Catalytic Ability Improvement of Phenylalanine Hydroxylase from *Chromobacterium violaceum* by N-Terminal Truncation and Proline Introduction ^S

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Phenylalanine hydroxylase from *Chromobacterium violaceum* (CvPAH) is a monomeric enzyme that converts phenylalanine to tyrosine. It shares high amino acid identity and similar structure with a subunit of human phenylalanine hydroxylase that is a tetramer, resulting in the latent application in medications. In this study, semirational design was applied to CvPAH to improve the catalytic ability based on molecular dynamics simulation analyses. Four N-terminal truncated variants and one single point variant were constructed and characterized. The D267P variant showed a 2.1-fold increased thermal stability compared to the wild type, but lower specific activity was noted compared with the wild type. The specific activity of all truncated variants was a greater than 25% increase compared to the wild type, and these variants showed similar or slightly decreased thermostability with the exception of the N-Δ9 variant. Notably, the N-Δ9 variant exhibited a 1.2-fold increased specific activity, a 1.3-fold increased thermostability and considerably increased catalytic activity under the neutral environment compared with the wild type. These properties of the N-Δ9 variant could advance medical and pharmaceutical applications of CvPAH. Our findings indicate that the N-terminus might modulate substrate binding, and are directives for further modification and functional research of PAH and other enzymes.

Keywords: Phenylalanine hydroxylase, semirational design, catalytic ability, thermostability, phenylketonuria, *Chromobacterium violaceum*

Introduction

Phenylalanine hydroxylase (PAH) is an iron-dependent enzyme and converts L-phenylalanine to L-tyrosine. PAH is a member of the family of aromatic amino acid hydroxylases, which also includes tyrosine hydroxylase and tryptophan hydroxylase [1]. The amino acid sequences of mammalian PAHs are highly homologous. PAH is a homotetramer, and each subunit comprises a catalytic core domain, an N-terminal regulatory domain and a C-terminal oligomerization domain. The coenzyme tetrahydrobiopterin (BH₄) and ferrous iron are required for the catalytic activity of PAH. The crystal structure, residues responsible for activity, substrate binding and tetramerization in mammalian

PAH are well studied [2]. PAH deficiency in human results in phenylketonuria (PKU), which is an inherited metabolic disease [2]. A phenylalanine-restricted diet was the only treatment for PKU patients until a novel therapeutic method, namely, BH₄-supplementation therapy, was developed for PKU patients who are BH₄ responsive [3, 4]. It is essential to molecularly characterize PAH deficiency for each patient because BH₄ supplementation is not effective for BH₄-nonresponsive PKU [5, 6].

PAH from *Chromobacterium violaceum* (CvPAH) was studied as a model of PAH since it is a monomer that has similar structure with the subunit of mammalian PAH. The CvPAH enzyme was first purified and characterized by Nakata *et al.* in 1979 [7]. It was cloned and overexpressed in

Escherichia coli [8], and then the crystal structure of CvPAH was determined and compared with mammalian PAHs [9]. It shared approximately 24% sequence identity with human PAH and displayed higher activity and better thermal stability. The active-site triad (His-His-Glu) was strictly conserved in CvPAH, but the N- and C-terminal regions of CvPAH were different from those of mammalian PAHs, which might represent the major contributor to the differences in stability between CvPAH and mammalian PAHs. The biochemical properties and catalytic mechanism of CvPAH have triggered scientific interest and have been analyzed for decades [10–12]. Moreover, the residues related to enzymatic activity and substrate binding in CvPAH were identified using molecular modification. Tyr179 plays a role in substrate binding and the rate-determining step in catalytic processes [13], and Asp139 contributes to stabilization of the transition state by direct binding with the cofactor [14]. CvPAH displayed low L-tryptophan hydroxylation activity, and the double variant L101Y/W180F exhibited a 5.2-fold increase in activity compared to the wild type [15]. Notably, purified CvPAH packaged into red blood cells efficiently decreased the phenylalanine level in the blood of mice, while encapsulated CvPAH was sustained in the circulation to metabolize phenylalanine for 10 days [16]. However, the application of CvPAH is limited by the low specific activity compared with the high level of phenylalanine in PKU patients' blood.

Molecular modification is a promising and advanced tool for improving the property of proteins. Herein, we aim to enhance the catalytic ability of CvPAH using truncation mutagenesis and site-directed mutagenesis on the basis of molecular dynamics simulation. After screening, we selected four truncated variants and one single point variant of CvPAH to further characterize their advantages in comprehensive properties.

Materials and Methods

Plasmids, Strains, and the Production of Variants

E. coli JM109 was used for plasmid construction, and the plasmid pET-24a was employed as an expression vector. The gene encoding CvPAH was cloned into the restriction enzyme sites of BamHI and HindIII, constructing pET24-*cvpah* plasmid. Luria-Bertani (LB) medium containing 50 µg/ml kanamycin was used for plasmid construction and propagation. The solid medium contained 1.5% agar.

The pET24-*cvpah* was used as the template to construct mutants. The N-terminal deletion was carried out by Megaprimer PCR of

Whole Plasmid (MEGAWHOP). The point mutation variants were constructed using the site-directed mutagenesis method. Primers used for site-directed mutation and truncation (Table S1) were synthesized by GENEWIZ Biotech Co., Ltd. (China). The sequences of the transformants were confirmed by GENEWIZ Biotech Co., Ltd.

Molecular Dynamics Simulation of CvPAH and Its Variants

Molecular dynamics (MD) simulations were carried out using the software NAMD 2.12 [17] with the Charmm27 force field [18]. The structure file of CvPAH (PDB ID: 1LTU) was downloaded from Protein Data Bank. Each protein was put into a cubic water box $70 \times 70 \times 73 \text{ Å}^3$ with a layer of water of at least 7 Å in each dimension. A cutoff of 12 Å for non-bonded interactions was applied. We performed 1 ns of water equilibration, 1,000 steps of minimization and 60 ps of heating from the 0 K up to 300 K or 325 K before each main MD. Then a 10 ns of classical Langevin MD simulation was carried out. The Langevin Piston Target was set to 1.01325. Each 10-ns long simulation calculates during 150 h on 16 Intel Xeon E5-2470 cores (32 with hyperthreading).

Expression and Purification of CvPAH and Its Variants

A single colony was cultivated into 5 ml of LB medium containing kanamycin at 37°C, with shaking at 200 rpm. Then overnight-cultured cells were transferred into 50 ml of LB medium with 1% inoculum and incubated at 37°C for cell growth. When OD_{600} reached 0.6–0.8, 0.6 mM IPTG (isopropylthio-β-galactoside) was added to induce the expression of CvPAH and its variants for 24 h at 24°C.

The purification of CvPAH and its variants was performed using an AKTA purifier (GE Healthcare UK Ltd.). Harvested cells were suspended in Tris buffer (20 mM, pH 7.5) and then were sonicated on ice. Cell lysates were precipitated by 50–70% ammonium sulfate precipitation. Samples were loaded onto a HiTrap DEAE column (GE Healthcare UK Ltd.) after being dialyzed against Tris buffer (20 mM, pH 7.5) and were eluted from the column using the concentration gradient buffer containing 0–1.0 M NaCl. The protein concentrations were determined by the Bradford method.

Activity and Kinetic Parameter Determination of CvPAH and Its Variants

CvPAH activity was determined by the increase of L-tyrosine, which was converted from L-phenylalanine. The reaction was performed in HEPES buffer (100 mM, pH 7.5) containing 1 mM L-phenylalanine, 5 mM DTT, 5 µM FeSO_4 , 0.2 mM DMPH₄ (6,7-dimethyl-5,6,7,8-tetrahydropterin) at 37°C. After 10 min, methanol was added to terminate the reaction. The concentrations of L-tyrosine and L-phenylalanine were detected by high-pressure liquid chromatography (HPLC) with a C18 column (La Chrom, 5 µm, $4.6 \times 250 \text{ mm}$), and the excitation and emission wavelength were 265 nm and 315 nm, respectively. One unit was defined as

the amount of CvPAH that produced 1 μmol L-tyrosine at 37°C per min.

Kinetic parameters of CvPAH and its variants were determined by the substrates over a concentration range from 0.2 to 1.0 mM in HEPES buffer (100 mM, pH 7.5) at 37°C. The values of K_m and k_{cat} were calculated through the Lineweaver-Burk method using the program GraphPad Prism 5. The values are presented as the means of three independent experiments.

Assay of Temperature and pH Effects on the Activity of CvPAH and Its Variants

The optimal temperatures for the activity of CvPAH and its variants were determined in Tris buffer (20 mM, pH 7.5). The activity of CvPAH and its variants was measured at various temperatures (20–60°C), and the highest activity was defined as 100%. To detect thermal inactivation, the purified CvPAH and its variants were analyzed by measuring the remaining activity after treatment at 50°C for the corresponding time. Analysis of the optimal pH was performed in corresponding pH buffers: pH 6.0–8.5 in 50 mM $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer, pH 9.0–10.0 in 50 mM $\text{Na}_2\text{CO}_3\text{--NaHCO}_3$ buffer. The values are presented as the means of three independent experiments.

Results

Semirational Design and Screening for CvPAH Variants with Improved Catalytic Ability

MD simulations (RMSD and RMSF) were performed on the basis of the crystal structure of CvPAH (PDB ID: 1LTU), and a higher value indicates more flexibility [19, 20]. RMSD results suggested that the CvPAH was unstable under 325 K compared with 300 K (Fig. 1A). Moreover, the average RMSF values indicated that the N-terminal region containing approximately 10 residues (MNDRADFVVP)

was very flexible (Fig. 1B). Subsequently, four N-terminal truncated variants of CvPAH, N- Δ 7, N- Δ 8, N- Δ 9 and N- Δ 10, were constructed as candidates to enhance the catalytic properties of CvPAH.

Proline substitution is an effective strategy to improve protein stability, since proline is less flexible than the other amino acids [21, 22]. Aiming to enhance the thermal stability of CvPAH, residues in the most flexible region and on the surface of the enzyme were selected to be replaced by proline based on the crystal structure and RMSF results (Fig. 1B). Seven variants of CvPAH with a single point mutation (A24P, I102P, H126P, A168P, A215P, T236P, and D267P) were constructed by sited-directed mutagenesis.

The expression levels of CvPAH and its variants were estimated by SDS-PAGE, and crude enzymes adjusted to have the same concentrations were used for screening. The results of screening showed that D267P and all truncation variants might exhibit improved catalytic ability (Table S2). Therefore, eleven mutants were constructed, and five variants were purified for further study of the biochemical characterization according to the screening results (Table S2).

Biochemical Properties of CvPAH Variants

To detect the effects of these mutations on the enzymatic activity, the wild-type CvPAH, four truncated variants and one single point variant were purified (Fig. 2A) and characterized. All of the CvPAH variants have the same optimum temperature of 40°C as the wild-type CvPAH (Fig. 2B). The results of thermal inactivation of CvPAH and its variants showed that the D267P variant followed by the N- Δ 9 variant exhibited the best thermal stability (Fig. 2C). The wild type retained 18% activity after treatment for

Fig. 1. MD simulation analysis of the wild-type CvPAH.

RMSD (A) and RMSF (B) analysis under the conditions of 300 K and 325 K based on the crystal structure (PDB ID: 1LTU). Residues selected for proline substitution were indicated.

Fig. 2. Effect of temperature and pH on the enzymatic activity of CvPAH and its variants.

(A) SDS-PAGE analysis of purified CvPAH and its variants. Lane M was the standard marker protein, and the molecular weight ranged from 20.1~200 kDa. (B) The optimum temperature was determined based on enzyme activity at the corresponding temperature, and the highest activity was plotted as 100%. (C) Thermostability was measured after treatment at 50°C for the indicated time, and the residual activity was plotted as a percentage of the initial activity. (D) The effect of pH on the activity of CvPAH and its variants was measured under the corresponding pH conditions, and the highest activity was plotted as 100%. The reactions were performed in corresponding pH buffers: pH 6.0–8.5 in phosphate buffer, pH 9.0–10.0 in carbonate buffer. Wild type: filled circle; N-Δ7: empty circle; N-Δ8: filled triangle; N-Δ9: filled star; N-Δ10: empty star; D267P: empty diamond.

30 min. D267P retained 38% activity, and N-Δ9 retained 23% activity.

Analysis of the optimum pH was performed in the corresponding pH buffers. The wild-type CvPAH and variants N-Δ7, N-Δ8, D267Ps showed their highest activity at pH 8.5 (Fig. 2D). Unexpectedly, the optimum pH of N-Δ9 and N-Δ10 shifted 0.5 units to 8.0. The N-Δ9 variant showed better activity under pH 7.0 and 7.5 than the other variants and the wild type, which should contribute to its application in the medical field as it has higher activity under the neutral conditions.

Analysis of Kinetic Parameters of CvPAH and Its Variants

The specific activity and kinetic parameters of the purified variants were determined and summarized in Table 1. The specific activity of the point mutation variant was decreased by 24% of that of wild type. The lower specific activity of the D267P variant was due to a slight decrease in catalytic efficiency; however, the K_m value of D267P was lower than that of the wild type, suggesting a better affinity for substrate. In contrast, the specific activity of all truncated variants was a greater than 25% increase compared with the wild type. Surprisingly, the N-Δ10

Table 1. Kinetic parameters of CvPAH and its variants.

	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	The specific activity (U/mg)
CvPAH	0.097 ± 0.0012	2.4 ± 0.3	24.7	377.2 ± 2.2
N-Δ7	0.152 ± 0.0014	3.9 ± 0.2	25.7	486.9 ± 1.6
N-Δ8	0.183 ± 0.0015	4.6 ± 0.4	25.1	491.0 ± 3.5
N-Δ9	0.249 ± 0.0014	6.8 ± 0.3	27.3	529.4 ± 1.3
N-Δ10	0.330 ± 0.0014	9.8 ± 0.3	29.7	472.4 ± 2.6
D267P	0.068 ± 0.0015	1.6 ± 0.1	23.5	323.7 ± 1.5

variant showed a markedly enhanced catalytic efficiency due to a 308% increase in k_{cat} . The N-Δ9 variant exhibited improved thermostability and better catalytic activity compared with the wild-type CvPAH, whereas the thermostability of the other truncated variants was slightly

decreased or similar compared with the wild type (Fig. 2C and Table 1).

Molecular Dynamics Simulation of CvPAH and Its Variants

The N-terminal loop containing 10 residues was located away from the spherical structure of the enzyme (Fig. 3A). To understand why N-terminal truncation resulted in improved catalytic activity, MD simulation of CvPAH was assayed. The N-terminus was constantly swinging during MD simulation (Fig. 3B), which was consistent with RSMF results (Fig. 3D). The conserved active site triad (His143-His138-Glu184) was located around a cavity (Fig. 3B) that was thought to be the entrance for the substrate and the product. The lack of an N-terminus might facilitate substrate and product traffic via the entrance cavity, causing the enzyme activity to increase. Additionally, MD

Fig. 3. Flexible structure of the N-terminal region.

(A) Structure of CvPAH. The N-terminal region containing 10 residues is presented in lavender, and the conserved active site triad (His143-His138-Glu184) was displayed with sticks in red. (B) Top view of the active center. The swinging of the N-terminal region during the MD simulation assay was represented by cyan. The 3D structure was illustrated using the program PyMOL based on its crystal structure. (C) RMSD analysis of N-Δ9 at 325 K compared with the wild type. (D) RMSF analysis of N-Δ9 at 325 K compared with the wild type.

simulations were performed on the N- Δ 9 variant to reveal the insight of its improved thermostability. RMSD results suggested that N- Δ 9 was more stable than the wild type at 325K (Fig. 3C), which was consistent with the results of

Fig. 4. MD simulation analysis of the variant D267P.

(A) RMSD analysis of D267P at 325 K compared with the wild type. (B) RMSF analysis of D267P at 325 K compared with the wild type, and the inner illustration indicated the region around the 267th residue. (C) 3D structure of D267P and the region around the Pro267 that is represented by sticks. The C-terminal loop was shown in red. The figure was prepared using the program PyMOL.

thermostability (Fig. 2C). The average RMSF values of N- Δ 9 were lower than that of the wild type, however, the N-terminal 22-26 amino acids of the N- Δ 9 variant became much more flexible (Fig. 3D).

Compared to the wild type, the single point mutant D267P exhibited reduced conformational flexibility at a high temperature (Fig. 4A). As shown in Fig. 4B, the region around the 267th residue in D267P was less flexible than that in the wild type. The mutated residue was located in the C-terminal loop next to an α -helix (Fig. 4C), which might be an indirect effect associated with the thermostability of D267P. The introduced proline increased the rigidity of the region and potentially limited the C-terminal loop movement, thereby further improving the thermal stability of D267P.

Discussion

In this study, we constructed CvPAH variants using two strategies, including N-terminal truncation and proline substitution. Among these variants, a single point mutant had better thermostability, and four truncated mutants had increased activity. The mutation-induced enhancement of the enzymatic activity was elaborated using MD stimulation.

Flexible residues on the surface were selected for proline substitution to enhance the stability of the enzyme given that proline restricts protein flexibility. In the case of CvPAH, seven flexible residues were chosen to be replaced by proline. Only one variant, D276P, had better thermal stability according to the screening results. It is possible that the proline substitution occurred in various CvPAH domains. The Asp267 residue is located in the C-terminal region, and the other target residues are located in the catalytic core domain (36-256 amino acids) or the N-terminal domain. Similar results have demonstrated that introducing proline substitutions in the different domains stabilized α/β -hydrolases at different denaturation states [23]. In addition, we found that the N- Δ 10 variant showed lower thermal stability than the N- Δ 9 variant likely also due to the deletion of the proline, the tenth residue at the N-terminus.

The N-terminal flexible loop of CvPAH was truncated to address its function for the first time. All of the truncated variants showed higher catalytic efficacy, indicating that the N-terminus modulates the catalytic activity but was not involved in the catalysis process. The N-terminal region of a protein or enzyme generally exhibits a function related to a regulatory role or substrate affinity. The N-terminal 10 residues of *Thermotoga maritima* CE7 acetyl esterase played

a role in substrate specificity and was important for thermal stability [24]. The N-terminal 36 residues of the murine 5-aminolevulinate synthase truncated product resulted in a lower thermal stability and increased k_{cat} value, and its N-terminus was involved in a cell-specific regulatory mechanism [25]. We proposed that the N-terminal 10 residues of CvPAH play a role in substrate binding or regulation given that the truncation decreased substrate affinity. The N-terminal deletion may have caused some distinct changes in the microenvironment of the access tunnel or the active site region, which led to a lower affinity value (K_m). The truncated variants exhibited enhanced catalytic efficiency due to the increase in k_{cat} , which offset poor affinity for the substrate (Table 1). Similar findings have been observed [23, 24], which sometimes occur in the molecular modification of enzymes. However, the observation that the catalytic ability of CvPAH increased in the N-terminal truncation needs to be further studied.

Two N-terminal truncated variants shifted the pH profile from 8.5 to 8.0. One variant, N-Δ9, possessed better catalytic activity even under the neutral environment and higher thermal stability, thereby improving its suitability and efficiency in medical and pharmaceutical applications.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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