Crystal Structure of PnpCD, a Two-subunit Hydroquinone 1,2-Dioxygenase, Reveals a Novel Structural Class of Fe$^{2+}$-dependent Dioxygenases*

Received for publication, June 19, 2015, and in revised form, August 11, 2015 Published, JBC Papers in Press, August 24, 2015, DOI 10.1074/jbc.M115.673558

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Background: Two-subunit hydroquinone 1,2-dioxygenase PnpCD is the ring cleavage enzyme in para-nitrophenol catabolism.

Results: The structures of apo-PnpCD and its complex with substrate analog (hydroxybenzonitrile) were determined.

Conclusion: PnpCD reveals a new class of Fe$^{2+}$-dependent dioxygenases.

Significance: PnpCD structure contains a pseudo “cupin” and a novel iron metallocenter in the catalytic PnpD, which adds to understanding of the ring cleavage mechanism of dioxygenases.

Aerobic microorganisms have evolved a variety of pathways to degrade aromatic and heterocyclic compounds. However, only several classes of oxygenolytic fission reaction have been identified for the critical ring cleavage dioxygenases. Among them, the most well studied dioxygenases proceed via catecholic intermediates, followed by noncatecholic hydroxy-substituted aromatic carboxylic acids. Therefore, the recently reported hydroquinone 1,2-dioxygenases add to the diversity of ring cleavage mechanisms. Two-subunit hydroquinone 1,2-dioxygenase PnpCD, the key enzyme in the hydroquinone pathway of para-nitrophenol degradation, catalyzes the ring cleavage of hydroquinone to γ-hydroxymuconic semialdehyde. Here, we report three PnpCD structures, named apo-PnpCD, PnpCD-Fe$^{3+}$, and PnpCD-Cd$^{2+}$-HBN (substrate analog hydroxybenzonitrile), respectively. Structural analysis showed that both the PnpC and the C-terminal domains of PnpD comprise a conserved cupin fold, whereas PnpC cannot form a competent metal binding pocket as can PnpD cupin. Four residues of PnpD (His-256, Asn-258, Glu-262, and His-303) were observed to coordinate the iron ion. The Asn-258 coordination is particularly interesting because this coordinating residue has never been observed in the homologous cupin structures of PnpCD. Asn-258 is proposed to play a pivotal role in binding the iron prior to the enzymatic reaction, but it might lose coordination to the iron when the reaction begins. PnpD also consists of an intriguing N-terminal domain that might have functions other than nucleic acid binding in its structural homologs. In summary, PnpCD has no apparent evolutionary relationship with other iron-dependent dioxygenases and therefore defines a new structural class. The study of PnpCD might add to the understanding of the ring cleavage of dioxygenases.

* This work was supported by National Natural Science Foundation of China Grant 31070655, Shandong Provincial Funds for Distinguished Young Scientists Grant JQ201307, and Program for Changjiang Scholars and Innovative Research Team in University Grant PCSIRT13028. The authors declare that they have no conflicts of interest with the contents of this article.

The atomic coordinates and structure factors (codes 4ZXA, 4ZXC, and 4ZXD) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: PNP, para-nitrophenol; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; SeMet, selenomethionine; MST, microscale thermophoresis; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; γ-HMSA, 4-hydroxymuconic semialdehyde; HBN, 4-hydroxybenzonitrile; HQDO, hydroquinone 1,2-dioxygenase; ITC, isothermal titration calorimetry.
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Cloning, Site-directed Mutagenesis, and Protein Preparations—Full-length pnpCD genes were amplified by PCR using genomic DNA isolated from Pseudomonas sp. strain WBC-3. The PCR product was cloned into a modified pET-15b vector with a PreScission protease cleavage site to remove the His tag. The recombinant plasmid was overexpressed in Escherichia coli BL21 (DE3). Point mutations were generated by a two-step PCR strategy and confirmed by DNA sequencing.

For protein purification, transformed cells were grown at 37°C to an absorbance (A$_{600}$) of 0.8, followed by overnight incubation with 0.12 mM isopropyl 1-thio-β-D-galactopyranoside at 15°C. Bacterial cells were harvested by centrifugation, sonicated in the lysis buffer (25 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.4 mM PMSF), and washed by sonication on ice. After centrifugation, the soluble proteins were purified to homogeneity by combining nickel affinity and size-exclusion chromatography (Superdex 200, GE Healthcare) in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 3 mM DTT. Selenomethionine (SeMet)-labeled PnpCD was expressed from E. coli BL21 (DE3) in M9 medium supplemented with selenomethionine. The induction condition and purification procedure were the same with native protein. Before the metal ion specificity assay, the purified protein was incubated with an appropriate amount of EDTA for at least 1 h (the molar ratio of PnpCD to EDTA was 1:10) and again subjected to a Superdex 200 column in buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl.

Enzyme Assays—Enzyme activity measurements were performed at room temperature using air-saturated buffer. The activity of PnpCD was determined in two steps according to the described method (9). First, the enzyme solution (50 μM) was incubated with FeSO$_4$ (1 mM) for 2 min. Next, enzyme activity was determined spectrophotometrically by measuring accumulation of the product γ-HMSA at 320 nm (ε$_{320}$ = 11.0 mM$^{-1}$ cm$^{-1}$) (18). The assays were performed in a total volume of 1 ml containing 20 mM BES buffer, pH 7.0, 500 μM hydroquinone, and 50 mM incubated enzyme solution. Activity is defined as the number of micromoles of semialdehyde produced per min per mg of protein. The assays were averaged over three independent measurements.

The metal ion specificity of PnpCD was determined by monitoring the activity of nonligated enzyme in the presence of FeCl$_2$, FeCl$_3$, MnCl$_2$, CoCl$_2$, NiCl$_2$, CuCl$_2$, or CdCl$_2$. Prior to reaction initiation, the enzyme solution (50 μM) was incubated with different metal ions (0.1 mM) for 2 min.

Limited Proteolysis and Crystallization—For crystallization assay, the native protein samples of PnpCD were concentrated to 15–20 mg/ml in 10 mM Tris-HCl, pH 8.0, and 100 mM NaCl. Preliminary screenings were performed with crystallization screen kits (Hampton Research) by sitting-drop vapor diffusion at 20°C. Crystals were grown under two conditions. The reservoir solutions of “condition 1” contained 1.8 M ammonium carbonate ditrahydrate, 0.1 M sodium acetate, pH 4.6, and the “condition 2” containing 0.2 M sodium thiocyanate, 20% w/v PEG 3350. After optimization, crystals in “condition 1” were of sufficient quality to collect ~3.0 Å (apo-PnpCD, 3.05 Å), and the crystals in “condition 2” did not diffract well with resolution lower than 4 Å.
It was reported that a proteolytic fragment or domain of a protein may crystallize more readily or form better diffracting crystals than the intact protein (19). We therefore chose to remove flexible segments of PnpCd by limited proteolysis. Full-length PnpCd were mixed with elastase in buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM NaCl (the mass ratio of PnpCd to elastase was 20:1). The mixture was incubated on ice for 1 h. The proteolytic PnpCd was purified by a Superdex 200 column in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 3 mM DTT. These procedures resulted in a truncated PnpD and intact PnpC, as judged by SDS-PAGE. The proteolytic fragment of PnpCd was concentrated to 15–20 mg/ml for preliminary screenings. Crystals of PnpCd in complex with HBN (an analog of hydroquinone) were of sufficient diffraction quality to collect at 3.0 Å (PnpCd-Cd2⁺, 3.05 Å).

Unfortunately, we were unable to obtain crystals of PnpCd-hydroquinone despite extensive effort. Instead, we grew the crystals of PnpCd in complex with HBN (an analog of hydroquinone) under the same condition as proteolytic PnpCd by cocRYStallisation (PnpCd-Cd2⁺-HBN, 2.5 Å). For crystallization preparation, the proteolytic PnpCd was mixed with CdSO₄ and HBN in a molar ratio of 1:3:3 for 30 min on ice. Crystals of proteolytic SeMet PnpCd complexed with HBN were obtained under the same condition as the native protein complex (PnpCd-SeMet, 3.3 Å).

Data Collection, Structure Determination, and Model Refinements—For data collection, crystals were flash-frozen in liquid nitrogen with 15–20% (v/v) ethylene glycol as the cryoprotectant. The x-ray diffraction data were all collected at 100 K on beam line BL17U at SSRF (Shanghai, China) equipped with an ADCS Q315r CCD detector. The diffraction data were all processed using the HKL2000 program suite (20). The PnpCd structure was solved by single-wavelength anomalous dispersion phasing. The initial heavy atom sites were generated by HKL2MAP (21). Refinement of the heavy atom substructure, phasing, and density modification was performed using SOLVE/RESOLVE (22, 23). The determined phase (3.3 Å, PnpCd-SeMet) was then subjected to phase extension of the high resolution native data (2.5 Å, PnpCd-Cd²⁺-HBN). Autobuilding of the initial model was carried out by ARPWARP. The 2.5 Å structure of PnpCd-Cd²⁺-HBN was further refined by PHENIX and COOT (24, 25). One monomer from the resulting structure was subsequently picked out as the search model for molecular replacement of apo-PnpCd (3.05 Å) and PnpCd-Fe²⁺ (3.05 Å) using PHASER (26). All of the R_{free} values are calculated using 5% of reflections randomly excluded from refinement.

Diffraction data statistics are listed in Table 1. The final model was checked and validated using PROCHECK (27). Values of the mean temperature factors for protein and solvent were calculated using BAVERAGE from the CCP4 program suite (28). Structure representations were generated with the PyMOL program (version 0.99rc6).

CD Spectroscopy—CD spectroscopy was performed at 25 °C on a J-810 spectropolarimeter (Jasco). All the proteins were prepared in buffer containing 10 mM Tris-HCl, pH 8.0, and 100 mM NaCl. CD spectra of the proteins at a final concentration of 14 μM were collected from 200 to 250 nm at a scan speed of 200 nm min⁻¹ and with a path length of 0.1 cm. The spectra were averaged over at least three independent measurements.

Isothermal Titration Calorimetry—Because of the rapid auto-oxidization of Fe²⁺ solution and the close nature between Fe²⁺ and Mn²⁺, MnCl₂ solution was used for the titration. The binding affinities of wild type PnpCd and the mutant proteins to Mn²⁺ were measured by an ITC-200 microcalorimeter (GE Healthcare) at 25 °C. All protein samples and MnCl₂ were prepared in buffer containing 10 mM Tris-HCl, pH 8.0, and 100 mM NaCl. Mn²⁺ concentrations were adjusted to obtain significant heats of binding. Centrifugation (14,000 × g, 8 min) was performed to remove possible precipitate and to de-gas the solution before use. Twenty injections were measured with a stirring speed of 1000 rpm and a time interval of 120 s for each sample. Except for the first injection, each titration contains 2 μl of MnCl₂ solution. The blank buffer was also titrated as a contrast. The titration data were analyzed with Origin software using a single-site binding model.

Microscale Thermophoresis—Binding of wild type PnpCd and its mutant E248Q to the substrate hydroquinone was measured by microscale thermophoresis (MST). Briefly, a constant concentration of dye NT647-labeled protein in 20 mM BES buffer, pH 7.0, containing 100 mM NaCl and 1% Tween 20 was incubated at room temperature with different concentrations of hydroquinone. Immediately afterward, 3–5 μl of samples were loaded into standard glass capillaries (Monolith NT Capillaries, NanoTemper), and the thermophoresis analysis was performed on a NanoTemper Monolith NT.115 instrument (20% LED, 80% MST power). A laser on-time of 30 s and a laser off-time of 5 s were used. The experiment was performed in three replicates, and the MST curves were fitted with the law of mass action using NT analysis software to obtain the K_d values.

Results

Structure of Apo-PnpCd, PnpCd-Fe²⁺, and PnpCd-Cd²⁺-HBN—PnpD and PnpC from Pseudomonas sp. strain WBC-3 are proposed to be the α- and β-subunits of hydroquinone 1,2-dioxygenase (HQDO), respectively, which catalyze the ring cleavage of hydroquinone to γ-HMSA with the consumption of stoichiometric amounts of molecular oxygen. Analysis of metal ion specificity indicated that PnpCd selectively utilizes Fe²⁺ for its catalytic reaction (Fig. 1). The crystal structure of proteolytic PnpCd was determined at 3.05 Å. The electron densities for the first 15 N-terminal residues of PnpD are not visible, probably because of the limited proteolysis of full-length PnpCd. There are two heterotetramers (αβ)₂ in the asymmetric unit. Two PnpD molecules contained undefined spherical densities, which were determined as ferric ions for the aerobic environment during protein purification and crystallization. This finding was in good agreement with ITC measurement, showing that the molar ratio of bound metal ion to purified PnpCd was 0.49 ± 0.01. We also obtained the structure of proteolytic PnpCd in complex with Cd²⁺ and hydroxybenzonitrile (HBN, an analog of substrate hydroquinone) at 2.5 Å, of which the crystal packing and modeled residues are the same with proteolytic PnpCd. Notably, all α-subunits in the asymmetric unit...
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contain fully occupied Cd^{2+} and HBN sites. We then solved the structure of full-length PnpCD at 3.05 Å, but PnpD residues 1–15 are still not available. Unlike proteolytic PnpCD, all α-subunits of the full-length structure contain no iron, indicating that the iron-containing PnpCD may have not been crystallized or gradually lost its binding of iron during crystallization. Details of the diffraction data and refinement statistics are given in Table 1. For clarity, we define the three solved structures as the binary PnpCD-Fe^{3+} complex, the ternary PnpCD-Cd^{2+}-HBN complex, and apo-PnpCD, respectively.

**PnpD Contains a C-terminal Catalytic Cupin and an Uncharacterized N-terminal Domain—PnpD, the large subunit of PnpCD, exhibits clear electron density for residues 16–339 in all solved structures. The PnpD monomer contains six α-helices and 16 β-strands, which can be described as a 152-residue N-terminal domain (residues 16–167) and a 172-residue C-terminal domain (residues 168–339) (Fig. 2A). A DALI server search (29) against the Protein Data Bank failed to identify any structures with significant similarities over the full-length peptides.

The central structure of the PnpD C-terminal domain comprises eight antiparallel β-strands (β9–16) with a “jelly roll” topology (Fig. 2, A and B), which was then designated as cupin fold through structural comparison using the DALI server. Residues 319–339 of PnpD form a short α-helix (α6), and residues 168–235 include mainly long loops, which play a vital role in mediating the interaction between PnpD and PnpC. The jelly roll of SfiI_3543 (PDB code 3D82, Z-score of 12.4, r.m.s.d. = 1.9 Å for 100 aligned Ca) is the structure most similar to the PnpD C-terminal domain, followed by 3-hydroxyanthranilate-3,4-dioxygenase (PDB code 1YFU, Z-score of 8.4, r.m.s.d. = 3.7 Å for 111 aligned Ca), auxin-binding protein-1 ABP1 (PDB code 1LR5, Z-score of 7.4, r.m.s.d. = 3.5 Å for 107 aligned Ca), and cysteine dioxygenase (PDB code 3ELN, Z-score of 7.3, r.m.s.d. = 2.3 Å for 101 aligned Ca). The characteristic cupin domain contains two conserved motifs, each corresponding to two β-strands, separated by a less conserved region composed of another two β-strands with an intervening variable loop (Fig. 3, A and B). Four residues at the conserved locations, including His-256, Asn-258, Glu-262 in motif 1, and His-303 in motif 2, are responsible for the metal ion binding of PnpD cupin (Fig. 3A). Notably, residue Asn-258 in motif 1 is not conserved according to the sequence alignment of all selected cupin proteins. This new coordination configuration might present new ideas about on the catalytic mechanism of cupin proteins.

The N-terminal domain of PnpD mainly consists of two antiparallel β-sheets (β1–β2/β3–β4/β5–β6–β7–β8), which are perpendicularly packed and connected by an α-helix (α1) (Fig.

**TABLE 1**

X-ray data collection and refinement statistics

Values in parentheses are for the highest resolution shell.

| Data collection         | Apo-PnpCD | PnpCD-Fe^{3+} | PnpCD-Cd^{2+}-HBN | PnpCD-SeMet |
|-------------------------|-----------|---------------|-------------------|-------------|
| Space group             | P2₁,2₁,2₁ | P2₁,2₁,2₁     | P2₁,2₁,2₁         | P2₁,2₁,2₁   |
| Cell dimensions (Å)     | 62.20, 114.01, 158.08 | 76.84, 181.74, 186.86 | 77.02, 181.05, 186.81 | 77.15, 181.54, 187.03 |
| Resolution (Å)          | 50.00–3.05 (3.16–3.05) | 50.00–3.05 (3.16–3.05) | 50.00–2.50 (2.59–2.50) | 50.00–3.30 (3.42–3.30) |
| Observed reflections    | 145,672 | 351,930 | 660,941 | 550,169 |
| Completeness (%)        | 99.4 (98.9) | 99.9 (100.0) | 100 (100) | 100 (100) |
| Redundancy              | 6.6 (6.4) | 6.9 (7.3) | 7.2 (7.1) | 13.7 (13.7) |
| I/σ(I)                  | 19.3 (4.6) | 28.6 (7.9) | 29.3 (5.5) | 27.0 (8.3) |
| R_{work} (%)            | 13.1 (49.3) | 13.8 (49.8) | 10.2 (47.8) | 17.6 (49.2) |
| Wilson B factor (Å²)    | 44.8 | 41.4 | 33.2 | 52.2 |

**Refinement**

| R_{work}/R_{free}        | 0.2018/0.2533 | 0.1877/0.2451 | 0.1865/0.2373 |
| Bond length (Å)          | 0.010 | 0.010 | 0.008 |
| Bond angles (°)          | 1.405 | 1.240 | 1.067 |
| Average B-factors (Å²)   | 49.410 | 38.318 | 33.829 |
| Protein                  | 23.905 | 41.080 | 40.055 |
| Metal ion                | 26.893 | 37.154 | 33.327 |
| Solvent                  | 37.154 | 25.099 | 33.327 |
| Ramachandran plot (%)    | 82.3 | 84.7 | 86.3 |
| Favored                  | 16.0 | 14.1 | 12.8 |
| Generally allowed        | 1.3 | 0.8 | 0.9 |
| Disallowed               | 0.4 | 0.4 | 0.4 |

* R_{free} = \frac{\sum_{kl} I(hkl) - \langle I(hkl) \rangle}{\sum_{kl} I(hkl)}, where \langle I(hkl) \rangle is the mean intensity of multiple recorded reflections.
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2, A and B). Residues 121–167 of PnpD form a helix-loop-helix structure (α3-loop-α4), whereas the loop of residues 16–31 makes a large number of contacts between PnpC and PnpD. To identify proteins with structural homology to the N-terminal domain of PnpD, a DALI server search was performed. The highest Z-score was obtained for the plant DNA repair protein WHY2 (PDB code 4KOP, Z-score of 8.6, r.m.s.d. = 2.9 Å for 104 aligned Ca), followed by the mitochondrial RNA-binding protein-1 MRP-1 (PDB code 2GJE, Z-score of 7.7, r.m.s.d. = 2.9 Å for 106 aligned Ca), and the plant transcriptional regulator PBF-2 (PDB code 1L3A, Z-score of 7.4, r.m.s.d. = 2.8 Å for 103 aligned Ca). Compared with the N-terminal domain of PnpD, none of the mentioned proteins shows a sequence identity higher than 12%.

Interestingly, all structural homologs mentioned above exhibit the nature to bind nucleic acids (single-stranded DNA for WHY2 and PBF-2 and gRNA for MRP-1) (30–32). However, because of the sterically close effect of the cupin domain, the N-terminal domain of PnpD cannot form a whirligig appearance as its homologs (Fig. 2D), thus leading to a lack of the concave-shaped surface for nucleic acid interaction. Moreover, the positively charged residues are absent in the potential binding surface of the PnpD β-sheet (β1-β2-β3-β4) (Fig. 2E). Therefore, the N-terminal domain of PnpD might have a function other than nucleic acid binding (described below).

PnpC Is a Cupin Protein Lacking Catalytic Site—As shown in Fig. 4, A and B, the small subunit PnpC is mainly formed by eight antiparallel β-strands (β1–β8). PnpC has also been characterized as a cupin fold by structural comparison using DALI (Fig. 3, C and D), the same as the PnpD C-terminal domain. However, the residues responsible for iron binding are not conserved in PnpC when compared with its structural homologs (Fig. 3C), suggesting that PnpC might not contain an active site for dioxygenation reactions. Consistently, in the solved structures, there is no metal ion observed in PnpC.

Subsequently, a DALI pairwise comparison was performed for the two cupins in PnpCD, which revealed that the structure of PnpC is highly similar to the PnpD C-terminal domain (Z-score = 11.3, r.m.s.d. = 2.3 for 121 aligned Ca, sequence identity = 14%). However, there are several clear differences that should be noted (Fig. 4C). First, steric occlusion by PnpC residues Phe-71 and Tyr-134 precludes formation of a competent metal binding pocket. Next, the PnpC peptide between β4 and β6 is significantly longer than the corresponding peptide of PnpD (between β13 and β15). Also, different conformations and lengths are observed for the C-terminal residues of PnpC(151–163) and PnpD(319–339). Residues of the above two regions block the entrance to the active site in PnpC but contribute to active site formation in PnpD, further explaining the reason why PnpC cannot bind metal ion.

Intimately Associated Biological Unit, Heterotetramer (αβ2)2—The crystal structures of PnpCD reveal the presence of a tightly associated heterotetramer (αβ2)2 made up of two heterodimers of PnpCD (αβ) (Fig. 5A). The heterotetrameric nature of PnpCD in solution was confirmed by gel filtration analysis (Fig. 5B). Combined with the previous reports (9, 11, 12), it can be inferred that the polymeric status (heterotetramer) of HQDOs is highly conserved.

As shown in Fig. 5D, extensive contacts are observed between PnpC and the C-terminal domain of PnpD, including hydrogen bond and hydrophobic interactions, which result in the formation of a cupin dimer with a buried surface area of 2900 Å$^2$ (~30% surface area of PnpC). These data strongly recommend a stable interaction between PnpC and PnpD, which is in agreement with the fact that these two subunits cannot be efficiently purified separately (data not shown). The purification results of its homologs from Pseudomonas putida Dll-E4 (12) and Burkholderia sp. strain SJ98 (11) also contribute to this inference.

Two PnpCD molecules in the heterotetrameric structure are related by a noncrystallographic 2-fold axis (Fig. 5A). The interaction between them is mainly mediated by the α-subunits, resulting in a surface interface of 1100 Å$^2$. Hydrophobic interactions play a vital role in forming the dimer of PnpCD,
which significantly reduces the hydrophobic residues in a solvent-accessible surface area and strongly contributes to the stability of PnpCD in solution. The hydrophobic residues include Ile-24, Tyr-44, Phe-45, Leu-60, Pro-62, Leu-109, Val-114, Pro-147, Phe-154, Phe-250, Ile-251, Val-272, Pro-283, and Tyr-306, whereas a few residues (Glu-22, Asp-42, Tyr-44, Arg-57, Glu-249, and Thr-308) are involved in the hydrogen bond networks (Fig. 5C). According to the sequence alignment result, most of these residues are conserved for HQDOs from different species (Fig. 6). As indicated above, residues from the N-terminal domain of PnpD are indispensable for the heterotetrameric structure formation.

**Characteristics of the Iron Coordination in PnpCD**—In the binary PnpCD-Fe$^{3+}$ structure, half the α-subunits (PnpD) were observed with a ferric ion in the active site located on the interface of the C-terminal cupin and the N-terminal domain. The single iron ion is penta-coordinated, with a trigonal bipyramidal molecular geometry (Fig. 7A). The equatorial positions are occupied by His-256 N, Glu-262 O, and His-303 N2, whereas the axial positions are occupied by Asn-258 O and the O atom of a water molecule. Notably, the fifth coordination site exhibits elongated density. This density was modeled as a water molecule because the density for the second atom of the possible oxygen is not clear, and binding of oxygen in the absence of substrate generally does not occur (33, 34). In the structure of PnpCD-Cd$^{2+}$-HBN complex, each α-subunit (PnpD) contains a fully occupied Cd$^{2+}$ and HBN. The metal ion also coordinates to five ligands in a trigonal bipyramidal geometry (Fig. 7B). By replacing the position of a water...
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Substrate-binding Site—HBN, an analog of substrate hydroquinone, was bound to the active site of PnpCD-Cd$^{2+}$-HBN structure with clearly visible electron density (Fig. 7D). Compared with the active site of PnpCD-Fe$^{3+}$, no obvious conformational changes have been observed upon HBN binding. By replacing the position of a water molecule, HBN coordinates to the active site metal via its nitrogen atom. The hydroxyl group of HBN is 2.41 Å from Glu-248 Oe2, forming the only hydrogen bond between the ligand and protein residue. Thus, this hydrogen bond interaction should have an indispensable effect on the proper location of substrate. The hydrophobic pocket of HBN is mainly formed by the cupin domain residues of PnpD, including Trp-230, Pro-232, Leu-252, Phe-264, Trp-273, Leu-313, and Val-315. The N-terminal domain residues Trp-76 and Phe-79 also contribute to the pocket formation. Based on the sequence alignment with other HQDOs, Glu-248 and these hydrophobic residues of PnpCD are all highly conserved (Fig. 6). As mentioned above, the only hydrophilic residue around the substrate of HQDOs is Glu-248, which explains why HQDOs selectively convert hydroquinone rather than phenol, catechol, resorcinol, or hydroxyhydroquinone (9, 10).

To assess the importance of the conserved residues around the active site, we designed following single point mutants: W76A, F79A, W230A, L252A, F264A, W273A, L313A, V315A, and E248Q. The protein mutants were purified and then subjected to enzyme activity assays. As indicated by circular dichroism (CD) spectroscopy, all protein mutants are well folded as the wild type PnpCD. Consistent with our structural analysis, all mutants exhibited decreased activity compared with the wild type protein (Fig. 8A). Mutants W76A, F79A, F264A, W273A, and L313A virtually did not produce γ-HMSA from hydroquinone, indicating that each of these hydrophobic residue mutations almost completely abolished the catalytic activity of PnpCD. W230A and L252A resulted in a 70% loss of enzyme activity, whereas the V315A mutant retained about 50% activity. Notably, the mutant E248Q almost resulted in a complete loss of enzyme activity, and its substrate affinity (with a $K_d$ of 203 μM), measured by microscale thermophoresis, was comparable with the wild type PnpCD (with a $K_d$ of 526 μM) (Fig. 8, F and G). This indicated that the charged Glu-248 may directly participate in the electron transfer during the catalysis.

Unfortunately, we failed to obtain the PnpD structure in complex with oxygen after several attempts. However, in both PnpCD-Fe$^{3+}$ and PnpCD-Cd$^{2+}$-HBN structures, we found a small hydrophobic pocket near the metal ion-binding site, which was suitable for oxygen binding (Fig. 9A). This pocket is located at the opposite position of Glu-262, and surrounded by the PnpD residues Trp-76, Phe-79, Val-83, Val-254, and His-256. It is worth noting that the homolog 3-hydroxyanthranilate-3,4-dioxygenase (Z-score of 8.4, r.m.s.d. = 3.7 Å for 111 aligned Ca) binds to an oxygen molecule at almost the same position proposed for PnpCD (33), providing an effective support for our hypothesis. Interestingly, we identified two tunnels in each α-subunit of PnpCD-Cd$^{2+}$-HBN by surface analysis (Fig. 9, B and C). These tunnels are located near the active site, and thus might be responsible for substrate entry and product exit. One tunnel is mainly hydrophobic, which is formed by the PnpD residues Trp-76, Phe-79, Pro-232, and Glu-317. The other tunnel is surrounded by residues Tyr-80, Arg-260, and Glu-317 from PnpD, generating a mainly hydrophilic environment. In the structure
of PnpCD-Fe^{3+}, a different conformation was observed for Phe-79 and Tyr-80, which blocks the entrance to the active site, thus leading to disappearance of the two tunnels, respectively (Fig. 9, B and C). The structural analysis suggests that Phe-79 and Tyr-80 might be important in determining the open/closed conformation of the active site.

**Discussion**

Ring cleavage dioxygenases take critical parts in the degradation of aromatic compounds by aerobic microorganisms. They even can be the key determinants of the fate of certain aromatic compounds, and in several instances their properties were shown to confine the specificity of a degradation pathway (35, 36). Many pathways converge to well studied catecholic intermediates, which are subject to *meta-* or *ortho-* cleavage by extradiol or intradiol dioxygenases, respectively. A number of degradation pathways also proceed via noncatecholic ring-cleaving substrates, including hydroquinone, hydroxyquinol, gentisate, and protocatechuate. Determination of the dioxygenases catalyzing the ring cleavage of these substrates has long been of interest to the scientists in this field. In this study, we reported the first crystal structure of two-subunit hydroquinone 1,2-dioxygenase PnpCD, which helps us with a better understanding of the diversity of dioxygenase-catalyzing ring cleavage reactions.

**Novel Iron Metallocenter and the Possible Role of Asn-258** — In the PnpCD structures, four residues (His-256, Asn-258, Glu-262, and His-303) are observed to coordinate the iron ion. A four-residue coordination environment is common in plant germin-like proteins (PDB codes 3D82, 1LR5, and 1FI2). However, it rarely exists in cupin dioxygenases that are generally represented as a 2-His + Asp/Glu facial triad coordination. These dioxygenases include 3-hydroxyanthranilate 3,4-dioxygenase (PDB code 1YFU), cysteine dioxygenase (PDB code 1YFU, cysteine dioxygenase (PDB code...
3ELN), 2,4-dihydroxyacetophenone dioxygenase (PDB code 4P9G), gentisate 1,2-dioxygenase (PDB code 3BU7), and \( \Delta^2 \)-ketoglutarate-dependent dioxygenase AlkB (PDB code 4QKB). To date, only two cupin dioxygenases, quercetin 2,3-dioxygenase (PDB code 1H1I) and acireductone dioxygenase (PDB code 4QGL), have been reported to coordinate Fe\(^{2+}\) with four residues. However, these two enzymes constitute an unusual group of metallo-dioxygenases that retain activity after a metal swap (37, 38), which are significantly different from PnpCD. The results above imply that the catalytic mechanism of PnpCD might be distinct from that of the reported cupin dioxygenases.

Interestingly, the axial coordinating ligand Asn-258 has never been observed in the structures of PnpCD cupin homologs (Fig. 3A). As illustrated in Figs. 6 and 8A, Asn-258 is highly conserved in different HQDOs and should play an important role in the catalytic mechanism of HQDOs.

**FIGURE 6.** Multiple sequence alignment of HQDOs harboring two subunits.

The alignment is carried out using T-Coffee, and the results are generated using ESPript. The residues conserved in all five sequences are highlighted in red. The four coordinating residues of PnpD are assigned with red triangles.

| A | B |
|---|---|
| PnpD | WBC-3 |
| PnpC2 | DLL-E4 |
| HqdB | TTNP3 |
| HapE | ACB |
| PnpE1 | SJ98 |

| 1 | 10 | 20 | 30 | 40 | 50 | 60 |
|---|---|---|---|---|---|---|
| MAPS | SAMT | SAA | PAPD | XQA | PAP | PAP |
| MAPS | SAVE | ASP | PDD | XASA | PAP | PAP |
| MAPS | SAWA | ASR | PDD | XASA | PAP | PAP |
| MAPS | SAVP | ASP | PDD | XASA | PAP | PAP |
| MAPS | SAVP | ASP | PDD | XASA | PAP | PAP |

| 70 | 80 | 90 | 100 | 110 | 120 | 130 |
|---|---|---|---|---|---|---|
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |

| 140 | 150 | 160 | 170 | 180 | 190 | 200 |
|---|---|---|---|---|---|---|
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |

| 210 | 220 | 230 | 240 | 250 | 260 | 270 |
|---|---|---|---|---|---|---|
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |

| 280 | 290 | 300 | 310 | 320 | 330 |
|---|---|---|---|---|---|
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |

| 1 | 10 | 20 | 30 | 40 | 50 | 60 |
|---|---|---|---|---|---|---|
| MAPS | SAMT | SAA | PAPD | XQA | PAP | PAP |
| MAPS | SAVE | ASP | PDD | XASA | PAP | PAP |
| MAPS | SAWA | ASR | PDD | XASA | PAP | PAP |
| MAPS | SAVP | ASP | PDD | XASA | PAP | PAP |
| MAPS | SAVP | ASP | PDD | XASA | PAP | PAP |

| 70 | 80 | 90 | 100 | 110 | 120 | 130 |
|---|---|---|---|---|---|---|
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |
| TQVR | RP | EQA | TAP | PAP | PAP | PAP |

| 140 | 150 | 160 | 170 | 180 | 190 | 200 |
|---|---|---|---|---|---|---|
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |
| VNP | VNP | VNP | VNP | VNP | VNP | VNP |

| 210 | 220 | 230 | 240 | 250 | 260 | 270 |
|---|---|---|---|---|---|---|
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED | VED |

| 280 | 290 | 300 | 310 | 320 | 330 |
|---|---|---|---|---|---|
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |
| VED | VED | VED | VED | VED | VED |

FIGURE 6. Multiple sequence alignment of HQDOs harboring two subunits. The alignment is carried out using T-Coffee, and the results are generated using ESPript. The residues conserved in all five sequences are highlighted in red. The four coordinating residues of PnpD are assigned with red triangles. WBC-3, Pseudomonas sp. strain WBC-3; DLL-E4, P. putida DLL-E4; TTNP3, Sphingomonas sp. strain TTNP3; ACB, Pseudomonas fluorescens ACB; SJ98, Burkholderia sp. strain SJ98.
role in the catalytic reaction of PnpCD. The ITC measurement of PnpCD and its mutants (N258D and N258A) indicated that Asn-258 should directly participate in the metal ion coordination other than crystal packing. Notably, in the structure of PnpCD-Fe$^{3+}$, the conformation of the Asn-258 side chain shows a slight difference between the iron-free or iron-bound state. Concerted conformational changes of other coordinating residues are also observed (Fig. 7C). The geometric position of the coordinating residues in iron-free state is similar to that of human homogentisate 1,2-dioxygenase (PDB code 1EY2). Also, it is noted that HBN is a bit longer than substrate hydroquinone. Based on those observations, we proposed a bold hypothesis for the role of Asn-258 in the reaction mechanism. Asn-258 should play a vital role in binding the iron before the enzymatic reaction. However, when the reaction starts, Asn-258 might not coordinate the iron any more. In the meantime, concerted conformational changes may occur on other coordinating residues, which would be suitable for catalyzing the reaction as the cupin dioxygenases with 2-His + Asp/Glu facial triad coordination. Although this new hypothesis still needs further verification, it might shed new light on the study of cupin coordination and catalytic mechanism of dioxygenases.

**Mechanistic Implications**—As mentioned above, PnpCD is a member of the non-heme Fe$^{2+}$-dependent dioxygenase family, and its coordinating residues include two histidines, one glutamic acid, and one asparagine. These features imply that the
enzymatic reaction of PnpCD may perform in a mechanism similar to extradiol dioxygenases. Based on the structures we have determined and studies of other extradiol dioxygenases (33, 39–41), we propose a general catalytic mechanism for PnpCD in the ring cleavage of hydroquinone (Fig. 10). (i) In the absence of Fe$^{2+}$ ion, the coordinating residues (His-256, Asn-258, Glu-262, and His-303) may exhibit two conformations (Fig. 7C), which have not been reported in other extradiol dioxygenases. (ii) Fe$^{2+}$ ion coordinates to the active site probably through the tunnels we identified (Fig. 9). The single Fe$^{2+}$ ion is penta-coordinated, with a trigonal bipyramidal molecular geometry (Fig. 7A). (iii) The substrate hydroquinone enters into the active site through the proposed tunnels. By replacing the axial water molecule, hydroquinone may coordinate to Fe$^{2+}$ in a position opposite to Asn-258 (Fig. 7B). It is then, probably, that Asn-258 no longer coordinates the iron. In the meantime, concerted conformational changes may occur on other coordinating residues, which would be suitable for catalyzing the reaction as the cupin dioxygenases with 2-His/Asp/Glu facial triad coordination. Subsequently, O$_2$ may bind to the hydrophobic pocket opposite to Glu-262 (Fig. 9A). These allow a concerted activation of substrate and O$_2$ by electron transfer via Fe$^{3+}$. His-256 is located near the possible O$_2$-binding site and may play an important role in stabilizing the Fe$^{2+}$-superoxide intermediate. (iv) Recombination of the above radicals may generate an alkylperoxo intermediate, which was proposed to break down via Criegee rearrangement to yield a lactone intermediate (40, 42). Glu-248 is positioned to serve as a proton acceptor for the alkylperoxo intermediate to promote heterolytic O–O bond fission. (v) The lactone would undergo hydrolysis via the oxygen atom from O$_2$ (42), thus leading to the formation of open ring product. The product could be proto-
nated by Glu-248 and finally released from the active site via the proposed tunnels.

*Cupin Dimer in PnpCD*—Both PnpC and the C-terminal domain of PnpD comprise a conserved β-barrel fold (Figs. 2A and 4A), which was then designated as cupin by structural comparison using the DALI server. Extensive contacts are observed between the two cupins (Fig. 5D), resulting in the formation of a stable cupin dimer with a buried surface area of 2900 Å². The cupin fold of PnpD contains the intact active site elements of non-heme iron enzymes, including a four-residue (2His-1Glu-1Asn) coordination system. While in PnpC, steric occlusion by residues Phe-71 and Tyr-134 precludes formation of a competent metal binding pocket (Fig. 4C). Combined with the observation that the homologous dioxygenase HQDO bound to iron ion in a 1:1 manner (9), we inferred that PnpC should be vestigial and does not participate in catalysis. A structural comparison was performed for the two cupins in PnpCD, which revealed that the structure of PnpC is highly similar to the PnpD C-terminal domain despite that their sequence identity is as low as 14%.

The above features of PnpCd are similar to that of some bicupin proteins, including quercetin dioxygenase (EC 1.13.11.24), gentisate 1,2-dioxygenase (EC 1.13.11.4), homogentisate dioxygenase (EC 1.13.11.5), and 1-hydroxy-2-naphthoate dioxygenase (EC 1.13.11.38). These bicupins only comprise a single active site in one of the two domains, with the other domain remaining as a nonfunctional vestigial remnant. The result of phylogenetic analysis suggested that two-domain cupins most likely evolve from gene duplication of a single-domain ancestor (43–45). Consequently, we speculate that PnpCD may also evolve in a similar way. One reason for this evolution might be to enhance the stability of the protein, which would efficiently improve the enzyme activity. In turn, the cupin with active site exposed to solvent might become less important, thus allowing a gradual loss of its enzyme activity.

**Intriguing N-terminal Domain of PnpD**—The N-terminal domain of PnpD mainly consists of two antiparallel β-sheets packed perpendicularly (Fig. 2A). No putative conserved domain has been detected via the blast search of the protein sequence. Considering that, we performed a structural comparison using a DALI server. Intriguingly, the structural homologs of the PnpD N-terminal domain exhibit the nature to bind nucleic acids. However, because of the steric effect of the cupin domain, the N-terminal domain of PnpD cannot form the characteristic whirligig appearance as its homologs (Fig. 2D). Moreover, the positively charged area is absent in the potential binding surface of PnpD (Fig. 2E). Therefore, the N-terminal domain of PnpD might have functions other than nucleic acid binding.

A structural analysis was performed on the tightly associated heterotetramer. The result showed that PnpD N-terminal domain plays a vital role in formation of the (αβ)₄ structure (Fig. 5, A and C). Further studies indicated that N-terminal
domain residues Trp-76 and Phe-79 are indispensable for the formation of active site pocket (Fig. 7D). Single mutants W76A and F79A almost resulted in a complete loss of enzyme activity (Fig. 8A). Moreover, Phe-79 and Tyr-80 of PnpD contribute to the formation of putative tunnels (Fig. 9, B and C), and thus they might play an important part in determining the open/closed conformation of the active site. Despite all these, are there other functions for the characteristic fold of PnpD N-terminal domain? This remains an open question and would be an interesting topic for future research.

New Structural Class of Fe²⁺-dependent Dioxygenases—Structural comparison using the DALI server failed to identify any homologs of full-length PnpD (α-subunit). As mentioned above, PnpD is composed of a C-terminal catalytic cupin and an intriguing N-terminal domain. Combined with the nonfunctional cupin of PnpC (β-subunit), PnpCD comprises a cupin dimer with single active site. Recently, the structure of another hydroquinone 1,2-dioxygenase (PcpA) was reported (46). Similar to PnpCD, PcpA is also a non-heme Fe²⁺ dioxygenase capable of cleaving the aromatic ring of hydroquinone. However, PcpA is composed of an ∼37-kDa subunit containing four similarly sized βαβαβ motifs connected by long loops. The overall fold of PcpA is significantly different from that of PnpCD. The PnpCD structure exhibits a four-residue coordination environment, whereas other cupin dioxygenases are generally represented as a 2-His + Asp/Glu facial triad coordination. Besides that, the axial coordinating ligand Asn-258 has never been observed in the homologous cupin structures. These coordinating features imply that the catalytic mechanism of PnpCD might be distinct from that of the reported cupin dioxygenases.

In summary, PnpCD has no apparent evolutionary relationship to other iron-dependent dioxygenases and therefore defines a new structural class. The study of PnpCD might shed new light on cupin coordination and the ring cleavage mechanism of dioxygenases, thus providing useful clues for enzyme engineering for a better catalytic efficiency or a broader specificity. Moreover, it will contribute to the functional identification of the less well characterized cupin proteins or other hypothetical proteins.

Author Contributions—S. L., L. Gu, and N. Z. designed the study. S. L., T. S., C. Z., W. Z., D. Z., J. S., K. W., T. W., Y. H., L. Gu, and S. X. performed the experiments. S. L. and L. Gu analyzed the data. S. L. wrote the original draft. S. L. T. S., L. Gu, and N. Z. edited the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank the staff at beamline BL17U1 at the Shanghai Synchrotron Radiation facility for assistance with data collection. We also thank Yao Wu at Institute of Microbiology, Chinese Academy of Sciences, for assistance with the MST (MicroScale Thermophoresis) experiment.

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