variants were included for further characterization. The successful variants were sequenced, and each unique sequence was expressed in pET28a/BL21 for his-tag purification and determination of k_{cat} and K_M values with PFPA.

Enantioselectivity and Conversion

A reaction was carried out in 100 mL of 225 mM NH_4Cl/NH_4OH buffer (pH 9.6) containing 20 mM PFPA, 22 mM glucose, 2 mM NAD^+, 17.5 mg PheDH-AmDH and 200 U of glucose dehydrogenase 103 at 25 °C. The conversion was allowed to continue for 48 h, after which the reaction mixture was adjusted to pH 13 with 5 mL of 10 N NaOH and extracted with methyl *tert*-butyl ether. The organic extract was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to give of FPPAm; yield: 212 mg (93.8% conversion, 73.9% yield).

The ^1H NMR spectrum of the isolated product was consistent with the structure of (*R*)-(−)-1-(4-fluorophenyl)-propyl-2-amine and agreed with reported data.[25] ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.26$–6.95 (m, 4H), 3.12 (m, 1H), 2.67 (dd, 1H, 6.3 Hz, 13.2 Hz), 2.49 (dd, 1H, 6.3 Hz, 13.2 Hz), 1.10 (d, 3H, 6.4 Hz).

Confirmation of the (*R*)-selectivity was achieved through polarimetry, since single enantiomers of (4-fluorophenyl)-propyl-2-amine were not available. Optical rotation was measured on a Rudolph Autopol III polarimeter at 589 nm resulting in a specific rotation of $[\alpha]_D^{25}$: −38.4 (*c* 0.46, MeOH).[25]

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