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Note

Discrimination of Aliphatic Substrates by a Single Amino Acid Substitution in *Bacillus badius* and *Bacillus sphaericus* Phenylalanine Dehydrogenases

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Replacement of glycine by serine at positions 123 and 124 of phenylalanine dehydrogenases from *Bacillus badius* and *Bacillus sphaericus* respectively strikingly decreased enzyme activity toward aromatic amino acids and resulted in an elevation of relative activity toward aliphatic amino acids. The mutant from *B. badius* preferentially dehydrogenated branched-chain amino acids, while that from *B. sphaericus* acted on amino acids with straight-chain amino acids.

Key words: phenylalanine dehydrogenase; site-directed mutagenesis; *Bacillus badius*; *Bacillus sphaericus*

Phenylalanine dehydrogenase (PheDH) [EC 1.4.1.20] is an NAD^+-dependent oxidoreductase that catalyzes the oxidative deamination of L-Phe and the reductive amination of related α-keto acid analogs. It belongs to a family of amino acid dehydrogenases that consists of glutamate dehydrogenase (GluDH), leucine dehydrogenase (LeuDH), and valine dehydrogenase (ValDH). We have found that PheDH from *Bacillus sphaericus* is an important catalyst for the synthesis of natural[1)] and non-natural amino acids, some of which are constituents of pharmaceuticals, by the reductive amination reaction.[2,3)]

Based on a molecular modeling using *Clostridium symbiosum* GluDH structure (PDB id, 1BGV),[4)] Gly-124 and Leu-307 residues in *B. sphaericus* PheDH have been proposed to cause the difference in substrate recognition between PheDH and LeuDH. The corresponding residue with Gly-124 of *B. sphaericus* PheDH is alanine in natural branched-chain amino acid dehydrogenases, LeuDH from *Bacillus stearothermophilus*[4,5)] and ValDH from *Streptomyces coelicolor*.[6)] We have constructed a single mutant of *B. sphaericus* PheDH, in which Gly-124 was replaced by Ala (Bs-G124A).[7)] This substitution enhanced enzyme activity toward aliphatic substrates as compared to the wild-type enzyme, but the single mutant still showed the highest activity toward L-Phe among the tested substrates, even though detailed steady-state kinetic analysis showed significantly higher specificity constants toward aliphatic substrates than aromatic ones.[8)]

Homocystinuria (HCU), caused by cystathionine β-synthase deficiency, is an important inborn error of metabolism leading to noticeable increases concentrations of L-methionine in blood and of L-homocystine in urine.[9)] In several countries, newborn screening for HCU is carried out using blood specimens as well as the diagnosis of phenylketonuria.[10)] PheDH from *Bacillus badius* has been successfully used in the diagnosis of phenylketonuria in Japan for more than a decade.[11–13)] We have screened for methionine dehydrogenase in nature with limited success,[14)] but generation of a novel amino acid dehydrogenase with substrate specificity for L-Met might become available for a newborn screening program for HCU.

Gly-124 of *B. sphaericus* PheDH is a critical residue for substrate discrimination. The sequence of *B. sphaericus* PheDH shows high homology to that of *B. badius* PheDH among PheDHs. Especially, position 124 of *B. sphaericus* PheDH is entirely identical with position 123 of *B. badius* PheDH. In this study, we constructed two single mutants, Gly123Ser of *B. badius* PheDH and Gly124Ser of *B. sphaericus* PheDH respectively, by site-directed mutagenesis, and evaluated the effects of these replacements on substrate specificity in detail. We herein report that the replacement of Gly by Ser dramatically improve enzyme activity toward aliphatic substrates as compared to single mutant Bs-G124A.

To produce protein fused with 6× His-tag at the N-terminus, the pUC18 vector was modified as follows: The fragment of pRSET-B vector (Invitrogen Co., Carlsbad, CA) was amplified by PCR using primers 5′-gccgaattcttaagaaggagatatacata-3′ and 5′-gctcggatccttatcgtcatcgtc-3′. The DNA fragment encoded a ribosomal binding site, an initiation codon, and the 6× His-tag region derived from pRSET-B with additional *Eco*R I and *Bam*H I sticky ends at the 5′ and 3′ termini. It was connected with pUC18 digested with *Eco*R I and *Bam*H I restriction enzymes. After sequence verification, it was designated pUC18His, and was used as an expression vector throughout this study. Wild-type PheDH genes from *B. badius*[15,16)] and *B. sphaericus*[17,18)] were subcloned into the *Bam*H I and *Pst* I sites of pUC18His, and these plasmids were named pBb and pBs, respectively.

Single mutants of Bb-G123S from *B. badius* and of Bs-G124S from *B. sphaericus* were constructed using a QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following primers and complements were used: Bb-G123S, 5′-cgtttctatacaagtactgatatggg-3′ and 5′-cccatatcagtacttgtatagaaacg-3′, and Bs-G124S, 5′-cgattttacacaagtactgacatgggg-3′ and 5′-ccccatgtcagtacttgtgtaaaatcg-3′. The products were transformed into *E. coli* JM109. The transformants were grown at 37 °C for 12 h in LB broth, pH 7.5, supplemented with 50 μg/ml of ampicillin, and then 0.5 mM of isopropyl-β-D-

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Table 1. Relative Activities of Wild-Type and Mutant PheDHs with Aromatic and Aliphatic Substrates

Substrates	Relative activity (%)				
	B. badius		*B. sphaericus*		
	Wild-type[14]	G123S	Wild-type[16]	G124A[7]	G124S
L-Phenylalanine	100 (71.6 U/mg)[b]	18 (2.16 U/mg)[b]	100 (114 U/mg)[b]	100 (4.0 U/mg)[b]	63 (1.09 U/mg)[b]
L-Tyrosine	9.0	—[a]	72	7.8	23
L-Leucine	3.0	100	1.3	57	67
L-Isoleucine	0.2	89	0.45	89	70
L-Norleucine	19	72	3.9	87	86
L-Valine	4.0	71	1.4	52	76
L-Norvaline	5.0	64	1.3	80	100
L-Methionine	8.0	23	3	67	98
L-Ethionine	7.0	6.4	3.1	—	39

Enzyme activity was measured at a concentration of 10 mM, except for L-tyrosine, at 4 mM.
[a]No enzyme activity for L-tyrosine was detected.
[b]Parentheses show specific activities.

thiogalactopyranoside was added to the medium and the cells were further cultivated for 12 h at 30 °C to induce the enzyme protein.

Each cell paste was suspended in 5 times the volume of 20 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl, 5 mM 2-mercaptoethanol and 25 mM imidazole (lysis buffer), and was disrupted by sonication for 20 min at 4 °C with a Kubota Ultrasonicator Insonator model 210 (19 kHz, Kubota, Tokyo). Cell debris was removed by centrifugation at 28,400 × *g* for 20 min at 4 °C, and the supernatant was used as a cell-free extract. The cell-free extract was applied to a Chelating-Sepharose Fast Flow column (column volume, 10 ml) equilibrated with lysis buffer. After a wash with 20 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl, 5 mM 2-mercaptoethanol, and 75 mM imidazole, the adsorbed enzyme was eluted with 20 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl, 5 mM 2-mercaptoethanol, and 500 mM imidazole. The enzyme was dialyzed against 20 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol overnight.

The oxidative deamination reaction, catalyzed by mutants, was assayed by measuring the reduction of NAD^+ at 340 nm in a 1 cm light path using a poly(methylmethacrylate) (PMMA) cuvette. The enzyme assay was performed as described previously.[17] One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min in oxidative deamination. The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), or from the absorbance at 280 nm with bovine serum albumin as the standard.

Bb-G123S and Bs-G124S were purified to electrophoretically homogeneous as His-tag fusions. In both cases, the molecular masses of the subunits were estimated to be 43 kDa by SDS–PAGE, and were higher than that of the original. The optimum temperature (65 °C), thermal stability (55 °C), and optimum pH (pH 10.4) of Bb-G123S were the same as those of the wild-type from *B. badius*.[15] On the other hand, these properties of Bs-G124S, optimum temperature (60 °C), thermal stability (50 °C), and optimum pH (pH 10.4), were slightly different from those of the wild-type from *B. sphaericus*.[17] Since these are relatively modest changes as compared to the wild-type enzymes, they have little impact on substrate specificities.

Table 2. Steady-State Kinetic Parameters of Wild-Type and Mutant of *B. badius* PheDH

Substrates	Wild-type[19]			G123S		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
L-Phenylalanine	0.087	39	448	227	1.55	0.0068
L-Tyrosine	8.6	34	3.9	ND[a]	ND[a]	ND[a]
L-Leucine	ND	ND	ND	3.3	1.54	0.47
L-Isoleucine	ND	ND	ND	3.8	1.48	0.39
L-Norleucine	ND	ND	ND	8.1	1.60	0.20
L-Valine	ND	ND	ND	10.7	1.67	0.16
L-Norvaline	ND	ND	ND	8.6	1.47	0.17
L-Methionine	ND	ND	ND	9.7	0.559	0.058

[a]ND, not determined.

Substrate specificity toward various aromatic and aliphatic amino acids of the single mutant enzymes was examined. The relative activities are shown in Table 1. Both single mutants, Bb-G123S and Bs-G124S, showed high activity toward a wide variety of aliphatic substrates. This tendency of Bb-G123S was more prominent than that of the single mutants, G124A and G124S, of *B. sphaericus*. The Bb-G123S mutant preferentially oxidized the longer aliphatic branched-chain amino acids (as for length, L-leucine > L-isoleucine > L-valine and L-norleucine > L-norvaline, and as for branching, L-leucine > L-isoleucine > L-norleucine, and L-valine > L-norvaline), while the Bs-G124S mutant preferentially oxidized the shorter straight-chain aliphatic amino acids (as for length, L-valine > L-leucine > L-isoleucine, and L-norvaline > L-norleucine, and as for branching, L-norleucine > L-isoleucine > L-leucine, and L-norvaline > L-valine).

To evaluate the substrate specificity in detail, we examined the steady-state kinetic parameters on both single mutants, as shown in Tables 2 and 3. A dramatic decrease in the specific constant (k_{cat}/K_m value) of Bb-G123S for L-Phe was caused by the increase in the K_m value for it (Table 2). On the other hand, the specific constants for aliphatic substrates, except for L-Met, appeared to depend on the side-chain length of their substrates as well as the relative activity. No significant difference in k_{cat} values was observed between L-Phe and aliphatic substrates, except for L-Met. Thus, a difference in the substrate specificity of this mutant is

Table 3. Steady-State Kinetic Parameters of Wild-Type and Mutants of *B. sphaericus* PheDH

Substrates	Wild-type[8]			G124A[8]			G124S		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
L-Phenylalanine	1.1	68	61.8	8.1	4.2	0.52	3.7	0.78	0.21
L-Tyrosine	1.3	68	52.3	ND[a]	ND[a]	0.11[b]	7.6	0.63	0.083
L-Leucine	0.55	0.70	1.3	0.057	1.0	18	0.18	0.60	3.3
L-Isoleucine	0.14	0.14	1.0	0.11	1.1	10	0.19	0.68	3.6
L-Norleucine	0.79	3.2	4.0	0.096	1.6	17	0.45	1.0	2.2
L-Valine	2.0	0.91	0.46	0.13	0.97	7.5	0.29	0.71	2.4
L-Norvaline	3.3	1.4	0.42	0.19	1.5	7.9	0.26	0.79	3.0
L-Methionine	2.7	1.2	0.44	0.73	1.4	1.9	0.45	0.64	1.4

[a]ND, not determined.
[b]Determined from the gradient of the linear part of the curve from a plot of activity against substrate concentration.

apparently determined by the substrate binding affinity for each substrate. The specific constants for the aromatic substrates, L-Phe and L-Tyr, in Bs-G124S also dramatically decreased as compared to the wild-type of *B. sphaericus* (Table 3). This alteration agrees with the result for the single mutant Bs-G124A.[8] In addition, the k_{cat} values for the aliphatic amino acids, including L-Met, in each mutant, G124A and G124S, were approximately of the same order. Nevertheless, the specific constants for the aliphatic amino acids in Bs-G124S showed only lower levels than those in Bs-G124A. These results provide experimental evidence that the enzyme activities and the specific constants of the two mutants, Bb-G123S and Bs-G124S, have broader specificity toward aliphatic substrates than those of the single mutant Bs-G124A.[7,8]

The K_m values for NAD^+ were estimated to be 1.55 mM for Bb-G123S and 0.18 mM for Bs-G124S. The K_m value for NAD^+ of Bb-G123S was 10-fold higher than that (0.15 mM) of the wild-type,[15] but that of Bs-G124S agreed with that (0.17 mM) of the wild-type.[17] This result strongly suggests that Gly-123 of *B. badius* PheDH is important not only for discrimination between aromatic amino acids and aliphatic amino acids, but also for access of NAD^+ to its binding site in the Rossmann-fold in the C-terminal region.[19]

The replacement of Gly by Ser at positions 123 and 124 of *B. badius* and *B. sphaericus* PheDHs, then, disrupts the affinity and the specificity toward aromatic amino acids, and results in a relative elevation of the enzyme activity toward aliphatic amino acids. Both enzyme activities for aliphatic amino acids depend on the side-chain length and the structural feature (straight or branched). In PheDH from *B. badius*, substitution at this position causes not only an alteration of substrate specificity, but also a striking disruption of cofactor binding affinity. Since the relative activity and the specificity for L-Met were especially improved in the mutant of *B. sphaericus*, further systematic and random screening programs based on molecular modeling of new mutants are in progress to achieve our final goal in the diagnosis for HCU.

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