ACS SyntheticBiology

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Development of an NADPH-Dependent Homophenylalanine Dehydrogenase by Protein Engineering

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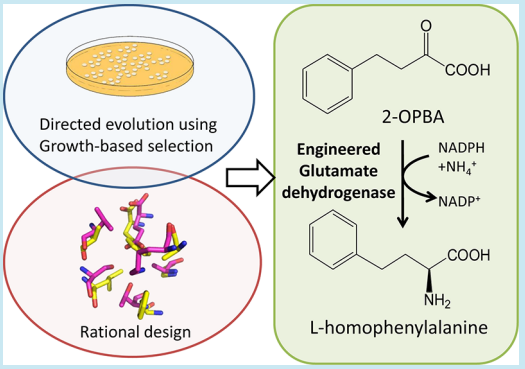
Supporting Information

ABSTRACT: L-Homophenylalanine is a nonproteinogenic amino acid and can be used as a versatile pharmaceutical intermediate. Production of L-homophenylalanine involves amination of the keto acid precursor 2-oxo-4-phenylbutyric acid (2-OPBA), which can be accomplished by bioenzymatic processes. Current biocatalysts for this reaction include transaminases and NADH-dependent phenylalanine dehydrogenases, which are not optimal for metabolic engineering of whole-cell biocatalysis. Here, we report the development of an NADPH-dependent homophenylalanine dehydrogenase by engineering the NADPH-dependent glutamate dehydrogenase (GDH) from *Escherichia coli*, which provides a new tool for *in vitro* catalysis and *in vivo* metabolic engineering. We took a stepwise substrate walking strategy: the first round directed evolution switched GDH's substrate specificity from its natural substrate 2-ketoglutarate to the intermediate target phenylpyruvate, which has similar structure as 2-OPBA; and the second round further improved the enzyme's catalytic efficiency toward the final target 2-OPBA. Compared to wild type GDH, the catalytic efficiency (k_{cat}/K_m) of the final mutant was ~100 fold higher for 2-OPBA and ~3000 fold lower for the original substrate 2-ketoglutarate. When overexpressed in *E. coli*, the engineered GDH aminated 2-OPBA to L-homophenylalanine more effectively than the transaminases and NADH-dependent phenylalanine dehydrogenase, possibly because it utilizes the strong anabolic driving force NADPH under aerobic condition.



KEYWORDS: *protein engineering, synthetic biology, L-homophenylalanine, glutamate dehydrogenase, reductive amination*

L-Homophenylalanine is a nonproteinogenic amino acid and can be used as a versatile pharmaceutical precursor of several angiotensin-converting enzyme (ACE) inhibitor drugs and a novel proteasome inhibitor drug, carfilzomib. Production of L-homophenylalanine can be accomplished by bioenzymatic methods,[1−6] one of which involves the stereoselective amination of 2-oxo-4-phenylbutyric acid (2-OPBA)[1−3] (Figure 1A).

Stereoselective amination of keto acids can be catalyzed by three categories of amination enzymes: transaminases, NADH-, and NADPH-dependent amino acid dehydrogenases (Figure 1A). Transaminases (also known as aminotransferases) are a large protein family which transfers the amino group between amino/keto acid pairs (Figure 1A). In microbial cells, transaminases typically catalyze the synthesis of various amino acids including valine, leucine, isoleucine, phenylalanine, tyrosine, and aspartate from only a few universal amino group donors such as glutamate. The transamination reaction is readily reversible *in vivo*, which is consistent with the need for the redistribution of the reduced nitrogen among different amino acids dynamically to fit the need of protein synthesis. Transaminases have been used for the synthesis of L-homophenylalanine *in vitro*.[1,3] However, a high level of amino group donor glutamate may need to be maintained for the reversible reaction to proceed in the desired direction *in vivo*[7] (Figure 1A). Different from transaminases, NADH- and NADPH-dependent amino acid dehydrogenases use free ammonia as the substrate and thus can dissimilate or assimilate ammonia (Figure 1A). Most NADH-dependent amino acid dehydrogenases such as phenylalanine dehydrogenases (PheDH) and leucine dehydrogenases (LeuDH) natively function as catabolic enzymes in amino acid degradation.[8−12] On the other hand, the NADPH-dependent amino acid dehydrogenases such as the NADPH-dependent alanine dehydrogenases (AlaDH) and glutamate dehydrogenases (GDH) operate virtually irreversible *in vivo* favoring the amino acid synthesis thanks to the relatively high intracellular NADPH/NADP ratio, which is consistent with their roles as the ammonia assimilation enzymes in metabolism.[13,14] Thus, an NADPH-dependent homophenylalanine dehydrogenase may be a useful tool for the whole cell biosynthesis of L-homophenylalanine. The pathway of 2-OPBA biosynthesis has been synthetically created[15] or characterized in native

Received: July 29, 2013
Published: September 20, 2013

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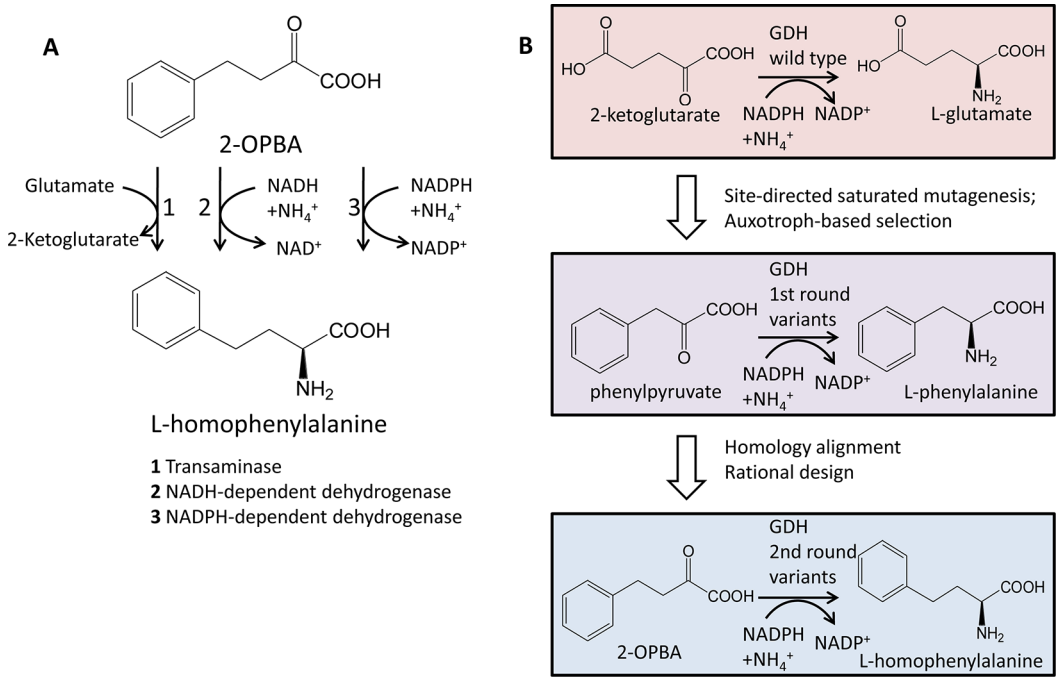

Figure 1. Amination strategy for L-homophenylalanine and outline of substrate walking protein engineering process. (A) The keto acid 2-oxo-4-phenylbutyric acid (2-OPBA) can be aminated to form L-homophenylalanine by three types of enzymes (1) transaminases, (2) NADH-dependent dehydrogenase, and (3) NADPH-dependent dehydrogenase. (1) requires amino donor such as glutamate. (2) and (3) use free ammonia as the substrate. (B) The stepwise substrate walking process first aimed to switch the substrate specificity of *E. coli* NADPH-dependent GDH from its native substrate 2-ketoglutarate to phenylpyruvate. A second round of protein engineering then improve the enzyme's activity toward the final product 2-OPBA.

organisms[5] recently, which represents important progress toward total biosynthesis of L-homophenylalanine from inexpensive feedstock such as glucose. In addition, since NADPH can be regenerated *in vitro* using various inexpensive substrates,[16−18] the NADPH-dependent homophenylalanine dehydrogenase may also provide more flexibility when designing the *in vitro* catalysis processes.

To develop an NADPH-dependent amination enzyme for L-homophenylalanine production, we used a stepwise substrate walking approach[19] (Figure 1B) by first adapting the enzyme to an intermediate substrate that mimics the final target, and then engineering the enzyme again for the final substrate. We chose the *E. coli* glutamate dehydrogenase (GDH) encoded by *gdhA* as the starting material, which is naturally NADPH-dependent but specific for 2-ketoglutarate. GDH serves as the major ammonia assimilation enzyme in *E. coli* under ammonia abundant condition, which is consistent with its relatively high catalytic activity and intracellular solubility. These features make it an attractive target for protein engineering and further application in *E. coli*. On the other hand, another strategy could be to switch the cofactor preference of the PheDH, which are specific for aromatic substrates but NADH-dependent. However, since the PheDHs are mostly from evolutionarily remote microbes, their heterologous overexpression in *E. coli* may need to be optimized. In the first round of protein engineering, we chose phenylpyruvate as an intermediate target, which is one carbon smaller than 2-OPBA. Phenylpyruvate was chosen not only because it has similar structure as the final target but also because the amination of phenylpyruvate yields an amino acid phenylalanine and thus generates a growth-selectable phenotype. We used site-directed saturated mutagenesis with an auxotroph selection scheme because the drastic switch of substrate preference from carboxylic acid side chain (2-ketoglutarate) to aromatic side chain (phenylpyruvate) may require extensive searching in protein sequence space at multiple sites, which could not be easily achieved by rational sampling and screening. Next, we used rational design guided by homologous structure and sequence alignment to increase the enzyme's activity toward the final substrate 2-OPBA (Figure 1B).

In the first round of directed evolution, a phenylalanine-auxotroph based selection platform was setup (Figure 2A). Transaminases encoded by *ilvE*, *aspC*, *tyrB*, and *avtA* in *E. coli* can catalyze the synthesis of phenylalanine from phenylpyruvate and were deactivated by gene knockout. The resulting strain, 4KO, cannot synthesize phenylalanine, as well as valine, leucine, isoleucine, aspartate, and tyrosine. As a result, in minimal medium supplemented with valine, leucine, isoleucine, aspartate, and tyrosine but not phenylalanine, the growth of 4KO cells can only be restored if the GDH mutant can aminate phenylpyruvate (Figure 2A). To create such a GDH mutant, an *E. coli* GDH structural model was built based on sequence comparison using the known structure of *Clostridium symbiosum* GDH (PDB: 1BGV).[20] The model identified several residues which are potentially important for substrate recognition (Figure 2B), including K92, A166, V377, and S380. These residues are randomized by site-saturated mutagenesis using degenerate codon NNK to yield the KAVS library. The DNA containing site-saturated mutagenesis library was transformed into 4KO cells and cultured in the selective condition described above.

From a library of ∼6 million independent transformants, 11 mutants were selected and confirmed, which gave 5 different GDH variants at the protein sequence level (Table 1). At each

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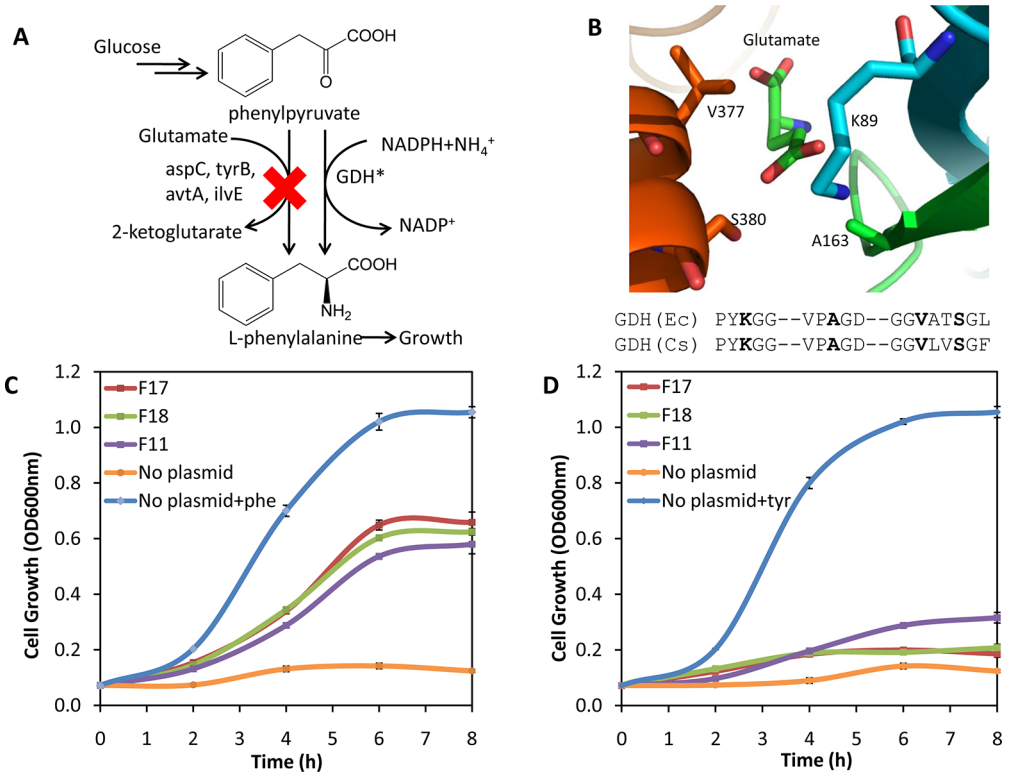

Figure 2. Directed evlution of glutamate dehydrogenase (GDH) using phenylalanine auxotroph selection. (A) The selection platform built in *E. coli* has several transaminases knocked out to block the formation of L-phenylalanine from phenylpyruvate. Mutant GDH that can use phenylpyruvate as the substrate would rescue growth in phenylalanine-free medium. (B) Sequence alignment of *Clostridium symbiosum* GDH (PDB: 1BGV) and *E. coli* GDH. (C) Mutants from the selection rescued growth of the phenylalanine auxotroph. F17, F18, F11: phenylalanine auxotroph harboring plasmdis containing mutant GDH. No plasmid: phenylalanine auxotroph strain with no plasmid. No plasmid+phe: phenylalanine auxotroph strain with no plasmid but with phenylalanine added in the culture. (D) Mutants from the selection did not rescue growth of the tyrosine auxotroph. The error bars represent the standard deviation of three independent repeats.

Table 1. Mutants Obtained from the Phenylalanine Auxotroph Selection

	Lys 92		Ala 166		Val 377		Ser 380	
	codon	residue	codon	residue	codon	residue	codon	residue
WT	AAA	K	GCA	A	GTC	V	TCG	S
F1	TGT	C	GGG	G	GCT	A	GCG	A
F2	TGT	C	GGG	G	GCT	A	TCT	S
F4	TGT	C	GGG	G	GCT	A	TCT	S
F7	GCG	A	GGG	G	GCT	A	AGT	S
F9	GCG	A	GGG	G	GCG	A	GCT	A
F11	TGT	C	GGG	G	GCG	A	GCG	A
F14	TCT	S	GGT	G	GCT	A	GCT	A
F15	GCG	A	GGG	G	GCG	A	TCT	S
F17	GCG	A	GGG	G	GCT	A	TCT	S
F18	GCG	A	GGG	G	GCG	A	GCT	A
F20	TCT	S	GGG	G	GCT	A	GCG	A

site, the mutations selected converged to a very limited set of replacement residues: K92 residue can only be replaced by C, A, and S; S380 residue can only stay unchanged or be replaced by A; for A166 and V377, the only replacement is G and A, respectively. These results suggested that (1) the selection scheme was relatively stringent and (2) the amino acid residues picked for mutagenesis were important for phenylpyruvate binding and thus were subjected to significant selection pressure. As a result, the mutations in all of the 11 GDH variants were relatively conserved while most of the other possible substitutes at the randomized positions were eliminated under the high selection pressure. At the nucleotide sequence level, the same amino acid substitutions were encoded by different codons in different mutants (Table 1), suggesting that they were independent hits. Analysis of these mutations based on the structure suggested the potential mechanism of the substrate specificity switch: changing of the K92 to uncharged residues may disrupt the interaction between the enzyme and the carboxyl group in the glutamate side chain; changing of A166 and V377 to smaller residues may relieve the steric hindrance between the substrate binding pocket and the bulky phenyl ring. All 5 different GDH variants rescued the

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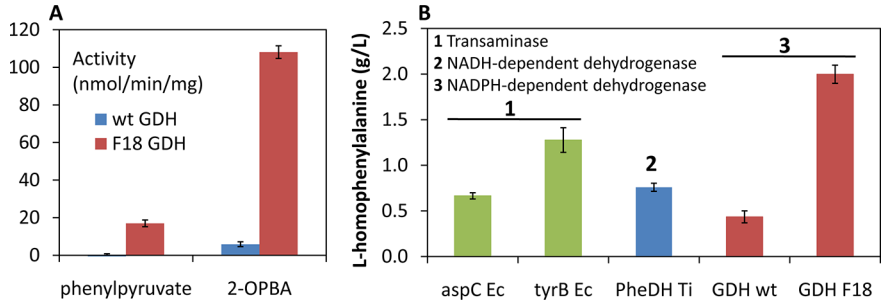

Figure 3. GDH mutant catalyzes reductive amination of 2-oxo-4-phenylbutyric acid (2-OPBA). (A) Reductive amination activity of wild type the F18 mutant GDH for phenylpyruvate and 2-OPBA measured using cell extract. (B) L-homophenylalanine production from 2-OPBA by *E. coli* cells with the overexpression of transaminases, a NADH-dependent phenylalanine dehydrogenase, and the NADPH-dependent wild type and F18 mutant GDH. The error bars represent the standard deviation of three independent repeats.

Table 2. Kinetic Parameters of the Wild Type and Mutant Glutamate Dehydrogenases

	wild type			F18			F18 T195A		
	K_m (mM)	k_{cat} (S^{-1})	k_{cat}/K_m (mM^{-1} S^{-1})	K_m (mM)	k_{cat} (S^{-1})	k_{cat}/K_m (mM^{-1} S^{-1})	K_m (mM)	k_{cat} (S^{-1})	k_{cat}/K_m (mM^{-1} S^{-1})
2-ketoglutarate	0.81 ± 0.02	167 ± 15	205.7	N.D.[a]	N.D.[a]	0.037 ± 0.001[b]	96.3 ± 4.1	5.73 ± 0.02	0.060
phenylpyruvate	7.98 ± 0.22	0.48 ± 0.11	0.061	0.20 ± 0.01	0.51 ± 0.02	2.61	0.47 ± 0.08	0.25 ± 0.05	0.53
2-OPBA	15.3 ± 0.03	4.50 ± 0.02	0.294	1.05 ± 0.03	7.24 ± 0.04	6.93	0.24 ± 0.01	6.79 ± 0.03	28.9

[a]N.D. not determined. [b]Catalytic efficiency was estimated by the use of the equation $v/[E_o]=(k_{cat}/K_m)[S]$ for $K_m \gg [S]$

growth in similar manner. The growth rescue data of 3 of them were shown as examples (Figure 2C). Curiously, although most of the aromatic transaminases have broad substrate range,[21,22] the GDH variant may be highly selective for phenylpyruvate over *p*-hydroxyphenylpyruvate, the keto acid precursor of tyrosine, as they did not rescue growth in minimal medium with glucose and phenylalanine, but with no tyrosine supplemented (Figure 2D).

One of the variants, F18, was selected for further characterization. Compared to the wild type GDH, the F18 GDH variant showed significant activity toward phenylpyruvate, as suggested by enzyme assays using crude cell extract (Figure 3A). Moreover, it also showed activity toward the target substrate 2-OPBA (Figure 3A). Kinetics parameters were also characterized using purified enzymes (Table 2). F18 mutant has ~43 fold higher NADPH-dependent catalytic efficiency (measured by k_{cat}/K_m) for the substrate phenylpyruvate compared to the wild type GDH. The increase in the catalytic efficiency was mainly atributed to the decreased K_m, which suggested the effective reshaping of the substrate pocket to fit the new substrate. Meanwhile, the F18 variant also has very low activity toward the original substrate 2-ketoglutarate (Table 2) with the K_m well beyond the tested substrate concentration range (0.1−200 mM). The decrease of catalytic efficiency toward the native substrate 2-ketoglutarate is important for eliminating the side reactions *in vivo* especially since 2-ketoglutarate is a central metabolite and present in relatively high level inside the cells. In terms of the cofactor specificity, enzyme assays using purified proteins showed that F18 GDH, as well as the wild type GDH, is NADPH-dependent for the target substrate 2-OPBA (Figure S1, Supporting Information).

To test its *in vivo* functionality as a 2-OPBA amination enzyme, keto acid feeding experiments were performed. The keto acid 2-OPBA feeding medium was M9 minimal medium with 4 g/L NH_4Cl, 5 g/L glucose, and around 3.5 g/L 2-OPBA (20 mM). No amino group donors such as glutamate or lysine were added. Wild type *E. coli* strains individually overexpressing F18 GDH, wild type GDH, aspartate transaminase AspC (*E. coli*), tyrosine transaminase TyrB (*E. coli*), and an NADH-dependent phenylalanine dehydrogenase (*Thermoactinomyces intermedius*),[23] respectively, were cultured in the keto acid feeding medium for 24 h before the L-homophenylalanine produced was measured (Figure 3B). The F18 GDH from the first round of protein engineering produced higher amount of L-homophenylalanine compared to the two previously used tranaminases AspC and TyrB and the NADH-dependent phenylalanine dehydrogenase from *T. intermedius*, which has also been shown to be highly soluble and functional when overexpressed in *E. coli*.[23] These results are consistent with the above-mentioned hypothesis that NADPH-dependent reductive amination might be a good strategy for *in vivo* L-homophenylalanine production with free ammonia as the nitrogen source. It is shown that, in anaerobic conditions, the intracellular NADH/$NADH^+$ ratio is high due to the low respiration and can serve as a robust driving force for reductive reactions.[24,25] However, in aerobic condition, which is commonly used in industrial amino acid production, intracellular NADH/$NADH^+$ ratio is only around 1/10 of the NADPH/$NADP^+$ ratio in *E. coli*.[26] The NADPH-dependent F18 GDH variant utilized the NADPH driving force and may highly favor the reductive amination direction of the reaction *in vivo*.

The selection based on a phenylalanine auxotroph platform yielded initial success in shifting GDH's substrate specificity toward aromatic compounds. Next, we performed rational mutagenesis to further sculpt the substrate binding pocket during the second round of protein engineering. The GDHs share significant homology with other amino acid dehydrogenases especially phenylalanine dehydrogenases and leucine dehydrogenases.[10,27,28] When superimposing the structure of *Rhodococcus* sp. M4 L-phenylalanine dehydrogenase (PDB: 1C1D)[29] and *C. symbiosum* GDH, we identified another residue, T193 in *C. symbiosum* GDH (corresponding to T195 in

dx.doi.org/10.1021/sb400093x | *ACS Synth. Biol.* 2014, 3, 13−20

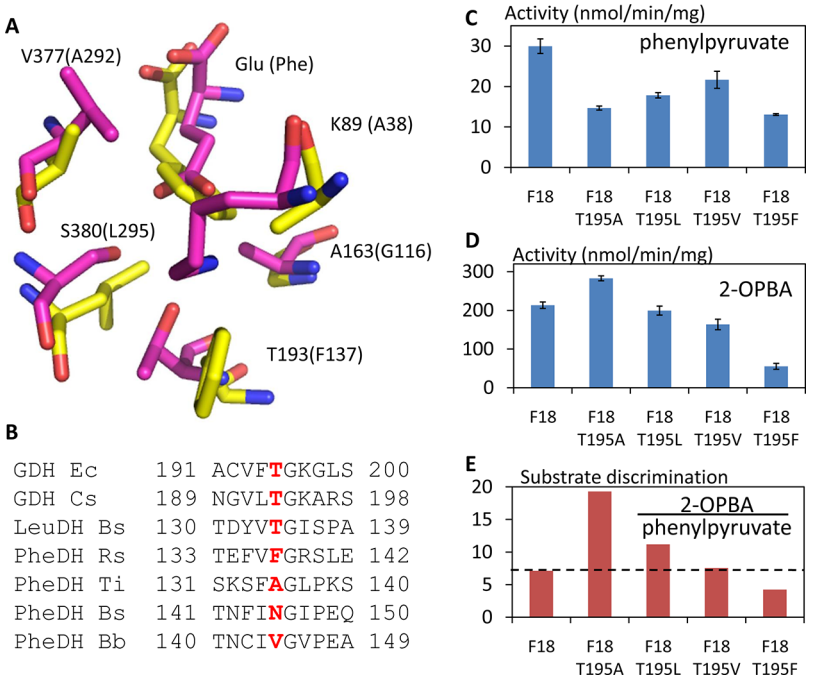

Figure 4. Improvement of the F18 mutant GDH using rational mutagenesis. (A) Structure alignment of the *Clostridium symbiosum* GDH (PDB: 1BGV) (magenta) and the *Rhodococcus* sp. M4 L-phenylalanine dehydrogenase (PDB: 1C1D) (yellow). The residue and number of the *Rhodococcus* sp. M4 L-phenylalanine dehydrogenase are in parentheses. (B) Sequence alignment of GDH, leucine dehydrogenase (LenDH), and phenylalanine dehydrogenases (PheDH). Ec, *E. coli*; Cs, *C. symbiosum*; Bs, *Bacilius sphaericus*; Rs, *Rhodococcus* sp. M4 ; Ti, *Thermoactinomyces intermedius*; Bb, *Bacillus badius*. (C) Activity of different F18-based secondary mutants for phenylpyruvate as measured using crude extract of *E. coli* cells overexpressing corresponding proteins. (D) Activity of different F18-based secondary mutants for 2-oxo-4-phenylbutyric acid (2-OPBA) as measured using crude extract of *E. coli* cells overexpressing corresponding proteins. (E) Substrate discrimination between phenylpyruvate and 2-OPBA. The discrimination value is obtained by divide the 2-OPBA activity value in D with the phenylpyruvate activity value in C. The error bars represent the standard deviation of three independent repeats.

E. coli GDH), which was at the base of the substrate binding pocket and could be the potential target for further engineering (Figure 4A). Protein sequence alignment showed that this conserved threonine residue in GDH and leucine dehydrogenase is replaced with less hydrophilic residues N, V, A, or F in phenylalanine dehydrogenase[28] (Figure 4B), indicating that T195 may also be important in substrate recognition. We mutated T195 into A, L, V, or F in the F18 GDH variant and tested the reductive amination activities toward phenylpyruvate and 2-OPBA using crude cell extract (Figure 4C, D, E). The data suggested the F18 T195A variant may have increased activity toward the final target 2-OPBA, while all the variants may have lower activities compared to the parent enzyme F18 for phenylpyruvate.

On the basis of the above results, we purified the F18 T195A variant and characterized it (Table 2). Compared with the first round variant F18, the F18 T195A GDH has ~4 fold improved catalytic efficiency (k_{cat}/K_m) toward 2-OPBA, which is largely due to the decreased K_m. In addition, the catalytic efficiency for the intermediate substrate phenylpyruvate was decreased by ~5 fold. These results suggested that the second round of protein engineering effectively walked the enzyme activity away from the first phase target and approached the final substrate. The mutation T195A increased the enzyme's discrimination between phenylpyruvate and 2-OPBA, possibly because it enlarges the binding pocket and increases its hydrophobicity, which would favor the van del Waals contact between the enzyme and the bigger aromatic substrate. Since phenylpyruvate is an intermediate in the biosynthetic route of 2-OPBA,[5,15] this discrimination might be important in the case of *in vivo* production to avoid formation of the shorter chain byproduct. Overall, the F18 T195A variant from the second round of mutagenesis has ~100 fold higher k_{cat}/K_m for 2-OPBA compared to wild type GDH. It is also highly specific for NADPH (Figure S1, Supporting Information). Keto acid feeding experiments were also performed using F18 T195A (Figure S2, Supporting Information), which showed that F18 T195A yielded higher level of homophenylalanine from 2-OPBA when overexpressed in *E. coli*. Future work is needed to improve the enzyme's efficiency for *in vivo* application, especially by increasing k_{cat}.

The relatively small K_m value of the engineered enzyme is promising because if L-homophenylalanine is to be produced from unrelated carbon and nitrogen source through biosynthesis, the concentration of the precursor 2-OPBA *in vivo* would be within a physiological range. However, the k_{cat} of the mutant enzymes for 2-OPBA is still much lower than that of the wild type enzyme for its native substrate. Similarly, in our previous study on another enzyme 2-isopropylmalate synthase (LeuA),[15] the substrate binding pocket of the engineered LeuA fits phenylpyruvate well based on the structure prediction. However, the k_{cat} was not optimal.[15] One hypothesis could be that it might be difficult for the bulky and rigid aromatic substrate to assume optimal orientation and proximity to the catalytic residues and the cofactor, because the binding pocket was only tailored for the phenyl side chain while the enzyme's catalytic center and the cofactor binding structures remain the same. Interestingly, all GDH variants tested have higher k_{cat} for 2-OPBA than for the one carbon shorter keto acid, phenylpyruvate. This could be due to the fact that the longer carbon

dx.doi.org/10.1021/sb400093x | *ACS Synth. Biol.* 2014, 3, 13−20

chain has higher degree of freedom in conformation and thus may adopt better conformation for catalysis.

In summary, we utilized a substrate walking approach to develop a NADPH-dependent reductive amination enzyme for *in vivo* production of L-homophenylalanine. The resulting variant out-performed the transaminases and NADH-dependent phenylalanine dehydrogenase in converting the keto acid precursor 2-OPBA to L-homophenylalanine. This enzyme is useful for metabolic engineering of strains for one-pot production of homophenylalanine from inexpensive raw materials. Further work is needed to improve the enzyme activity, particularly the turnover rate. Amino acid residues that do not directly participate in substrate interaction may need to be mutated to support optimal orientation for catalysis.

METHODS

Plasmid Construction. All cloning and plasmid preparation were done using *E. coli* XL1-blue cells (Stratagene, La Jolla, CA). Restriction enzymes were purchased from New England BioLabs (Ipswich, MA). The Rapid DNA ligation kit was from Roche (Mannheim, Germany). KOD DNA polymerase was from EMD Chemicals (San Diego, CA). Oligonucleotides were purchased from IDT (San Diego, CA). Detailed information about plasmids and primers used in this study can be found in Supporting Information Tables S1 and S2.

Briefly, *gdhA* (*E. coli*), *aspC* (*E. coli*), *tyrB* (*E. coli*), and *pdh* (*Thermoactinomyces intermedius*) were amplified from corresponding genomic DNA and inserted at the Acc65I and XbaI sites of the pZElac vector[7] to yield plasmids pZElac_GDH, pZElac_aspC, pZElac_tyrB, and pZElac_PheDH, respectively. The wild type and mutant GDH from *E. coli* was amplified and inserted in the Acc65I and XbaI sites of pZElac-his vector[30] to yield plasmid pZElac_hisGDH, pZElac_hisF18, and pZElac_hisF18TA, respectively.

To change T195 residue of F18 mutant GDH to A, two PCR reactions were performed using primer sets GDH Acc65I fwd/T195A rev and T195A fwd/GDH XbaI rev, respectively, with F18 GDH plasmid as the template. The resulting fragments were assembled using "splicing by overhang extension" (SOE) PCR and inserted into the Acc65I and XbaI site of pZElac. Similar strategy was used to change T195 residue to V, L, and F, respectively.

Construction of the Phenylalanine Auxotroph Strain. Gene deletion of *ilvE*, *aspC*, *tyrB*, and *avtA* from *E. coli* strain BW25113 chromosome was performed using P1transduction and the strains used for the P1 transduction were obtained from the Keio collection.[31] Colonies containing the correct deletions were transformed with plasmid pCP20 to remove the kanamycin resistance marker. The resulted strain was named 4KO.

Library Construction and Selection. The K92, A166, V377, and S380 residues of *E. coli* GDH were randomized by degenerate codon NNK (N is A,T,G,C; K is G,T). Briefly, the *gdhA* gene in *E. coli* was divided into four fragments and amplified from the genomic DNA separately using the primer sets GDH Acc65I fwd/K92 rev, K92 fwd/A166 rev, A166 fwd/VS rev, and VS fwd/GDH XbaI rev, respectively. The resulting four fragments were assembled using Splicing by overhang extension (SOE) PCR and inserted into the Acc65I and XbaI site of pZElac. The ligation product was transformed into electrocompetent ElectroMAX DH10B cells (Invitrogen), yielding ~6 million independent transformants. The plasmid DNA from the pooled transformants was isolated and named KAVS library.

KAVS library DNA was transformed into 4KO strain by electroporation and yielded ~6 million independent transformants. Pooled transformants were plated on the selection plates containing M9 miminal medium supplemented with 2 g/L glucose, 200 mg/L aspartate, 40 mg/L each of isoleucine, leucine, valine, and tyrosine, 200 mg/L ampicillin, and 0.1 mM isopropyl-b-D-thiogalactoside (IPTG). The plates were incubated at 30 °C. Approximately, 10−20 colonies formed on one plate, which was plated with ~2 million cells. Colonies with relatively big size were picked randomly and restreaked on the selection plates. Single colonies were picked and grown in LB medium to purify the plasmid, which was used to retransforme freshly grown 4KO strain and tested for rescue of growth.

Growth Curve Characterization. The growth curve was characterized using the same selection medium as described above. Newly transformed 4KO cells harboring plasmids of different GDH variants were first cultured in LB medium with 200 mg/L ampicillin at 30 °C overnight. The cells from the overnight culture were then harvested and washed three times with M9 minimal medium. The washed cells were then resuspended in 3 mL selection medium with 200 mg/L ampicillin and 0.1 mM IPTG in test tubes with the initial OD600 nm of ~0.05. As positive control, 40 mg/L phenylalanine was added. As negative control, 4KO cells with no plasmid were used and ampicillin was omitted. Cultures were incubate at 30 °C and the optical density was measure every 2 h. Similar method was used to characterize growth curve for tyrosine auxotroph rescue.

Enzyme Assays Using Crude Cell Extract. *E. coli* strain XL1 Blue was transformed with different GDH variants in pZElac vector. Single colonies were picked to inoculate in 5 mL LB medium with 0.1 mM IPTG and incubate at 37 °C for 24 h. Cells from 4 to 5 mL culture were harvest and resuspend in 1 mL 0.1 M Tris-Cl pH = 8.0. Cell lysates were prepared by bead beating followed by centrifugation at 10 000 × g for 20 min at 4 °C. 10 μL cell lysate was used in 200 μL reaction system, which also contained 100 mM Tris-HCl, pH = 8.0, 200 μL NADPH, 40 mM NH_4Cl, and 20 mM keto acid substrates (or no substrate for negative control). The reaction was started by adding the substrate, and the OD_{340} was monitored. The soluble protein concentrations in cell lysates were quantified using Quick Start Bradford Protein Assay (Bio-Rad, CA) according to manufacturer's instructions.

L-Homophenylalanine Production with Keto Acid Feeding. *E. coli* strain BW25113 was transformed with different pZElac vectors harboring wild type and F18 mutant of *gdhA* (*E. coli*), *aspC* (*E. coli*), *tyrB* (*E. coli*), and *pdh* (*Thermoactinomyces intermedius*). Single colonies were picked to inoculate in LB medium and incubate at 37 °C overnight. Cells were then washed and resuspended in 3 mL M9 minimal medium in test tubes supplemented with 4 g/L NH_4Cl, 5 g/L glucose, 0.1 mM IPTG, and around 3.5g/L homophenylpyruvate (20 mM). The initial $OD_{600\ nm}$ is around 1. After incubation at 37 °C for 24 h, 190 μL culture was taken from each sample and mixed with 10 μL 10N HCl. The acidified samples were analyzed as o-phthaldialdehyde (OPA) derivatives by HPLC.

Kinetic Parameter Characterization. The enzymes GDH, GDH F18, and GDH F18 T195A were purified as follows: plasmid pZElac_hisGDH, pZElac_hisF18, and pZElac_hisF18TA were transformed in XL1 Blue cells. The

transformants were cultured in 40 mL LB medium containing 100 mg/L ampicillin. After the cells reached midlog phase, 1 mM IPTG was added to induce protein expression followed by incubation at 30 °C overnight. The cells were collected by centrifugation and the recombinant proteins were purified using His-Spin Protein Miniprep kit (Zymo research Corporation, CA) according to the manufacturer's instructions. The purified proteins were checked by SDS-PAGE for homogeneity and quantified by Bradford assay (Bio-Rad, Hercules, CA).

The dehydrogenase activity was measured and the kinetics parameters were obtained using previously reported methods.[7]

■ ASSOCIATED CONTENT

Supporting Information

The plasmids and primers used in this study (Table S1 and S2). Enzyme assays showing the cofactor specificity of the GDH variants (Figure S1). The performance of F18 T195A in keto acid feeding experiemtns (Figure S2). This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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