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Decomposition of aromatic hydrocarbon intermediates by recombinant hydroxyquinol 1,2-dioxygenase from *Arthrobacter chlorophenolicus* A6 and its structure characterization

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ABSTRACT

This study was carried out to characterize hydroxyquinol 1,2-dioxygenase from *Arthrobacter chlorophenolicus* A6 (*Ar* 1,2-HQD). The *cphA-I* gene encoding *Ar* 1,2-HQD was cloned and the enzyme was overexpressed for subsequent purification of the recombinant protein by Ni^{2+}-NTA affinity chromatography and fast protein liquid chromatography (FPLC). Purification of the enzyme by Ni^{2+}-NTA affinity chromatography increased the substrate activity for various aromatic hydrocarbon intermediates by 9.4- to 12.9-fold. Subsequent purification of the enzyme by FPLC increased the activity by 51.7- to 65.2-fold. The substrate specificity of *Ar* 1,2-HQD indicated that the catalytic function of this enzyme is similar to that of the type-II catechol 1,2-dioxygenase (1,2-CTD). The deduced 304 amino acid sequence analysis also revealed that *Ar* 1,2-HQD is an intradiol dioxygenase and it is related closely to 1,2-CTD. Its structure consisted of 5 α-helices in the N-terminal domain and 13 β-sheets in the C-terminal domain. The ferric ion was coordinated to two histidine residues and two tyrosine residues. The results of this study suggest that the highly purified *Ar* 1,2-HQD can be used as a key enzyme in the biodegradation of aromatic hydrocarbon contaminants.

1. Introduction

Phenolic compounds have been used in many industrial production processes, including synthetic resin, dyes, pharmaceuticals, perfumes, pesticides, tanning agents, solvents or lubricating oils (Veenagayathri and Vasudevan, 2011). Given their toxicity and persistence in the environment, these compounds can pose serious risks to living organisms, including humans (Park et al., 2013). One of the well known phenolic ecotoxins is chlorophenol, which is genotoxic, mutagenic, and carcinogenic. Those who consume drinking water contaminated with chlorophenols can suffer from infections, dermatitis, irritation of the digestive tract, and extreme exhaustion (Michałowicz and Pol, 2005). Biodegradation of these hazardous compounds has been suggested as an effective approach for their removal (Wen et al., 2006). There are two different pathways for aerobic metabolism of chlorinated aromatic compounds: (i) aromatic compounds consisting of one or two chlorines can be transformed to chlorocatechols and further degraded via *ortho* cleavage, or (ii) aromatic compounds containing more than two chlorines can be transformed to hydroxyquinol or chlorohydroxyquinol (Daubaras et al., 1996; Ferraroni et al., 2004).

Hydroxyquinol is an intermediate found in the central pathway of biodegradation of various aromatic compounds by yeast, fungi, and bacteria (Hatta et al., 1999; Murakami et al., 1999; Ferraroni et al., 2005; Travkin et al., 2006). Hydroxyquinol is generated from the breakdown of resorcinol, benzoate, 4-hydroxybenzoate, protocatechuate, salicylate, and gentisate by *Trichosporon cutaneum* (Sze and Dagley, 1984). Rieble et al. (1994) reported that the fungus *Phanerochaete chrysosporium* degrades vanillate via the intermediate hydroxyquinol. Hydroxyquinol is also an intermediate during the degradation of mononuclear hydroxyaromatics, amino-

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hydroxyaromatics, hydroxylated biaryl ethers, and nitro group aromatics by various bacteria (Ferraroni et al., 2005; Travkin et al., 2006).

Hydroxyquinol and its chlorinated derivatives can be decomposed by hydroxyquinol 1,2-dioxygenase (1,2-HQD). Microorganisms from which HQDs can be purified and characterized include fungi (*P. chrysosporium*; Rieble et al., 1994), yeast (*T. cutaneum*; Sze and Dagley, 1984), Gram-negative bacteria such as *Burkholderia cepacia* AC1100 (Daubaras et al., 1996), *Azotobacter* sp. GP1 (Latus et al., 1995), and *Ralstonia pickettii* DTP0602 (Murakami et al., 1999), and Gram-positive bacteria such as *Nocardioides simplex* 3E (Ferraroni et al., 2005), *Arthrobacter* sp. strain BA-5-17 (Murakami et al., 1999), and *Arthrobacter chlorophenolicus* A6 (Nordin et al., 2005). The function of HQD is the cleavage of aromatic rings of hydroxyquinol and its chlorinated derivatives (Daubaras et al., 1996; Murakami et al., 1999; Nordin et al., 2005).

The crystal structure of 1,2-HQD from *N. simplex* 3E (*Ns* 1,2-HQD) indicated that this enzyme contains two catalytic irons that are coordinated to the polyhedron side chains of Tyr-164, Tyr-197, His-221, and His-223; the resulting structure resembles the structures of other intradiol ring cleavage dioxygenases that are dimeric and each subunit of them has one iron (Ferraroni et al., 2005). The intradiol dioxygenases have a trigonal bipyramidal ferric iron site with two tyrosine and two histidine residues (Guzik et al., 2011). The interaction of ferric iron with phenolate moieties of tyrosine residues plays a significant role in enzyme function, and stabilizes the active site geometries of dioxygenases (Palaniandavar et al., 2006). The catalytic reaction mechanism of the intradiol dioxygenase starts with the binding of the substrate as a dianion and the production of an iron-alkylperoxo intermediate. In the next step, the Criegee rearrangement and O–O bond cleavage involve the acyl migration to yield the cyclic anhydride and iron-bound oxide or hydroxide. Then, hydrolysis of the anhydride takes place and this yields the ring-opened product (Vaillancourt et al., 2006). However, the structure of *Ar* 1,2-HQD has not been characterized yet.

The objectives of this study were to clone, overexpress, and purify *Ar* 1,2-HQD and to characterize its structure by modeling. Specific activities of enzyme products obtained from the purification process were examined by using hydroxyquinol, catechol, and its analogs as substrates. The amino acid sequence of *Ar* 1,2-HQD was deduced as a part of its structural characterization. The predicted 3-D structure of *Ar* 1,2-HQD was compared with those of *Ns* 1,2-HQD, catechol 1,2-dioxygenase (1,2-CTD), and chlorocatechol 1,2-dioxygenase (1,2-CCD).

2. Materials and methods

2.1. Microorganisms and media

A. chlorophenolicus strain A6 was obtained from the American Type Culture Collection (ATCC 700700). The cells were enriched in a mineral medium (2.1 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.5 g of NH_4NO_3, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.023 g of $CaCl_2 \cdot 2H_2O$, 2 mg of $FeCl_3 \cdot 6H_2O$, and 5.0 g of yeast extract per L of Milli-Q water) supplemented with 0.15 g/L of 4-chlorophenol as the sole carbon source. Quick Start Bradford reagents, ampicillin sodium salt, and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Bio-Rad (Hercules, USA). The plasmid vector pET-11a used for gene cloning was obtained from Novagen (Darmstadt, Germany). *Escherichia coli* BL21 (DE3) was used as the host strain for gene transformation and overexpression of the target enzyme. The Luria–Bertani (LB) medium was used for cell harvesting, and the mineral medium was used for washing the cells and enzymes. All media were sterilized prior to use.

2.2. Cloning and overexpression of enzyme

The procedure for cloning and overexpression of *Ar* 1,2-HQD was adopted from Lee et al. (2013) and modified in this study. Briefly, the *cphA-I* gene was amplified by PCR by using genomic DNA of *A. chlorophenolicus* A6 as a template using *Pfu* DNA polymerase (Daemyung Science, Seoul, Korea) and the two primers $P_{forward}$ (5′-ggg ggg cat atg acg acc cgt caa gta gcc cca-3′) and $P_{reverse}$ (5′-ggg ggg gga tcc tca ctt cag atc agg att agg-3′). The *Nde*I and *BamH*I sites in these primers are underlined. The amplified PCR product was digested with the *Nde*I and *BamH*I endonucleases (Promega, Madison, USA). The gene was cloned into the pET-11a vector modified with a His-tag to construct the recombinant plasmid. The cloned vector was then transformed into *E. coli* BL21 (DE3), and DNA sequencing was performed to confirm the successful cloning of the *cphA-I* gene. The *E. coli* harboring the recombinant plasmid was grown in an LB medium containing 50 μg/mL-ampicillin at 37 °C on an incubating shaker operated at 180 rpm until the OD_{600} of the culture reached a value of 0.6. Then IPTG was added at various concentrations in the range of 0.1–0.6 mM, and the cells were incubated at various temperatures of 15 °C–37 °C. Depending on the temperature, the induction period was adjusted in the range of 2–24 h with stirring at 130 rpm. The internal sequence of the *cphA-I* gene was compared with sequences available at the National Center for Biotechnology Information (NCBI) base followed by multiple sequence alignment using CLC Free Workbench software (CLC Bio A/S, Aarhus, Denmark).

2.3. Enzyme purification

Cells of *E. coli* BL21 (DE3) that overexpressed the target enzyme were lysed in 30 mL of lysis buffer (150 mM of NaCl, 20% of glycerol, 0.024% of 2-mercaptoethanol, and 25 mM of Tris–HCl buffer at pH 7.5) by sonication (on for 20 s and off for 30 s; 36 times), and the resulting extracts were centrifuged at 9000× *g* for 45 min. The enzyme was further purified by passing the supernatant through a Ni^{2+}-NTA His•Bind column (Novagen) after incubation of 30 min. The column was washed with 30–50 mL of washing buffer to remove residual proteins. Two washing buffers were used: washing buffer 1 (150 mM of NaCl, 20% of glycerol, 15 mM of imidazole, 25 mM of Tris–HCl buffer at pH 7.5) and washing buffer 2 (150 mM of NaCl, 20% of glycerol, 20 mM of imidazole, and 25 mM of Tris–HCl buffer at pH 7.5). The target enzyme was then eluted by application of an elution buffer (150 mM of NaCl, 20% of glycerol, 300 mM of imidazole, 25 mM of Tris–HCl buffer at pH 7.5). Finally, the target enzyme was further purified by fast protein liquid chromatography (FPLC). The fraction obtained by FPLC was stored in a deep freezer at −80 °C prior to use. Protein purity and concentration were examined and quantified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration analysis. SDS-PAGE was performed on 10% polyacrylamide slab gels. The purified fractions were loaded on the gel and the enzymes after electrophoresis were visualized using PageBlue Protein Staining Solution (Thermo Fisher Scientific, Chicago, USA).

2.4. Enzyme activity assay

The enzyme activities specific to the substrates tested (hydroxyquinol, catechol, 4-chlorocatechol, and 3-methylcatechol) were measured separately. The enzymes contained in crude extracts, obtained from the first Ni^{2+}-NTA His•Bind column, and obtained from the second FPLC step, were mixed with each of the substrates in the presence of H_2O_2 as an oxygen source (molar ratio