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Searching for Substituted Residues to Narrow NADPH-Reaction Specificity in *p*-Hydroxybenzoate Hydroxylase: Combination of a Single Mutant Database and Cluster Analysis

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Abstract. To design *p*-hydroxybenzoate hydroxylase (PHBH) mutants that show narrower NADPH-reaction specificities than the wild-type *Pseudomonas fluorescens* do, we substituted 53 conserved residues with a high degree of conservation, at 389 sites among 92 PHBH enzymes with other amino acids. These substitutions produced a mutant database with 365 active and stable single mutants including 64 mutants that showed a narrower NADPH-reaction specificity than the wild-type do. In particular, some R44X, Y181X, L199X, or L299X substitutions resulted in a considerably narrower NADPH-reaction specificity than the specificity shown by the wild type. Fifty-three of the conserved residues could be classified into 4 groups: α, β, γ, and δ. Subsequently, a qualitative and quantitative analysis regarding the effects of the substitution of the conserved residues on the NADPH-reaction specificity and the main activity indicated that the R44, Y181, L199 and L299 sites belonged in the β, γ, or δ groups. The experimental results validate the use of the mutant database for the conserved residues, and suggest a new strategy, using a combination of the mutant database plus a rational design method, for searching target conserved residues for substitution to improve the properties of a given protein.

Keywords: Mutant database, *p*-hydroxybenzoate hydroxylase, NADPH-reaction specificity.

1. Narrowing NADPH-reaction Specificity of *p*-hydroxybenzoate Hydroxylase

p-Hydroxybenzoate hydroxylase (PHBH) catalyzes the hydroxylation of *p*-hydroxybenzoate to protocatechuate (main activity) (Fig. 1) [1, 2]. The main activity of PHBH is similar to that of other FAD-containing aromatic compound oxygenases (FAOs) [3]. On the other hand, from a clinical viewpoint, various assay methods to diagnose liver function have been proposed. One popular method includes the determination of *p*-hydroxybenzoate, the reduction product of cholinesterase, using PHBH [4]. However, PHBH also exhibits protocatechuate-dependent NADPH/NADH oxidase activity (sub-activity) through an unknown mechanism (Fig. 1), which reduces the accuracy of the method. It is thus desirable to reduce the sub-activity of the enzyme, i.e., to narrow the NADPH-reaction specificity.

Recently, we developed a new method for designing simultaneously multiple properties of a given protein by using a mutant database with a combination of selected mutants [5]. In the previous study, we substituted the Cys/Met residues of PHBH with naturally occurring amino acids to construct a mutant database combining systematically selected mutants, which produced quadruple mutants with improved enzymatic properties including NADPH-reaction specificity. However, a problem that remained was that the NADPH-reaction specificity was not necessarily fully reduced.

In this study, we inferred conserved residues of PHBH to narrow the NADPH-reaction specificity, substituted these conserved residues with other amino acids to obtain a mutant database, and thereby produced unexpected single mutants with narrower NADPH-reaction specificity compared to the wild type. Subsequently, we summarized the effects of location and other properties of the conserved residues on the

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properties of the mutants to discover a novel strategy for searching for substituted target residues based on the mutant database.

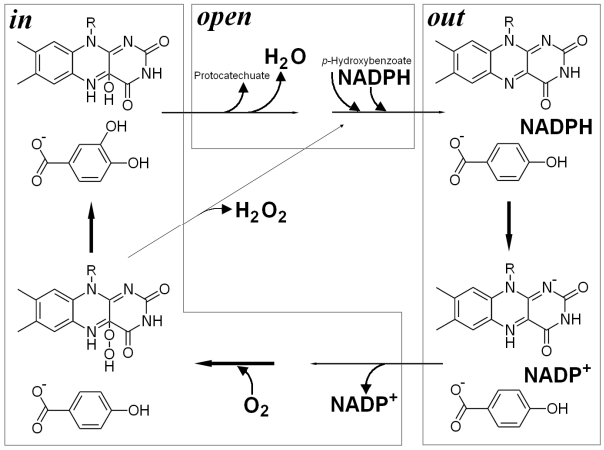

Fig. 1: Conversion of p-hydroxybenzoate to protocatechuate. The figure represents the chemical reactions and the ligand interactions in the catalytic cycle of PHBH. The boxes illustrate 3 types of open, out, and in conformations for the PHBH molecule.

2. Selection of the Conserved Amino Acid Residues

To identify conserved residues among the amino acid sequences of naturally occurring PHBH, we retrieved PHBHs from the DDBJ, which yielded 92 PHBH sequences. An unrooted phylogenetic tree was constructed from 389 residues of the 92 PHBH sequences by the maximum likelihood method using MEGA software (Fig. 2). This revealed 4 clades, which clearly indicated the separation of PHBHs originating from 3 classes of proteobacteria group, i.e., α, β, and γ proteobacteria, following the evolutionary branching of proteobacteria and other bacteria.

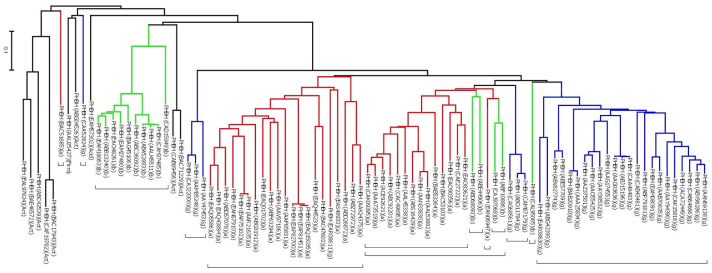

Fig. 2: An unrooted phylogenetic tree for PHBHs. Branches with PHBH derived from α proteobacteria are in red, from β proteobacteria in light green, from γ proteobacteria in blue, and from other microorganisms such as δ proteobacteria, and Gram-positive bacteria in black. The tree was illustrated using MEGA software.

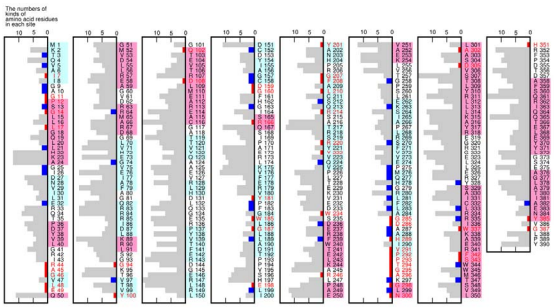

Fig. 3. Naturally occurring substitutions in PHBH amino acid sequences. Amino acid residues in the χ, ψ and other groups are in red, blue, and gray, respectively. α-Helices and β-sheets are in pink and light blue, respectively.

The 92 PHBH sequences were aligned using MEGA software, which indicated 49 sites where only 1 type of amino acid was observed, and 43 sites where 2 types of amino acids were present (Fig. 3). To construct a single mutant database of substitutions with naturally occurring amino acids, we selected all 49 residues in the *X* group and 4 residues (G9, L199, S212, and L299) from the *Ψ* group as target positions, and designated these residues as "conserved residues". These conserved residues consist of 12 Gly, 3 Ala, 1 Val, 5 Leu, 1 Ile, 2 Ser, 1 Gln, 3 Pro, 2 Phe, 5 Tyr, 3 Trp, 4 Asp, 2 Glu, 2 His, no Lys, and 5 Arg.

We analyzed the distances among the 53 conserved sites with a cluster analysis method using Rstudio software, which yielded 3 taxa: the α, β, and γ groups (Fig. 4). Five residues outside these 3 groups were combined to create the δ group. Fig. 5 illustrates the separate locations of the 53 conserved residues in the α,

β, γ, and δ groups for a monomer of PHBH, which usually forms a dimer. Fourteen conserved residues in the α group were observed to cluster around FAD; 14 in the β group appeared to aggregate around a substrate-binding part; and 20 in the γ group were mainly located around the P293-loop. However, no correlation of the conserved residue distribution between the FAD-binding domain and the α group or between the substrate-binding domain and the β group was observed. For example, 18 conserved residues observed on the FAD-binding domain were divided as follows: 9 residues from the α group, 7 from the γ group, and 2 from the δ group.

We believe that presumably functional residues that are directly/indirectly related to the activity, conformation, or other functions of a given enzyme exist, although they may not be conserved residues. We thus selected the presumable functional residues based on previous studies on PHBH. We selected 5 residues (S13, E32, R33, R42, and V47) and combined them into the ε group, because R44, A45, G46, Q102, D286, L299, and N300 had already been selected as conserved residues. Nine residues, A34, S35, A36, D37, Y38, V39, Q40, G41, and I43, were named the ζ group and 5 residues from the η groups were selected in addition. Fig. 5 depicts the locations of 19 presumable functional residues.

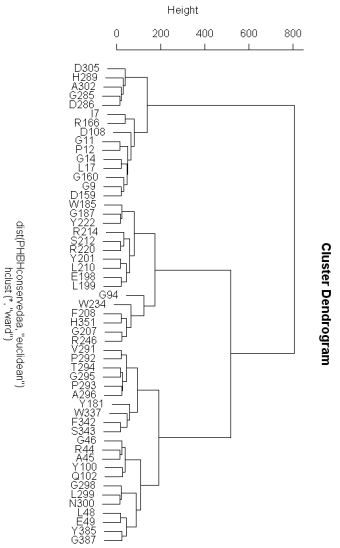

Fig. 4: A dendrogram of 53 conserved residues based on the position of any 2 residues in the tertiary structure of PHBH. This dendrogram was conditioned as euclidean and ward by using Rstudio software.

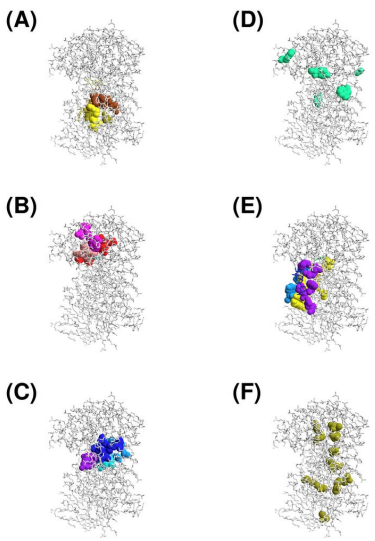

Fig. 5: Location of 53 conserved residues or 19 presumable functional residues in the tertiary structure of PHBH. (A) Conserved residues in the sub-taxa, a, b, and c of the α group, are shown in yellow, dark yellow, and brown, respectively; (B) sub-taxa a, b, and c of the β group in red, pink, and light pink; (C) sub-taxa a, b, c, and d of the γ group in light blue, blue, dark blue, and purple, respectively; and (D) the δ group in mint green. (E) Presumable functional residues in the ε, ζ, and η groups are shown in yellow, light blue, and purple, respectively. (F) Cys and Met residues are in ocher. The PHBH molecule is shown based on 1pbe. The sequence logo was generated using 92 PHBH amino acid sequences extracted from DDBJ. Graphics were illustrated using DeepView software.

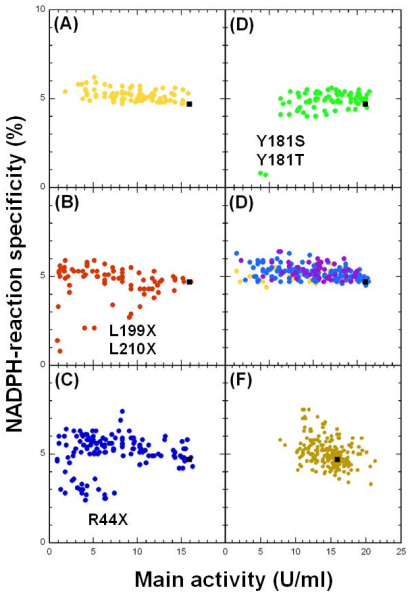

Fig. 6: Distribution of the main activity and the NADPH-reaction specificity of single mutants. In the distribution diagrams, (A) single mutants at sites in the α group are in yellow; (B) the β group in red; (C) the γ group in blue; (D) the δ in mint green; (E) the ε, ζ, and η groups in yellow, light blue, and purple, respectively; and (F) Cys and Met residues in ocher.

3. Construction of a Single Mutant Database

We used PHBH from *Pseudomonas fluorescens* NBRC 14160 as the model enzyme for our analysis. Substitution of each conserved residue with one of the other 19 naturally occurring amino acids resulted in 365 active and stable single mutants. The main activity and sub-activity of the single mutants were examined and the NADPH-reaction specificity was calculated, providing a database of single mutants for the conserved residues. Fig. 6 depicts the distribution of the main activity and the NADPH-reaction specificity of the single mutants for the conserved residues in the α, β, γ, and δ groups, for the presumable functional residues in the ε, ζ, and η groups, and the Cys/Met residues. All possible substitutions of the 14 conserved residues belonging to the α group were carried out, resulting in 79 active and stable single mutants. No single mutant at G14 or G160 was obtained, and only 1 single mutant at G9, G11, and D286 was acquired. Among the single mutants was obtained, no mutant exhibited a main activity higher than the activity of the wild-type enzyme, and no mutant exhibited an NADPH-reaction specificity narrower than specificity exhibited by the wild type.

With regard to the NADPH-reaction specificity, the single mutant database for the Cys/Met sites contained 65 variants with NADPH-reaction specificity, which was narrower than that of the wild type; the narrowest NADPH-reaction specificity value was 2.1% compared to 4.7% of the wild type. In contrast, substitutions at L199, L210, R44, and Y181 provided useful mutants that could be used as templates for further improvement of the NADPH-reaction specificity value to less than 2%. The single mutant database for the conserved residues revealed some unpredictable behavior, such as for some unexpected mutants, e.g., Y181S and Y181T, which had NADPH-reaction specificity that was narrower than the specificity exhibited by the wild type. The mutant database constructed on the basis of the conserved sites demonstrated the possibility of narrowing the NADPH-reaction specificity. Indeed, it was found that even substitution of the conserved residues would disrupt important properties, i.e., the main activity or conformational stability.

Hence, 64 single mutants of 365 active and stable single mutants exhibited NADPH-reaction specificity narrower than that of the wild type. In particular, single mutants at positions L199, L210, R44, and Y181 exhibited considerably narrower NADPH-reaction specificity: i.e., Y181S (0.7%), Y181T (0.8%), and L199Y (0.8%). These 4 residues that exhibited the NADPH-reaction specificity narrower than that of the wild type belonged in the β, γ, or δ classes, suggesting that cluster analysis of conserved residues would be useful for searching target residues from the conserved residues before substitution, because such a technique would be able to reduce size of the mutant library containing mutants with desired properties.

4. Three-dimensional (3D) Structures of the Conserved Residues in Mutants

Using DeepView software, we examined the structural characteristics of the residues that interacted with FAD, NADPH, and *p*-hydroxybenzoate, or Y181 as shown in Fig. 7A, B, C. Although Y181 is a conserved residue and the substitution of Y181 resulted in considerable alteration of the main activity or the NADPH-reaction specificity, no report regarding Y181 has been published. Studies were therefore focused on the conformations and interactions of Y181 and residues around Y181. These studies revealed that Y181 is present at the end of the β-sheet14 as *in* and *open* conformations; hydrogen bonds are formed not only between a side chain of Y181 and a side chain of S270 but also between a main chain of the Y181 and a main chain of L268; and that locations of the β-sheet15 differ considerably between *in* and *open* conformations (Fig. 1, Fig. 7C). We analyzed the interaction between given the amino acids at Y181 and the residues around Y181 by using computational analysis, indicating that the substitution of Y181 with Ser eliminated the hydrogen bond between OH_{Y181} and OG_{S270}, subsequently resulting in the formation of new hydrogen bonds: $OG1_{S181}$ and O_{L268}, and $OG1_{S181}$ and O_{F183}. This result suggested that the substitution of Y181 with Ser stimulates the stabilization of the *in* conformation when compared to that of the wild type, leading to H_2O_2 being prevented from generating as an *open* conformation, NADPH from combining in the *out* conformation, and finally, improving the NADPH-reaction specificity.

5. A Strategy to Search Substituted Residues

Ideally, a single mutant database should be constructed using comprehensive substitutions for all residues of the given enzyme. However, our previous and present studies on a single mutant database for non-conserved and conserved residues indicated that the substitution of selected residues could produce a

useful mutant database, and thus, knowledge of how target substituted residues should be selected is important to improve enzyme properties. In this study, some residues in the δ group could be substituted and enable production of mutants with diverse properties, suggesting that it would be more effective to search for target substituted residues by searching for conserved residues located in the external region of the protein molecule by using cluster analysis method.

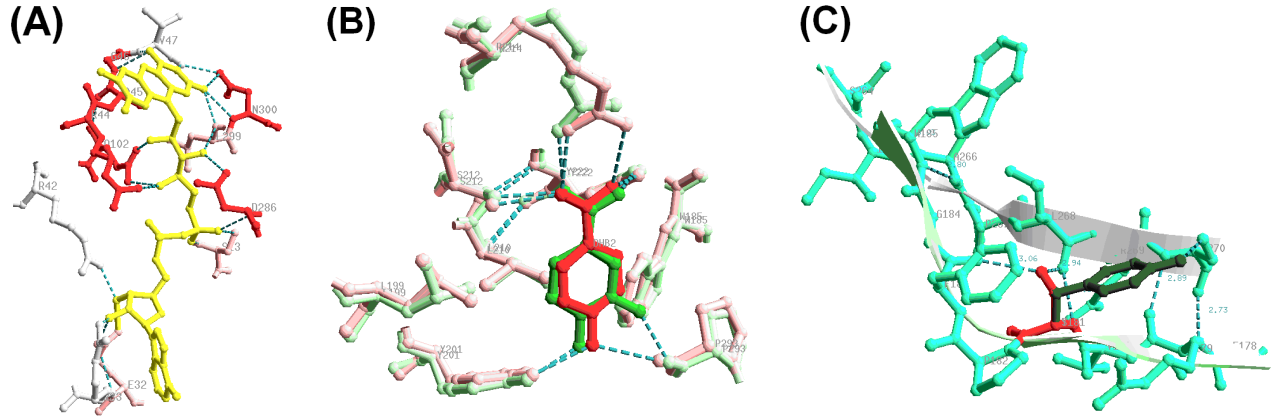

Fig. 7: Interaction of conserved amino acid residues and other components. (A) Interactions between FAD and residues. The FAD molecule is shown in yellow; R44, A45, G46, D286, and N300 residues are in red; S13, E32, and L299 residues are in pink; and R33, R42, and V47 residues are in white. Hydrogen bonds are shown in light blue. (B) Interactions between *p*-hydroxybenzoate or protocatechuate and residues. W185, L199, Y201, L210, S212, R214, Y222, P293 residues (pink) and *p*-hydroxybenzoate (red) in PHBH (1pbe) are shown, and the same residues (light green) and protocatechuate (green) in PHBH (1phh) are also depicted. (C) Interactions between Y181 (dark green) and S181 (red) residues and other residues (mint green) around Y181. Hydrogen bonds are shown in light blue with distance values. Figures were illustrated using DeepView software.

6. Materials and Methods

Comprehensive substitutions of the conserved residues in PHBH were carried out using our previous method [4]. MEGA software was used to align the amino acid sequences of 92 PHBHs or other enzymes with PHBH-like structure [6]. DeepView software was used to carry out the 3D-simulations of the substituted residues in the PHBH molecules [7].

7. References

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