Functional role of residues involved in substrate binding of human 4-hydroxyphenylpyruvate dioxygenase

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Abbreviations: HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPP, 4-hydroxyphenylpyruvate; HG, homogentisate; HPA, 4-hydroxyphenylacetate; 2-OG, 2-oxoglutarate; ITC, isothermal titration calorimetry; QM/MM, Quantum Mechanics/Molecular Mechanics; DSF, differential scanning fluorometry; MRE, mean residue of ellipticity;
Abstract

4-Hydroxylphenylpyruvate dioxygenase (HPPD) catalyzes the conversion of 4-hydroxylphenylpyruvate (HPP) to homogentisate, the important step for tyrosine catabolism. Comparison of the structure of human HPPD with the substrate-bound structure of A. thaliana HPPD revealed notably different orientations of the C-terminal helix. This helix performed as a closed conformation in human enzyme. Simulation revealed a different substrate-binding mode in which the carboxyl group of HPP interacted by a H-bond network formed by Gln334, Glu349 (the metal-binding ligand), and Asn363 (in the C-terminal helix). The 4-hydroxyl group of HPP interacted with Gln251 and Gln265. The relative activity and substrate-binding affinity were preserved for the Q334A mutant, implying the alternative role of Asn363 for HPP binding and catalysis. The reduction in $k_{cat}/K_m$ of the Asn363 mutants confirmed the critical role in catalysis. Compared to the N363A mutant, the dramatic reduction in the $K_d$ and thermal stability of the N363D mutant implies the side-chain effect in the hinge region rotation of the C-terminal helix. The activity and binding affinity were not recovered by double mutation; however, the 4-hydroxyphenylacetate intermediate formation by the uncoupled reaction of Q334N/N363Q and Q334A/N363D mutants indicated the importance of the H-bond network in the electrophilic reaction. These results highlight the functional role of the H-bond network in a closed conformation of the C-terminal helix to stabilize the bound substrate. The extremely low activity and reduction in Q251E’s $K_d$ suggest that interaction coupled with the H-bond network is crucial to locate the substrate for nucleophilic reaction.
Introduction

4-Hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) belongs to the non-heme Fe(II)/2-oxoacid dependent oxygenase family [1, 2]. It catalyzes the oxidation of 4-hydroxyphenylpyruvate (HPP) to homogentisate (HG) by using a single substrate with an internal α-keto acid moiety. The reaction is the first committed step in the tyrosine catabolic pathway (Scheme 1) [3, 4]. Hereditary defects of enzymes in this pathway may lead to metabolic diseases, including tyrosinemia, hawkinsinuria, and alkaptonuria [5-7]. Clinical treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), a potent inhibitor of HPPD, prevents the accumulation of toxic metabolites in patients with type I tyrosinemia [8-10]. HPPD is also a target for herbicide development because the reaction product HG is a critical precursor in the biosynthesis of cofactors of plastoquinones and tocopherol for photosynthesis [11-16].

Human HPPD is a dimeric protein with two domains per monomer [3, 4]. The active site is located in the C-terminal domain [17]. It possesses a conserved motif of 2-His-1-carboxylate facial triad, which is buried in a β-barrel structure at the active site for ferrous ion coordination [18-23]. Regarding the different coordination positions in the active site, the three metal-binding ligands have different catalytic roles [24]. The active site is covered by the terminal α-helix, which has a flexible conformation and functions as a gate to shield the substrate from the solvent during catalysis [19, 25].

Spectroscopy, computation, and crystal structure studies have revealed that the HPP substrate forms a bidentate coordination with the ferrous ion through the carboxylate and α-keto groups of the substrate [23, 26, 27]. The association with the substrate activates dioxygen for oxidative decarboxylation [26, 28]. This reaction generates 4-hydroxyphenyl acetate (HPA) and a highly reactive Fe(IV)-oxo intermediate, followed by aromatic ring hydroxylation in HPA and migration of the acetyl group to form HG [4, 29-32]. The side chain binding mode of HPA is critical for the oxidative reaction by the Fe(IV)-oxo on the aromatic ring of the substrate [27, 30, 31]. The 4-hydroxyl group in the aromatic side chain of HPP is essential for electron delocalization [30, 33]. A single mutant of P214T or N241S in S. avermitilis HPPD generated a new product of oxepinone or quinolacetic acid in which the two residues were located in the substrate-binding pocket, implying the involvement of an epoxide or arenium cation intermediate in the catalytic reaction [34, 35]. The model of substrate binding in P. fluorescens HPPD indicated that the 4-hydroxy group interacts with Gln272 and Gln286 residues. The interactions were confirmed with site-directed mutagenesis and QM/MM theoretical calculation [21, 33]. Recently, the substrate-bound structure of A. thaliana HPPD was elucidated (PDB code: 5XGK), and, in contrast to a previous report, the 4-hydroxyl group of the substrate was discovered to
form a hydrogen bond with the Asn423 side chain on the C-terminal helix [23]. Although site-directed mutagenesis also indicated the role of Asn423 in substrate binding and catalysis, the binding mode of the substrate remains unclear due to the conformation of the active site.

The crystal structures of *A. thaliana* HPPD displayed a similar conformation in the presence of the substrate or inhibitors or in the absence of the substrate (PDB code: 1TFZ and 1SQD), particularly for the C-terminal helix [22, 23]. When comparing the structures of *P. fluorescens* HPPD and *S. avermitilis* HPPD with a bound inhibitor of 2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione (NTBC), the C-terminal α-helix displayed a dramatic shift in the presence of the inhibitor [18, 21]. The C-terminal helix is assumed to gate the access to the active site for catalysis [19, 25]. Molecular dynamics simulation and free-energy calculations demonstrated the open–closed transition of the C-terminal helix on substrate binding [36], indicating that this favored orientation may be protein dependent, as revealed by the free-energy calculations and the elucidated crystal structures of *A. thaliana* and *Z. mays* HPPD [19, 36]. The differences might arise from low sequence similarity between different species and consequently affect the contact of the C-terminal α-helix with the active site that stabilizes the closed conformation of the protein (Fig. S1) [36].

No substrate-bound structure of human HPPD has been elucidated. Comparison of the structure of human HPPD (PDB code: 3ISQ) with the substrate-bound structure of *A. thaliana* HPPD revealed notably different orientations of the C-terminal helix (Fig. 1A) [20, 23]. The C-terminal helix in human HPPD had a closed conformation. Accordingly, the substrate-binding mode in the active site of human HPPD may differ from that of *A. thaliana* HPPD [36]. A simulation of the substrate-bound human HPPD revealed the interaction of the hydrogen bonds between the 4-hydroxyl group of the substrate and the side chains of Gln251 and Gln265 [25]. A notable hydrogen bond network bridged by the Gln334 side chain was observed, which interacted with the Glu349 side chain (the ferrous ion binding ligand), Asn363 side chain from the C-terminal helix, and the carboxyl group of HPP (Fig. 1B). These residues are strictly conserved across the HPPD families, although a low sequence similarity (~24%) was noted (Fig. S1). To reveal the role of these residues in catalysis, we performed site-directed mutagenesis and simulation analysis, which indicated the critical role of Gln251 in stabilizing the orientation of the aromatic side chain of HPP by participating in the initial nucleophilic reaction. Asn363 interacted with the substrate in the Q334A mutant and preserved relative activity and substrate binding affinity of the enzyme. The substitution of Asn363 confirmed its critical role in substrate binding and catalysis. The Asn363-substrate interaction also highlights the functional role of the C-terminal helix through the H-bond network to stabilize the substrate-binding during catalysis.
reaction.

Materials and Methods

Preparation of variant HPPD

Single and double mutants of Q334A, Q334N, N363A, N363D, N363Q, Q334A/N363D, Q334A/N363Q, and Q334N/N363Q were generated using the QuikChange mutagenesis system (Stratagene, San Diego, CA, USA) with the vector of pTrc-HPPD as template [25]. Table S1 presents the sequences of primers that were used for PCR. The mutant-containing vectors were transformed into E. coli DH5α-competent cells, and the desired mutations were confirmed through complete DNA sequencing.

Protein purification

Overexpression and purification of variant HPPD were performed as reported previously [25]. The supernatant of crude cell lysate was loaded onto a Q-Sepharose anion exchange column, which was equilibrated in buffer A (50 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, and 1 mM DTT). Ammonium sulfate solution was then titrated into the pooled fractions to a final concentration of 1.2 M before being loaded onto the Source™ 15PHE column. The column was equilibrated in buffer A containing 10% (v/v) glycerol and 1.2 M ammonium sulfate. The fractions pooled from the gradient were loaded onto an S-100 Sephacryl column (equilibrated with 50 mM Tris-HCl buffer, pH 7.5). On the basis of the SDS-PAGE, the fractions with the highest purity were pooled and concentrated. Protein concentrations were determined using the Bradford method [37].

Enzyme activity measurements

Oxygraph and HPLC assays were used to determine HPPD activity by measuring oxygen consumption and HG product formation in the reaction, respectively [25]. The reaction mixture, containing 0.2 mM ascorbate, 0.2 mM FeSO₄, and enzyme in 0.1 M Tris-acetate buffer (pH 6.5), was added to the oxygraph apparatus equipped with a Clark-type electrode (Hansatech DW1 Oxygraph System, Norfolk, UK). After incubation for 1 min at 37 °C, 1 mM HPP was added to the mixture to initiate the measurement. The reaction rate without enzyme was used for correction.

For the HPLC assay, reaction mixtures containing 1 mM ascorbate, 1 mM FeSO₄, 1 mM HPP, and enzyme in 0.1 M Tris-acetate (pH 6.5) were incubated for 5 min at 37 °C with shaking. The reaction was stopped through centrifugation at 8000g (Amicon Ultra-0.5, 10 kDa) to remove the protein. The filtered product was analyzed using HPLC on a C18 column (4.6 × 250 mm, 5-μm particle size; ODS HYPERSIL) at a flow rate of 1 mL/min. The elution conditions were as
reported previously [25]. Product elution was monitored at 230 nm [29].

**CD studies**

The CD spectra were recorded in a Jasco J-815 spectropolarimeter equipped with a Peltier temperature controller accessory. Proteins (0.2 mg/mL in 20 mM sodium phosphate buffer, pH 7.4) in a 1-mm-path-length cell were measured in the far-UV region (180–260 nm) at 25 °C. All spectra were averaged from five accumulations and were buffer-corrected. The fractions of secondary structure were estimated through the DICHROWEB server (http://www.dichroweb.cryst.bbk.ac.uk/html/home.html) using the CDSSTR method [38, 39]. The ellipticity at 222 nm was recorded for the thermostability of the secondary structure from 25 to 85 °C at a heating rate of 1 °C/min. The mid-point temperature ($T_m$, °C) for the protein unfolding transition was calculated using the sigmoidal fit of the curve.

**ITC measurements**

The binding affinity of the HPP substrate with the wild-type and mutant HPPD was determined using iTC200 calorimeters (MicroCal, Northampton, MA, USA) at 298 K in 50 mM Tris-HCl buffer (pH 7.5). Measurements were performed through stepwise titrations of 1.1 or 1.5 μL of HPP into the solution, which contained a 1:1 mixture of HPPD and CoCl₂, at intervals of 200 or 450 s, respectively. The control experiments for the heat of dilution were measured independently and subtracted from the experimental heat measured for the binding reaction. The integrated heat released from each injection was analyzed using MicroCal Origin software v7.0 by fitting the data to a one-site binding model.

**DSF**

The DSF experiment was performed using a thermocycler (C1000 Thermocycler, CFX96 Real-Time System; Bio-Rad, CA, USA). Proteins alone or in mixture with equal moles of FeSO₄ were diluted to 0.2 mg/mL in 50 mM Tris-HCl buffer (pH 7.5) containing 1:1000 SYPRO Orange dye (Sigma). All samples were prepared in triplicate by heating from 25 to 95 °C at a ramp rate of 0.3 °C/min. The melting temperature ($T_m$) was determined using CFX Maestro software v1.1 (Bio-Rad).

**Simulations**

The complexed structure of human HPPD (PDB code: 3JSQ) [20] was modeled using Discovery Studio 2019 software (Biovia); the ferrous ion was substituted with an Fe(III)-O$_2^{-}$ species and coordinated by HPP. This model was used as the template to generate the models of the HPPD mutant using the Built Mutants protocol followed by QM/MM minimization in the CHARMm force field. The quantum center (87 atoms) includes Fe(III)-O$_2^{-}$, HPP, and the side chains of His183, His266, and Glu349. Atom constraints were set for residues that were located outside 5 Å of Fe(III)-O$_2^{-}$ and HPP. The quantum calculation was performed using the
Perdew–Burke–Ernzerhof (PBE) functional. The spin multiplicity was set as “smart” in the Dmol3 settings, and thermal smearing of electronic occupancy was used in the calculation. The other parameters used in the calculation were run in a default setting.

Molecular dynamics simulations were performed on the complexed structure of HPPD in which an explicit periodic boundary solvation model was applied to the structure. The system includes water molecules and counter ions in the orthorhombic shape, with a minimum distance between the protein and box boundary of 7 Å. Harmonic restraint was set for all atoms in the protein complex followed by energy minimization of the whole system for 1000 steps of the steepest descent and 2000 steps of the adopted basis Newton-Raphson algorithm. The system was heated from 50 to 300 K for a 4-ps simulation, and a 10-ps simulation was run at 300 K for equilibration. The final steps for production simulation were conducted in a constant temperature and pressure type with no restraints. The particle mesh Ewald method was used to treat the long-range electrostatics, and the shake algorithm was applied to constrain covalent bonds involving H-atoms in the simulation. Conformations that reached equilibrium after MD simulation were selected for QM/MM calculations in which Fe(III)-O$_2^*$, HPP, and the side chains of His183, His266, Glu349, Gln251, Gln265, Gln334, and Asn363 were defined as the quantum atoms. The quintet spin state was set in the calculation as that reported previously [28, 33, 40].

The QM/MM calculated model was used to generate the model of HPPD with Fe(IV)-oxo and bound HPA by replacing HPP with 4-hydroxymandelate (HMA) through a superimposition with the 4-hydroxymandelate synthase (HMS)-HMA complex structure (PDB code: 2R5V) as that reported previously [33, 41]. HMS and HPPD are homologous enzymes that catalyze the same initial step for oxidative decarboxylation of the HPP to form the HPA and the Fe(IV)-oxo intermediate. The steps for HG and HMA product formation are different for HPPD and HMS involving in the aromatic hydroxylation and side chain migration of the HPA, and the hydroxylation at the benzylic carbon of the HPA, respectively [3, 42]. Although the sequence identity between human HPPD and A. orientalis HMS is less than 30%, the overall structures are highly similar (~2.3 Å in the C$_\alpha$ rms deviations) [41]. The coordination of Fe(III)-O$_2^*$ and HMA in HPPD structure was modified to Fe(IV)-oxo and HPA, followed by a rigid body translation and rotation to enhance the interaction of the 4-hydroxy group of HPA with the side chains of Ser226 and Asn241 [33, 41, 43]. The QM/MM calculation was applied to the model using the quantum atoms including Fe(IV)-oxo, HPA, and the side chains of His183, His266, Glu349, Ser226, Asn241, Gln334, and Asn363. The spin multiplicity was set as “quintet” as that reported previously [31, 33, 40].
Results

Construction of mutant HPPD and protein purification

To investigate the effect of the hydrogen bond network of Glu349–Gln334–Asn363 and interactions of Gln265 and Gln251 with the substrate related to the function of HPPD, the following single and double mutants were constructed by site-directed mutagenesis: Q334A, Q334N, N363A, N363D, N363Q, Q251E, Q265E, Q334A/N363Q, Q334N/N363Q, and Q334A/N363D.

The recombinant wild-type and mutant HPPD were overexpressed and purified through an anion exchanger, hydrophobic interaction, and size exclusive chromatography to near homogenous, as judged by SDS-PAGE (Fig. S2). Far-UV circular dichroism (CD) spectroscopy was used to analyze the secondary structure of the variant purified proteins. Those proteins displayed a similar spectrum and estimated secondary structure contents, suggesting that the overall secondary structures of the proteins were not affected by mutation (Fig. S3 and Table S2).

Activity of wild-type and variant HPPD

The specific activity of HPPD was analyzed with the oxygen consumption and HG product formation in the reaction. The results revealed that the activity of the Q334N and Q334A mutants was approximately 4% and 50% of the wild-type enzyme, respectively, suggesting the importance of the Gln334 in HPPD activity (Table 1). However, the significant loss in the specific activity for mutants of N363Q, N363A, and N363D (approximately 10%, 30%, and 10% of those of the wild-type enzyme, respectively) indicated the important role of this residue in the function. For the Q251E and Q265E mutants, the observed activity was less than 0.5% and 5% of that of the wild type, respectively; this suggested that the interaction from Gln251 is crucial in the function of HPPD.

The activities for double mutants of Q334N/N363Q, Q334A/N363Q, and Q334A/N363D were approximately 5%, 10%, and 14% that of the wild-type enzyme, respectively, as analyzed with the oxygraph assay (Table 1). When the activity was measured using the HPLC assay, it was found that the activities of Q334N/N363Q and Q334A/N363D were approximately 3- and 10-fold lower than those measured using the oxygraph assay (Table 1). The differences in the activity of the two assays suggested an uncoupled reaction. From the HPLC elution profile, a new peak that eluted at approximately 13 mL in addition to the HG product (eluted at approximately 10 mL) was observed for the Q334N/N363Q and Q334A/N363D mutants (Fig. 2). The elution site of this peak was identical to that of HPA. The specific activity calculated from the peak area of HPA was approximately 0.02 ± 0.02 nmol/min/µg for those double mutants.
**Steady-state kinetic analysis**

The steady-state kinetics of the variant HPPD as a function of HPP concentration was determined using the oxygen consumption assay (Table 2 and Fig. S4). Compared with the wild-type enzyme, the $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values decreased approximately 33- and 33-fold, respectively, in the Q334N mutant and 2- and 3-fold, respectively, in the Q334A mutant. The significant reduction in the catalytic efficiency of the Q334N mutant implies the critical role of Gln334 in the catalytic function. However, the half activity retained for the Q334A mutant implies the involvement of alternative residues that interact with the substrate.

The $K_m$ values of HPP were increased by approximately 2 to 3-fold for the N363Q, N363A, N363D mutants, and the Q334N/N363Q, Q334A/N363Q, and Q334A/N363D double mutants (Table 2 and Fig. S4). The $k_{\text{cat}}$ values were decreased by approximately 7-, 3-, and 7-fold for the single mutants, respectively, and approximately 22-, 10-, and 10-fold for the double mutants, respectively. Consequently, the $k_{\text{cat}}/K_m$ values were reduced by approximately 14-, 5-, and 17-fold, and 50-, 33-, and 20-fold for those single and double mutants, respectively. The decrease in the catalytic efficiency for the single mutants indicated the critical role of Asn363 in catalysis. Notably, the catalytic efficiency was reduced by approximately 8- and 13-fold for the single and double mutants, respectively, when the Asn363 mutation was incorporated in the Q334A mutant (double mutants of Q334A/N363D and Q334A/N363Q). By contrast, it was increased by approximately 5- and 3-fold in the Q334A/N363Q and Q334N/N363Q double mutants, respectively, compared with that of the N363Q mutant. The results indicated that the catalytic efficiency of N363Q can be recovered by Gln334 substitution. However, no improvement in the catalytic efficiency was observed in the Q334A/N363D double mutant compared with that of the N363D mutant.

The kinetic analysis was not available for the Q251E mutant because its activity was barely detectable. For the Q265E mutant, the $K_m$ and $k_{\text{cat}}$ values of HPP were increased and decreased by approximately 5- and 20-fold, respectively, compared with the wild-type enzyme, resulting in 50-fold reduced catalytic efficiency (Table 2 and Fig. S4). The result suggests that the interactions of Gln251 and Gln265 with the 4-hydroxyl group of the substrate are critical to stabilize the substrate for the enzyme’s catalytic function.

**Isothermal titration calorimetry analysis**

The binding properties of the HPP substrate with wild-type and mutant HPPD were analyzed with isothermal titration calorimetry (ITC). The measurements were performed by titrating HPP into the solution, which contained an equal molar ratio of monomeric enzyme with cobalt ion. The Co$^{2+}$ ion was used in this binding experiment to eliminate the precipitation of
Fe$^{2+}$ ion in the buffer solution, which can interfere with the heat measurement. Titration of HPP into wild-type HPPD-Co$^{2+}$ complex revealed an exothermic reaction (Fig. 3). The resulting heat data obtained by integrating the area of each injection was well fitted to a one-site binding model. Notably, the enthalpy ($\Delta H$) (~87 kcal/mol) calculated from the binding isotherm of the wild type was very high compared with those of the mutant enzymes (~32, 29, 22, 0.5, 1.5 and 1.1 kcal/mol for Q334N, Q334A, N363A, N363D, Q251E and Q265E, respectively). This might be due to the extra heat released from the enzymatic reaction [24]. Thus, the dissociation constant ($K_d$) was the only parameter used for comparison in this experiment. $K_d$ of the wild type was approximately 0.6 $\mu$M (Table 3). The binding affinity of HPP for Q334N, Q334A, N363A, N363D, Q251E, and Q265E mutants was decreased by approximately 18-, 4-, 4-, 93-, 15-, and 10-fold, respectively, compared with that of the wild-type enzyme. The calculated free energies for substrate binding were decreased by approximately 1.7, 0.9, 0.9, 2.7, 1.6, and 1.4 kcal/mol, respectively, after subtraction from that of the wild-type enzyme. Significant reductions in the substrate-binding affinity were observed from Q334N, N363D, Q251E and Q265E mutants, suggesting the crucial role of these residues in substrate binding.

**Structural stability of HPPD**

The structural stability of the variant enzymes under thermal stress was analyzed by changing the ellipticity at 222 nm in CD spectroscopy. It showed a two-state unfolding transition as a function of temperature for those proteins (Fig. S5). Compared with the wild-type protein, the $T_m$ values of Q334A, N363D, Q334A/N363Q, Q334A/N363D, and Q251E were reduced by 3, 4, 3, 7, and 2 °C, respectively (Table 4), suggesting reduced stability of the secondary structure by those substitutions.

Thermal denaturation of HPPD in the absence and presence of ferrous ions was investigated using differential scanning fluorometry (DSF), which measures the fluorescence enhancement of the probe upon binding to the exposed hydrophobic surface of the unfolded protein (Fig. S6) [44]. The estimated $T_m$ values of the wild-type enzyme were 52.6 °C and increased by approximately 2 °C in the presence of metal ions (Table 4). This indicates a conformation change of the protein structure by binding ferrous ions in the active site to increase thermal stability. For the Q334A, N363D, Q334A/N363Q, Q334A/N363Q, Q334A/N363D, and Q251E mutants, the $T_m$ values were decreased by approximately 2, 2, 2, 5, and 4 °C, respectively, compared with that of the wild type, suggesting the exposure of the hydrophobic area by substitution. These values were increased for all the mutants in the presence of ferrous ions. Notable increases in the $T_m$ values were observed for Q334N, N363A, Q251E, and Q265E mutants after binding with ferrous ions, suggesting that a conformational change of the active site increased the structural stability.
However, the estimated $T_m$ values of the Q334A, N363D, and Q334A/N363D mutants in the presence of ferrous ions were still smaller than that of the wild type by approximately 3, 4, and 5 °C, respectively. Thus, in the active site, these mutants underwent a conformational change to reduce the structural stability.

**Simulation of wild-type and mutant HPPD**

Spectroscopic analysis revealed the charge transfer feature upon the binding of HPP to HPPD-Fe$^{3+}$ [26, 31, 45]. The bidentate coordination of HPP with the iron would activate dioxygen for nucleophilic reaction on substrate [45]. To reveal the effect of residue replacement on substrate binding and the reaction mechanism, a model of HPPD with a bound Fe(III)-O$_2^-$ group and HPP substrate was generated. This model was used to demonstrate the nucleophilic attack of the activated dioxygen at the carbonyl carbon of HPP. MD simulation was applied to the models that were geometrically minimized using QM/MM calculation. After the simulation, the stable conformations from the wild type and the Q334A, N363D, and Q265E mutants were selected to run another QM/MM minimization, which included additional residues of Gln251, Gln265, Gln334, and Asn363 in the quantum region (Fig. 4, Fig. S7 and S8). The other mutants were excluded from further analysis due to the geometrical change of the active site at the beginning of the MD simulation.

The spin population, bond length and charge on the iron and dioxygen as shown in Table S3 suggested an oxygen activation state in the six-coordinated complex, consistent with the studies using DFT and QM/MM calculation for 2-oxoglutarate-dependent and cysteine dioxygenase enzymes [28, 46, 47]. The optimized geometries of wild-type or mutant HPPD with a bound Fe(III)-O$_2^-$ group showed a suitable orientation of HPP substrate for the nucleophilic reaction (Fig. 4 and S8). The bond length for Fe-O1 and O1-O2, and the charge and spin state on the iron and dioxygen were similar between wild-type and mutant enzymes (Table S3) [28, 31].

The QM/MM calculations revealed possible interactions from the residues with HPP in the active site to stabilize the substrate for the nucleophilic reaction. For the wild type, the amide side chain of Gln334 played a central role by interacting with the carboxyl group of Glu349, the HPP substrate, and the amide group of Asn363 from the C-terminal helix (Fig. 4A). This H-bond network seems to stabilize the bidentate chelation from the carboxyl and keto groups of HPP with metal ions for the nucleophilic reaction by the peroxide anion. Notably, the distance of the oxygen atom of the peroxide anion to the carbonyl carbon of HPP was approximately 2.46 Å, indicating the possible mode for the reaction. In such conditions, the 4-hydroxyl group of the phenyl side chain of HPP was stabilized by Gln265 and Gln251. The binding manner is similar to the proposed ternary complex model of *P. fluorescens* and *A. thaliana* HPPD [21, 33].
In the Q334A mutant, the Asn363 side chain was oriented to interact with the carboxyl groups of HPP so that the aromatic side chain of HPP could direct the 4-hydroxyl group to interact with Gln265 and Gln251 and thus stabilize the substrate binding (Fig. 4B). In this mode, the peroxide anion, which was approximately 2.4 Å from the carbonyl carbon of HPP, demonstrated a possible catalytic reaction for the Q334A mutant (Table 1). However, due to the interaction, the Asn363 side chain obliged to lose the interactions with the carbonyl group of Leu332 and Leu323 in the β-sheets of the active site (Fig. 4A and B).

The protonated form of the substituted residue in the N363D mutant might be due to the hydrophobic environment around the substituted residue (Fig. 4C). Although the interaction of Gln334 with Asp363 was lost, it still retained the H-bond interactions with Glu349 and HPP. In this binding mode, the 4-hydroxyl group of the substrate interacted with Gln251 and the peroxide anion was approximately 2.67 Å from the carbonyl carbon of HPP, demonstrating the possible catalytic reaction for the N363D mutant (Table 1).

The protonated form of the substituted residue was also observed in the Q265E mutant, highlighting the necessity of active site hydrophobicity for substrate binding (Fig. 4D). Interactions of the H-bond network were retained, which stabilized the bidentate-chelated substrate for the reaction by the peroxide anion at approximately 2.54 Å to the carbonyl carbon of HPP in the Q265E mutant model. However, the aromatic side chain of HPP was oriented, which led the 4-hydroxyl group to interact with Gln251.

**Discussion**

The binding of the substrate in the structure of human HPPD remains elusive, although the substrate-bound structure of A. thaliana HPPD was elucidated recently [20, 23]. The orientations of the C-terminal helix in the two proteins affect the active site conformation for substrate binding. The C-terminal helix functions as a gate to monitor the active site integrity for catalysis [19, 25]. We proposed a possible substrate-binding model based on the structure of human HPPD. The substitution and simulation experiments demonstrated the vital role of Gln251 in the initial nucleophilic reaction by interacting with the 4-hydroxy group of the substrate (Table 1 and Fig. 4). The functional role of the H-bond network in substrate binding and catalysis was also identified (Tables 3 and 4). Among them, Asn363 from the C-terminal helix played a crucial role by contributing to the interaction to stabilize substrate binding for catalysis.

Replacement of Gln251 with glutamate resulted in activity loss and reduction in the substrate binding affinity, indicating the functional role of this residue in catalysis. The result is consistent with the activity loss by using phenylpyruvate as a substrate in S. avermitilis HPPD
It indicated that Gln251’s interaction with the 4-hydroxyl group of HPP is crucial to locate the substrate in a precise position for the nucleophilic reaction by the peroxide anion \([48]\). Q251E mutant stability increased in the presence of ferrous ion, suggesting a conformational change to accommodate the bidentate binding of the substrate in the active site. The result supported the critical location of this residue at the entrance of the active site, which forms a bifurcate interaction with the C-terminal helix and substrate to bury the active site (Fig. 4A). A reduction in the binding affinity and activity was observed for the Q265E mutant, in which the 4-hydroxyl group of HPP interacted with both of the Gln265 and Gln251 or rotated to interact with the Gln251 performed a suitable conformation for nucleophilic reaction (Fig. 4). It suggests that both residues are required to stabilize the bound substrate for catalysis, but the role of Gln251 is crucial.

Because Gln334 is at the center of the hydrogen bond network, it links the ferrous ion binding center, terminal helix, and substrate in the active site of HPPD. The lower thermostability of the Q334A mutant compared with that of the wild type in the presence of ferrous ion indicates its critical role in stabilizing the active site conformation (Table 4). Notably, the relatively high activity and substrate-binding affinity preserved in this mutant suggested the involvement of Asn363 in substrate binding and catalysis (Tables 1 and 3). This was supported by the simulation model, which demonstrated the interaction of Asn363 with the substrate in the Q334A mutant (Fig. 4B). The role of Gln334 in substrate-binding stabilization but not catalytic function was consistent with the report in \(A.\) \(thaliana\) HPPD [33]. The Asn363-substrate interaction directs the 4-hydroxyl group of HPP to interact with Gln251 and Gln265, in which the HPP showed a suitable conformation for nucleophilic reaction by the activated dioxygen (Fig. 4B). The interaction also implied the role of the C-terminal helix, which forms a closed conformation of the active site for the H-bond interactions to stabilize the substrate binding for catalysis. This may explain why the C-terminal helix in a different conformation exhibits a different substrate binding from that reported in the structure of \(A.\) \(thaliana\) HPPD [23].

Previous report had indicated that the C-terminal helix gates the entrance of substrate into the active site through rotation of the hinge region around Asn363 [19]. It suggested the functional role of this residue in the open-closed state of the active site. Asn363 substitution decreased the catalytic efficiency, suggesting its critical role in catalysis (Tables 2 and 3). In the simulation model, Asn363 interacted with the \(\beta\)-sheet in the \(\beta\)-barrel structure of the active site and with the Gln334 from the C-terminal \(\alpha\)-helix to link the H-bond network (Fig. 4A). The interaction between Asn363 and Gln334 was disrupted in the N363D mutant model; however, the
interactions of Gln334 with the substrate and Glu349 were retained (Fig. 4C). The substitution result indicated that the interaction was crucial to stabilize the substrate for the initial nucleophilic reaction. However, the dramatic reduction in substrate-binding affinity and thermal stability of the N363D mutant but not for the N363A mutant suggested that the active site conformation was changed to reduce substrate-binding stability and increase the substrate dissociation rate (Tables 3 and 4). The result also implies the effect of the side chain structure of this residue with the hinge region rotation of the C-terminal helix. The conformation of the C-terminal helix may affect the substrate binding and structural stability [19, 25, 36].

The catalytic efficiency, substrate-binding affinity, and thermal stability were not recovered through the double mutation of Gln334 and Asn363, suggesting that an appropriate arrangement of the two residues is critical for a functional H-bond network. Notably, HPA production from the uncoupled reaction that catalyzed by Q334N/N363Q and Q334A/N363D mutants suggested that the electrophilic attack step by the Fe(IV)-oxo intermediate was not efficient in these double mutants [4, 29-31]. The optimized structure of human HPPD-Fe(IV)-oxo-HPA complex revealed that the bound HPA intermediate was stabilized through an H-bond network mediated by the interaction of Gln334 with the carboxyl group of HPA. It also showed that the 4-hydroxyl groups of HPA formed interactions with Ser226 and Asn241, similar to that proposed in A. thaliana and P. fluorescens HPPD (Fig. 4E and S8) [33, 43]. In such a binding manner, the C1-atom of the aromatic side chain of the substrate was appropriately located for the electrophilic reaction by the Fe(IV)-oxo intermediate (~3.47 Å), as judged by the spin population, bond length and charge of the iron-oxo species (Table S3) [31, 43]. The stability of bound HPA for the reaction may have been disturbed due to these double mutations, leading to its accumulation. This highlights the importance of the H-bond network in stabilizing the bound conformation of the substrate or intermediate for catalysis.

In conclusion, the chelated-bound HPP substrate or HPA intermediate in the active site were stabilized by a H-bond network, which included interactions from the side chain of Gln334 with the carboxyl group of HPP or HPA, the Glu349 in the metal-binding ligand, and Asn363 in the C-terminal helix. In the absence of interaction from Gln334, the Asn363 could involve in the substrate-binding and catalysis. Substitution of Asn363 decreased the catalytic efficiency of the enzyme. The dramatic reduction in the substrate-binding affinity and structure stability of Asp363 implies the side-chain effect in the hinge region rotation of the C-terminal helix. These results indicate the function role of the H-bond network in a closed conformation of the C-terminal helix, which stabilizes the bidentate bound HPP or HPA in an appropriate position to locate the 4-hydroxyl group of the substrate to interact with Gln251 and G265. The interaction
with Gln251 facilitated the accurate position of the substrate for the initial nucleophilic reaction. These results supported the proposed substrate-binding model in the active site of human HPPD.

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Data availability statement
All supporting data are included within the main article and its supplementary files. The structures of A. thaliana and human HPPD (PDB codes of 5XGK and 3ISQ, respectively) were used for the structural data analysis in the manuscript.

Conflict of interest statement
The authors declare that there is no conflict of interest.
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### Table 1: Specific activity of wild-type and mutant HPPD.

| Protein       | O$_2$ consumption (nmol/min/μg) | HG production (nmol/min/μg) |
|---------------|---------------------------------|---------------------------|
| WT            | 2.2 ± 0.3                       | 2.0 ± 0.1                 |
| Q334N         | 0.09 ± 0.01                     | 0.06 ± 0.01               |
| Q334A         | 1.1 ± 0.1                       | 0.9 ± 0.1                 |
| N363Q         | 0.2 ± 0.01                      | 0.08 ± 0.01               |
| N363A         | 0.7 ± 0.06                      | 0.5 ± 0.04                |
| N363D         | 0.2 ± 0.01                      | 0.3 ± 0.04                |
| Q334N/N363Q   | 0.1 ± 0.01                      | 0.03 ± 0.01               |
| Q334A/N363Q   | 0.2 ± 0.02                      | 0.4 ± 0.05                |
| Q334A/N363D   | 0.3 ± 0.06                      | 0.03 ± 0.001              |
| Q251E         | < 0.01                          | 0.01 ± 0.001              |
| Q265E         | 0.1 ± 0.01                      | 0.08 ± 0.01               |

*These data are the mean ± S.D. of three independent experiments.

### Table 2: Apparent kinetic parameters of wild-type and mutant HPPD enzymes.

| Protein       | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$) |
|---------------|------------|---------------------|-----------------------------------|
| WT            | 0.2 ± 0.01 | 2.0 ± 0.2           | 10 ± 1                            |
| Q334N         | 0.2 ± 0.04 | 0.06 ± 0.01         | 0.3 ± 0.01                        |
| Q334A         | 0.3 ± 0.1  | 1.0 ± 0.2           | 4 ± 1                             |
| N363Q         | 0.4 ± 0.07 | 0.3 ± 0.03          | 0.7 ± 0.04                        |
| N363A         | 0.3 ± 0.02 | 0.6 ± 0.04          | 2 ± 0.02                          |
| N363D         | 0.5 ± 0.1  | 0.3 ± 0.01          | 0.6 ± 0.1                         |
| Q334N/N363Q   | 0.5 ± 0.1  | 0.09 ± 0.01         | 0.2 ± 0.01                        |
| Q334A/N363Q   | 0.6 ± 0.04 | 0.2 ± 0.03          | 0.3 ± 0.03                        |
| Q334A/N363D   | 0.5 ± 0.07 | 0.2 ± 0.05          | 0.5 ± 0.04                        |
| Q265E         | 0.9 ± 0.05 | 0.1 ± 0.01          | 0.2 ± 0.01                        |

*These data are measured using the oxygraph assay and are the mean ± S.D. of three independent experiments. The kinetic parameters for the Q251E mutant were not detectable.
Table 3: Binding analysis of HPP with the wild-type and mutant HPPD-Co\textsuperscript{2+} complex

| Proteins  | $K_d$ (μM) | $\Delta G$ (kcal/mol) |
|-----------|------------|-----------------------|
| WT        | 0.6 ± 0.1  | 8.5 ± 0.1             |
| Q334N     | 11 ± 3     | -6.8 ± 0.2            |
| Q334A     | 2.6 ± 0.5  | -7.6 ± 0.1            |
| N363A     | 2.8 ± 1    | -7.6 ± 0.3            |
| N363D     | 56 ± 7     | -5.8 ± 0.1            |
| Q251E     | 9 ± 1      | -6.9 ± 0.1            |
| Q265E     | 6 ± 1      | -7.1 ± 0.1            |

*These data are measured using the ITC and are the mean ± S.D. of three independent experiments.

Table 4: Thermostability of wild-type and mutant HPPD

| Proteins | $T_{m}$ (°C)$^1$ | $T_{m}$ (°C)$^2$ (with Fe\textsuperscript{2+}) |
|----------|-----------------|---------------------------------------------|
| WT       | 57.1 ± 0.3      | 54.3 ± 0.5                                  |
| Q334N    | 57.9 ± 1.3      | 55.4 ± 0.2                                  |
| Q334A    | 54.2 ± 0.9      | 51.8 ± 0.2                                  |
| N363Q    | 59.3 ± 0.2      | 55.0 ± 0.5                                  |
| N363A    | 56.9 ± 1.0      | 55.6 ± 0.9                                  |
| N363D    | 53.0 ± 0.7      | 50.0 ± 0.2                                  |
| Q334N/N363Q | 56.6 ± 0.8   | 53.2 ± 0.2                                  |
| Q334A/N363Q | 53.9 ± 0.6   | 52.6 ± 0.3                                  |
| Q334A/N363D | 50.2 ± 0.4   | 49.7 ± 0.4                                  |
| Q251E    | 55.4 ± 0.8      | 54.7 ± 0.2                                  |
| Q265E    | 57.9 ± 0.7      | 59.8 ± 0.2                                  |

$^1$These values were calculated from the curves in Fig. S5, which measured the ellipticity at 222 nm in CD spectroscopy.

$^2$These values were calculated from the curves in Fig. S6, which measured the fluorescence intensity by DSF in the absence and presence of equal molar of ferrous ion, respectively.
Figure legends

Figure 1: Structural model of HPPD with bound HPP. (A) Superimposition of the structures of human and Arabidopsis thaliana HPPD. The proteins are shown as cartoon models and colored gray and cyan, respectively. The HPP substrate is present as a stick model and colored yellow and green, respectively. (B) Overall structure and a closed view of the active site of human HPPD. The N- and C-terminal domains are colored as magenta and white, respectively. The Cterm helix represents the C-terminal helix. The hydrogen bond and electrostatic interactions are shown as dashed green and orange lines, respectively.

Figure 2. HPLC analysis of the product. The protein concentrations used in the experiment were 5, 160, 8, 60, 10, 17, 110, 25, 200, 266, and 90 µg for the wild type, Q334N, Q334A, N363Q, N363A, N363D, Q334N/N363Q, Q334A/N363Q, Q334A/N363D, Q251E, and Q265E, respectively. The control assay includes all reaction mixtures except the enzyme. The light and thick arrows indicate the elution sites for HG and HPA, respectively.

Figure 3. ITC analysis of the binding of HPP to the HPPD-Co^{2+} complex. The upper panels represent the raw heat changes measured during the injection of 1, 4, 5, 4.5, 4, 3, and 5 mM HPP into 20, 100, 30, 70, 74, 50, and 50 µM, respectively, of the wild type and the Q334N, Q334A, N363A, N363D, Q265E, and Q251E mutants of HPPD, respectively, in the presence of equal molar of Co^{2+}. The lower panels show the integrated binding isotherm together with the fitted binding curve.

Figure 4. Simulation models of HPPD. (A-D) Wild-type, Q334A, N363D, and Q265E enzymes, respectively, in complex with Fe(III)-O_2^- and HPP. (E) Wild-type enzyme in complex with Fe(IV)-oxo and HPA. These structures were optimized with the QM/MM calculation. The metal ion is shown as sphere and the HPP, peroxy anion or oxo groups are represented by stick models. The 2D diagrams show the key interactions between the substrate and the surrounding residues in the active site. The hydrogen bond, electrostatic, and hydrophobic interactions are shown as dashed green, orange, and pink lines, respectively.
Scheme 1. The tyrosine catabolic pathway

L-tyrosine

\[ \text{tyrosine aminotransferase} \]

\[ \text{fumarate + acetoacetate} \rightarrow \text{fumarylacetoacetate} \]

\[ \text{4-hydroxyphenylpyruvate dioxygenase} \]

\[ \text{O}_2 \rightarrow \text{CO}_2 \]

\[ \text{fumarylacetoacetate} \rightarrow \text{maleylacetoacetate} \]

\[ \text{maleylacetoacetate isomerase} \]

\[ \text{maleylacetoacetate} \rightarrow \text{homogentisate} \]

\[ \text{1,2-dioxygenase} \]

\[ \text{homogentisate} \rightarrow \text{4-hydroxyphenylpyruvate} \]

\[ \text{4-hydroxyphenylpyruvate dioxygenase} \]

\[ \text{O}_2 \rightarrow \text{CO}_2 \]

\[ \text{OH} \rightarrow \text{OH} \]

\[ \text{O} \rightarrow \text{O} \]

\[ \text{OH} \]

\[ \text{COO}^- \]

\[ \text{Downloaded from http://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20210005/912974/bcj-2021-0005.pdf by guest on 01 June 2021} \]
Fig. 1. Structural model
Fig. 2. HPLC assays.
Fig 3. ITC analysis.

- **WT**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio

- **Q334N**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio

- **Q334A**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio

- **N363A**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio

- **N363D**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio

- **Q265E**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio

- **Q251E**
  - Time (min) vs. kcal mol$^{-1}$ of injectant vs. Molar ratio
Fig. 4. Simulation

(A) WT

(B) Q334A

(C) N363D

(D) Q265E

(E) WT-HPA
Scheme 1. The tyrosine catabolic pathway

L-tyrosine

\[ \text{tyrosine aminotransferase} \]

\[ \text{4-hydroxyphenylpyruvate dioxygenase} \]

\[ \text{O}_2 \rightarrow \text{CO}_2 \]

\[ \text{homogentisate} \]

\[ \text{fumaryl acetoacetase} \]

\[ \text{fumarate + acetoacetate} \]

\[ \text{maleylacetoacetate isomerase} \]

\[ \text{maleylacetoacetate} \]

\[ \text{homogentisate 1,2-dioxygenase} \]

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Fig. 1. Structural model

(A) (B)
Fig. 2. HPLC assays.
Fig 3. ITC analysis.
Fig. 4. Simulation

(A) WT

(B) Q334A

(C) N363D

(D) Q265E

(E) WT-HPA