Biosci. Biotechnol. Biochem., **76** (3), 567–574, 2012

Molecular and Catalytic Properties of 2,4′-Dihydroxyacetophenone Dioxygenase from *Burkholderia* sp. AZ11

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Received November 14, 2011; Accepted December 9, 2011; Online Publication, March 7, 2012
[doi:10.1271/bbb.110867]

The gene *dad* encoding 2,4′-dihydroxyacetophenone (DHAP) dioxygenase was cloned from *Burkholderia* sp. AZ11. The initiation codon GTG was converted to ATG for high-level expression of the enzyme in *Escherichia coli*. The enzyme was moderately thermostable, and the recombinant enzyme was briefly purified. The enzyme (M_r = 90 kDa) was a homotetramer with a subunit M_r of 23 kDa. It contained 1.69 mol of non-heme iron, and had a dark gray color. On anaerobic incubation of it with DHAP, the absorption at around 400 nm increased due to the formation of an enzyme-DHAP complex. Multiple sequence alignment suggested that His77, His79, His115, and Glu96 in the cupin fold were possible metal ligands. The apparent K_m for DHAP and the apparent V_{max} were estimated to be 1.60 μM and 6.28 μmol/min/mg respectively. 2-Hydroxyacetophenone was a poor substrate. $CuCl_2$ and $HgCl_2$ strongly inhibited the enzyme, while $FeSO_4$ weakly activated it.

Key words: *Burkholderia*; dihydroxyacetophenone; dioxygenase; metalloenzyme

Microorganisms in soil degrade various aromatic compounds. Bisphenol A (2,2-bis(4-hydroxyphenyl)-propane), a zenobiotic that has weak endocrine disrupting activity,[1,2] is cleaved to 4-hydroxybenzaldehyde and 4-hydroxyacetophenone by various bacteria.[3–7] Both compounds are further degraded in such a way that they can enter the central metabolic pathway. The former is oxidized to a key intermediate, 4-hydroxybenzoate.[4] The latter is converted to 4-hydroxyphenyl acetate by 4-hydroxyacetophenone monooxygenase (EC 1.14.13.84), and subsequently is hydrolyzed to hydroquinone.[8–10] Hydroquinone is cleaved to 4-hydroxymuconic semialdehyde and metabolized further.[11] Besides these metabolic intermediates, a small amount of 2,4′-dihydroxyacetophenone (DHAP, 2-hydroxy-1-(4-hydroxyphenyl)ethanone) is formed as a dead-end product, which is not metabolized any further but accumulates in the culture broth of bisphenol A-assimilating bacterium.[4] Hopper *et al.* found that *Alcaligenes* sp. 4HAP grown on 4-hydroxyacetophenone does not degrade hydroquinone, but oxidizes DHAP to 4-hydroxybenzoate.[12,13] In this strain, a hydroxylase converts 4-hydroxyacetophenone to DHAP, and then DHAP dioxygenase (DAD, EC 1.13.11.41) cleaves the hydroxyacetyl group of DHAP to yield 4-hydroxybenzoate and formate. DAD adds the two oxygen atoms of O_2 into two cleavage products one by one.[14] Thus bisphenol A is completely assimilated in a collaboration of bisphenol A-degrading bacteria and DHAP-degrading bacteria. DAD has been purified as a nonheme-iron-containing metalloenzyme from *Alcaligenes* sp. 4HAP with a view to cloning the gene *dad* encoding DAD.[15] However, much about the basic biochemical properties of DAD, including substrate specificity, kinetic constants, cofactor requirements, inhibitors, optimum pH, stability, and catalytic mechanism, has not been fully determined. Crystals of DAD from *Alcaligenes* sp. were prepared by Bowyer, but its three-dimensional structure was not determined.[16] Although many genes showing high degrees of homology to *dad* have been found in various bacterial strains, no biochemical investigation of DAD has been reported. In this paper, we explain the molecular and catalytic properties of DAD from DHAP-degrading bacterium *Burkholderia* sp. strain AZ11, which appears to be irrelevant to the metabolism of bisphenol A.

Materials and Methods

Chemicals. DHAP was synthesized from 2-bromo-4′-methoxyacetophenone by the method of Robertson and Robinson,[17] and identified by ^{1}H-NMR. All other chemicals were commercially available and of analytical grade.

Microorganisms. DHAP assimilating bacterium strain AZ11 was isolated from a soil sample in Gifu, Japan, by enrichment culture with DHAP as carbon and energy source. Isolation and cultivation of AZ11 was done with a liquid medium (pH 7.0) containing 4.48 g of $Na_2HPO_4 \cdot 12H_2O$, 1.7 g of KH_2PO_4, 3.0 g of $(NH_4)_2SO_4$, 0.15 g of $MgSO_4 \cdot 7H_2O$, 15 mg of $CaCl_2 \cdot 2H_2O$, 0.25 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.5 mg of $FeSO_4 \cdot 7H_2O$, 50 μg of $CuSO_4 \cdot 5H_2O$, 25 μg of $CoSO_4 \cdot 7H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 0.2 mg of $MnSO_4 \cdot 4H_2O$, 150 mg of yeast extract, and 100 mg of DHAP in 1 L of tap water. It was aerobic, Gram-negative, oxidase positive, motile, and rod-shaped. The genomic DNA of strain AZ11 was prepared, and the 16S rRNA gene was amplified by polymerase chain reaction with 27f and 1525r primers, as described by Shinoda *et al.*[18] The nucleotide sequence (1,494-bp long, DDBJ/EMBL/GenBank databases accession no. AB636680) showed high homology (99.9%) to those of *Burkholderia*. Based on these results, AZ11 was identified as *Burkholderia*. It was maintained by growth on 1.5% w/v solid agar slant, and stored at 4 °C.

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Abbreviations: DAD, 2,4′-dihydroxyacetophenone dioxygenase; DHAP, 2,4′-dihydroxyacetophenone; IPTG, isopropyl β-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PPB, potassium phosphate buffer

It was subcultured monthly. For large-scale culture, reciprocal culture with 500-mL Sakaguchi flasks was done at 30 °C for 2 d. *Escherichia coli* XL1-Blue MRA and XL1-Blue MRF′ obtained from Stratagene (La Jolla, CA) and *E. coli* JM109 obtained from Takara (Ohtsu, Japan) were used for gene cloning.[19,20] For gene expression, *E. coli* JM109 harboring plasmid pUDP1.3m with a *dad* insert was cultured at 37 °C in 1.4 L of LB medium (polypeptone 20 g, yeast extract 10 g, and NaCl 20 g per 1 L distilled H_2O, pH 7.0) containing 50 μg/mL ampicillin. When A_{660} reached about 0.15, 1 mM isopropyl β-thiogalactopyranoside (IPTG) was added, and culturing was continued for a further 9 h. Bacterial cells were collected by centrifugation at 4 °C, washed once with 50 mM potassium phosphate buffer (PPB) (pH 7.0), and stored at −25 °C until needed.

Enzyme purification. Unless otherwise noted, all procedures were performed at 0–4 °C with 50 mM PPB (pH 7.0).

(i) *Purification from* Burkholderia *sp. AZ11.* The bacterial cells (about 1.7 g wet weight) obtained from 2 L of liquid culture were suspended in about 8 mL of PPB and disrupted with a Sonifier (Branson, Danbury, CT). The solution was clarified by centrifugation. The supernatant (crude extract) was loaded onto a column (3 × 25 cm) of Superdex 200 (GE Healthcare, Buckinghamshire, UK) equilibrated with PPB. The active fractions were pooled and applied to a column (1.0 × 12 cm) of Q-Sepharose (Pharmacia, Uppsala, Sweden) previously equilibrated with PPB. After the column was washed with 200 mL of PPB, the enzyme was eluted with a linear gradient established with 50 mL of PPB and 50 mL of PPB containing 1 M NaCl. The active fractions were pooled. Solid $(NH_4)_2SO_4$ was dissolved in the solution to 1.5 M. The solution was loaded on a column (2.2 × 4.5 cm) of Phenyl Sepharose (GE Healthcare) previously equilibrated with PPB containing 1.5 M $(NH_4)_2SO_4$. The column was washed with 40 mL of equilibration buffer, followed by a linear gradient established with 50 mL of the equilibration buffer and 50 mL of PPB. After the column was washed with 60 mL of PPB, the enzyme was eluted with PPB containing 0.4% Lubrol PX. The active fractions were pooled and concentrated with a collodion bag (Sartorius, Göttingen, Germany), and stored at −25 °C.

(ii) *Purification from recombinant* E. coli. Cells of *E. coli* JM109 harboring pUDP1.3m (about 3.5 g wet weight) obtained from 1.4-L culture were suspended with 14 mL of the buffer and disrupted as above. Cell debris was removed by centrifugation, and the volume of the supernatant was adjusted to 140 mL with the buffer (crude extract). The solution was maintained at 60 °C for 10 min. The denatured protein was removed by centrifugation. Then the solution was treated with $(NH_4)_2SO_4$. The precipitate obtained from 30–55% saturation was dissolved in 6 mL of the buffer and applied to Superdex 200 column chromatography, followed by the ion-exchange column chromatography with Q-Sepharose, as described above. The active fractions were pooled and stored at −25 °C.

Determination of enzyme activity. DHAP showed an absorption peak at 280 nm in 50 mM PPB (pH 7.0), while the reaction products, 4-hydroxybenzoate and formate, scarcely absorbed at this wavelength. Hence the enzyme activity was determined at 25 °C by measuring the decrease in A_{280} ($\varepsilon_{280} = 11\ mM^{-1}\ cm^{-1}$) with spectrophotometer UV-300 or UV1600 (Shimadzu, Kyoto, Japan). The reaction was carried out at 25 °C in a cuvette (light path 1 cm). The standard reaction mixture (3 mL) contained 50 mM PPB (pH 7.0), 30 μM DHAP, and the enzyme. The activity of each fraction from column chromatography was briefly measured with Flying Spot Scanner (Shimadzu) equipped with a 96-well plate. The reaction mixture (200 μL) containing 50 mM PPB (pH 7.0), 210 μM DHAP, and the enzyme was incubated at room temperature for 10 min to measure the decrease in A_{280}. Enzyme activity was also determined at 25 °C by measuring O_2 consumption with Biological Oxygen Monitor 5300 (Yellow Springs Instrument, Yellow Springs, OH). The reaction mixture (4.0 mL) contained 50 mM PPB (pH 7.0), 0.35 mM DHAP, and the enzyme. One unit of enzyme activity was defined as the amount catalyzing the degradation of 1 μmol of substrate per min under the assay conditions. Specific activity was expressed as units per mg of protein. Protein was measured by the method of Lowry *et al.*, with bovine serum albumin as standard.[21] When the diluted protein sample was treated, the protein was precipitated with trichloroacetic acid and collected on a nylon membrane (pore size 0.45 μm) (Millipore, Concord, MA) before determination.[22]

To determine the effects of various reagents on enzyme activity, the enzyme purified from *E. coli* JM109 carrying pUDP1.3m was incubated at 4 °C for 1 d in the absence and the presence of 0.4% w/w Lubrol PX. Then it was preincubated at 25 °C for 5 min with various reagents (1 mM each) in 50 mM PPB (pH 7.0), and residual activity was measured spectrophotometrically under standard conditions.

SDS-polyacrylamide gel electrophoresis (PAGE) and amino acid sequence. SDS–PAGE was done with a 12.5% gel following Laemmli.[23] Coomassie Brilliant Blue R-250 or a silver staining kit (Bexel, Union City, CA) was used for protein staining. Electroblotting to a polyvinyridene difluoride membrane was done with semi-dry blotting apparatus HorizBLOT AE-6675 (Atto, Tokyo). The protein band, stained with Coomassie Brilliant Blue R-250, was excised, and the N-terminal amino acid sequence was determined by Edman degradation with a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA). The N-terminal amino acid residue was identified as dancyl amino acid by the method of Gray.[24]

Molecular mass. The molecular mass of the enzyme was determined by gel filtration on Superdex 200 (column size, 1.8 × 22 cm) previously equilibrated with 50 mM PPB (pH 7.0) and calibrated with ferritin (M_r = 450 kDa), bovine liver catalase (230 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and bovine pancreas α-chymotrypsin (23.5 kDa).

TLC. After the enzymatic reaction, the mixture was acidified with HCl and centrifuged to remove denatured proteins. The supernatant was extracted with ethyl acetate and analyzed by TLC with silica gel 60 F_{254} (Merck, Darmstadt, Germany). Toluene-$CHCl_3$-acetone (8:5:7, v/v/v) was used as solvent. The spots were visualized by UV. The following R_f-values were obtained: DHAP, 0.51; 4-hydroxybenzoate, 0.38; 2-hydroxyacetophenone, 0.69; benzoate, 0.55.

Analysis of metal and inorganic sulfide. Enzyme-bound iron and copper were determined with a polarized absorption spectrophotometer Z-5310 (Hitachi, Tokyo). Non-heme iron was also estimated colorimetrically with 0.53–0.58 mg of the purified enzyme by the method of Fish.[25] Inorganic sulfide was determined with 1.3 mg of the purified enzyme by the method of Fogo and Popowsky, as modified by Lovenberg *et al.*[26,27]

Instrumental analysis. Reversed-phase HPLC was conducted at 45 °C with a LC-9A liquid chromatograph (Shimadzu) equipped with a Cosmosil column 5C_{18}-MS-II (4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan), a SPD-6AV UV-Vis detector, a CTO-2A column oven, and a C-R6A data processor. The flow rate was 1 mL/min. Formate was detected at 210 nm, and the other compounds at 254 nm. Elution was done with MeOH-20 mM PPB (pH 7.0) (1:4, v/v). Under these conditions, formate, 4-hydroxybenzoate, benzoate, DHAP, and 2-hydroxyacetophenone were eluted at about 2.1, 3.2, 6.2, and 9.2 min respectively. For quantitative determination of formate, the deproteinized reaction mixture (2.5 μL) was analyzed by HPLC. For determination of aromatic carboxylates, the reaction mixture (3 mL) was acidified with HCl and extracted 3 times with ethyl acetate. The extract was evaporated at 25 °C, the residue was dissolved in 200 μL of elution buffer, and 10 μL was analyzed by HPLC. Absorption spectra and ^{1}H-NMR were measured by UV300 (Shimadzu) and by JNM-ECX400P (JEOL, Tokyo) respectively.

DNA preparation and manipulation. Genomic DNA of *Burkholderia* sp. AZ11 was prepared as described by Smith,[28] except that proteinase K treatment (1 mg/mL, 37 °C, 30 min) was also performed after lysis of the bacterial cells. All oligonucleotides for hybridization, DNA sequencing, and site-directed mutagenesis were obtained from Rikakken (Nagoya, Japan). An oligonucleotide OL26 (5′-GACAAAGCCGTATCCGAATTCTGGCA-3′), designed based on the N-terminal amino acid sequence of DAD purified from strain AZ11 and the nucleotide sequence assumed as *dad* in *B. cenocepacia* J2315 (Genbank accession no. AM747721, locus tag BCAM0037), was labeled at the 3′-terminal with digoxigenin using a DIG-tailing label kit

(Boehringer Mannheim, Mannheim, Germany), and used as a probe for hybridization. Southern and colony hybridization were done at 56 °C. Hybridization signals on nylon membranes were detected with a DIG luminescent detection kit (Boehringer Mannheim) following the manufacturer's protocols. All enzymes used in DNA manipulation were obtained from Takara. Plasmids were isolated from recombinant *E. coli* with a QIAprep kit (Qiagen, Hilden, Germany). Extraction of DNA from agarose gel was done with an Easy Trap (Takara). DNA digestion, ligation, and transformation were done as described by Sambrook *et al.*[29]

Nucleotide sequence. Deletion mutants for DNA sequencing were prepared with a Kilo-sequence deletion kit (Takara) after digestion with appropriate restriction enzymes. Both strands were sequenced on denatured double-stranded DNA templates with a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and appropriate primers (usually 25mer). The nucleotide sequence was determined with an ABI 3100 DNA sequencer (Perkin-Elmer, Norwalk, CT). The nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank databases under accession no. AB636681.

Computational tools. The nucleotide sequence and the deduced amino acid sequence were analyzed using the DNASIS program (Hitachi Software Engineering, Yokohama, Japan). Multiple sequence alignment was done using ClustalW (http://www.ddbj.nig.ac.jp/). The domain was located with the Pfam domain data base (http://pfam.sanger.ac.uk/), and hydrophobicity was judged with the Sosui WWW server (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html).

Cloning and expression of dad*.* The genomic DNA of *Burkholderia* sp. AZ11 was digested with *Kpn*I. Agarose gel electrophoresis followed by Southern hybridization with a DIG-OL26 gave a single hybridization signal on a 8.0-kb DNA fragment. The fragment was extracted from agarose gel and ligated into Charomid 9–36 (Nippon Gene, Tokyo), which was previously digested with *Kpn*I. After *in vitro* packaging with a Lambda inn (Nippon Gene) and infection of *E. coli* XL1-Blue MRA, colony hybridization produced positive colonies. The plasmid, designated pCKK8.0, was isolated from one of these, and digested with *Pst*I. A 5.8-kb DNA fragment with a hybridization signal was isolated and introduced into pUC18 (Takara) to obtain plasmid pUPP5.8 (Fig. 2). After digestion with *Kpn*I and *Bam*HI, deletion mutants of pUPP5.8 were prepared to determine the nucleotide sequence of the *Pst*I 5.8-kb region. One of the deletion mutants, designated pUDP1.3, contained an entire *dad* without any other open reading frames (ORFs) in the 1.3-kb DNA insert, and was used to express *dad* in *E. coli* JM109. Site-directed mutagenesis to convert initiation codon GTG to ATG was performed with a PrimSTAR mutagenesis kit (Takara) and PCR primers, forward primer (5′-ACAAACCATGGTCGACAAAGCCGTAT-3′) and reverse primer (5′-TCGACCATGGTTTGTCTCCTTGCATG-3′) (the nucleotides to be mutagenized are underlined). Mutagenesis was confirmed by DNA sequencing with appropriate sequencing primers.

Results

Isolation and identification of a DHAP degrading microorganism

Strain AZ11 was isolated from a soil sample by enrichment culture with DHAP, and was identified as *Burkholderia*. DAD was induced by cultivating strain AZ11 with DHAP as carbon and energy source, but bacterial growth was poor. The crude extract showed high activity (0.042 ± 0.009 μmol/min/mg) for DAD. The addition of 4-hydroxybenzoate and that of glucose to the culture medium improved bacterial growth, but not the production of DAD. Strain AZ11 did not grow on bisphenol A or its intermediary metabolite 4-hydroxyacetophenone. Crude extract prepared from DHAP-induced cells was incubated at 25 °C with a small amount (0.35 μmol) of DHAP in 4 mL of air-saturated 50 mM PPB (pH 7.0), until O_2 consumption, monitored with an O_2 electrode, stopped. HPLC analysis of the reaction mixture indicated that 0.35 μmol of DHAP and 0.34 ± 0.02 μmol of O_2 were consumed, and that 0.33 ± 0.01 μmol of 4-hydroxybenzoate and 0.35 ± 0.04 μmol of formate were formed. Formation of 4-hydroxybenzoate was also confirmed by silica gel TLC. These results conformed to the stoichiometric relationship of the DAD reaction:

$$(p)\text{HO-C}_6\text{H}_4\text{-COCH}_2\text{OH} + \text{O}_2 \rightarrow (p)\text{HO-C}_6\text{H}_4\text{-COO}^- + \text{HCOO}^- + 2\text{H}^+$$

Purification of DAD from Burkholderia *sp. AZ11*

The results of enzyme purification from strain AZ11 grown on DHAP are summarized in Table 1A. They showed about 39-fold purification with a yield of 10%. The purified enzyme preparation showed a single protein band at about 23 kDa on SDS–PAGE (Fig. 1). The M_r of the enzyme was determined to be about 90 kDa by gel filtration on Superdex 200 (data not shown). The N-terminal amino acid sequence of the enzyme was determined to be VDKAVSEFWQNIPAIANPFK by Edman degradation. This is 55% identical to that of DAD from *Alcaligenes* sp. 4HAP,[15] and coincides to the one deduced from the nucleotide sequence of *B. cenocepacia* J2315 (Fig. 3). The enzyme showed high affinity toward DHAP: the apparent K_m was estimated to be about 1.2 μM. Further investigation of the enzyme was hampered by low yield, and preparation of the recombinant enzyme was required.

Cloning and expression of dad *in* E. coli

A *Pst*I 5.8-kb DNA fragment containing *dad* was cloned from the genomic DNA into pUC18 to give plasmid pUPP5.8 (Fig. 2). Deletion mutants of pUPP5.8 were prepared to determine the nucleotide sequence of the *Pst*I 5,755-bp long-insert DNA. There were four ORFs with appropriate ribosome-binding sequences in this region. ORF1 and ORF3 encoded a 291-amino acid protein (calculated M_r, 32,680 Da) and a 326-amino acid protein (calculated M_r, 35,384 Da) respectively. These proteins had the helix-turn-helix motif for binding to DNA, and appeared to be transcriptional regulators. ORF2 encoded a 521-amino acid protein (calculated M_r, 55,772 Da) having a FAD-dependent

Table 1. Purification of DAD from *Burkholderia* sp. AZ11 (A) and *E. coli* JM109 Carrying pUDP1.3m (B)

Step	Protein (mg)	Sp. activity (U/mg)	Activity (U)	Purification (-fold)	Yield (%)
(A)					
Crude extract	97.2	0.0375	3.65	1	100
Superdex 200	34.3	0.0871	2.99	2.3	82
Q-Sepharose	2.92	0.423	1.24	11	34
Phenyl Sepharose	0.25	1.48	0.37	39	10
(B)					
Crude extract	371	0.322	119	1	100
Heat	170	0.545	92.7	1.7	78
$(NH_4)_2SO_4$	38.6	1.58	61.0	4.9	51
Superdex 200	13.1	3.25	42.6	10	36
Q-Sepharose	6.0	5.49	32.9	17	28

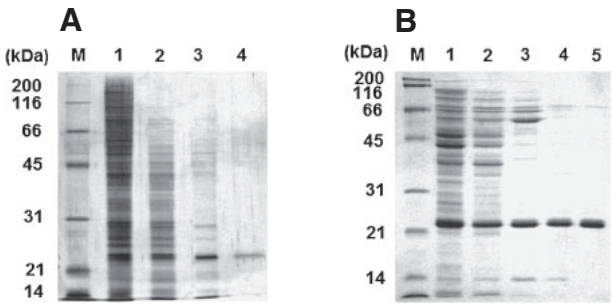

Fig. 1. SDS–PAGE of the Purification of DAD from *Burkholderia* sp. AZ11 (A) and *E. coli* JM109 Carrying pUDP1.3m (B).

A, lane M, marker proteins; lane 1, crude extract (2 μg); lane 2, Superdex 200 (1 μg); lane 3, Q Sepharose (0.2 μg); lane 4, Phenyl Sepharose (0.07 μg). Proteins were visualized by silver staining. B, lane M, marker proteins; lane 1, crude extract (50 μg); lane 2, heat treatment (25 μg); lane 3, ammonium sulfate (15 μg); lane 4, Superdex 200 (3 μg); lane 4, Q-Sepharose (3 μg). Proteins were stained with Coomassie Brilliant Blue R-250.

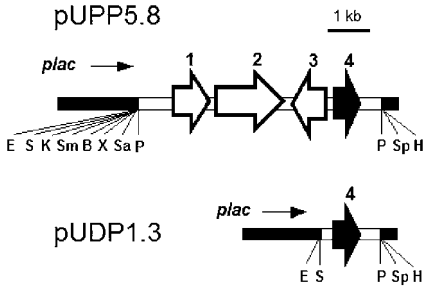

Fig. 2. Construction of Recombinant Plasmids.

The solid line shows vector pUC18, and the hollow line, the insert DNA of pUPP5.8 (above) and its deletion mutant pUDP1.3 (below). ORFs 1-3 are shown by white arrows, and ORF4 (*dad*) by black arrows. The direction of the *lac* promoter of pUC18 is shown by arrows (*plac*). *Abbreviations*: E, *Eco*RI; S, *Sac*I; K, *Kpn*I; Sm, *Sma*I; B, *Bam*HI; X, *Xba*I; Sa, *Sal*I; P, *Pst*I; Sp, *Sph*I; H, *Hin*dIII.

Fig. 3. Multiple Sequence Alignment of DAD.

Sequences: Bsp, *Burkholderia* sp. AZ11(GenBank AB636681); Bce, *B. cenocepacia* J2315 (RefSeq YP_002232670); Asp, *Alcaligenes* sp. 4HAP (GenBank AJ133820);[15] Pfl, *Pseudomonas fluorescens* Pf-5 (RefSeq YP_260468); Nar, *Novoshingobium aromaticivorans* DSM 12444 (RefSeq YP_497143); Rsp, *Roseobacter* sp. SK209-2-6 (RefSeq ZP_01753062); Hse, *Herbaspirillum seropsedicae* SmR1 (RefSeq YP_003774012); Bma, *B. mallei* ATCC 23344 (RefSeq YP_105997); Bgl, *B. glumae* BGR1 (RefSeq YP_002908823); and Vsp, *Vibrio* sp. Ex25 (RefSeq YP_003287777). Numbers to the right correspond to residue numbers. Cupin motifs are underlined. Positions identical in all sequences are marked by arrows. Amino acid residues marked by both arrows may be important for metal binding. Conserved residues and similar residues are indicated by double daggers and daggers respectively.

oxidoreductase domain. ORF4 (*dad*) was 534-bp long and encoded a 177-amino acid protein (calculated M_r 20,221 Da).

One of the deletion mutants, designated pUDP1.3, had an entire *dad* as sole ORF in a 1,368-bp long insert DNA (Fig. 2). An appropriate ribosome binding sequence, GAGA, and initiation codon GTG specify *dad*. The nucleotide sequence of hybridization probe OL26 coincided perfectly with the real sequence (nucleotide nos. 383–408). A possible promoter sequence (TTGAAT for the −35 sequence and TCCTGT for the −10 sequence) was found in the region (nucleotide nos. 112–139) upstream of *dad*. The first 20 amino acid sequence deduced from the nucleotide sequence coincided with that obtained by Edman degradation of the purified enzyme, except that the N-terminal residue was deleted. The amino acid sequence of DAD contained a typical β-barrel fold of the cupin superfamily at positions 74–131 (Fig. 3).[30] Multiple sequence alignment of DAD from 10 bacterial strains indicated that DAD from *Burkholderia* sp. AZ11 was highly homologous to that from *B. cenocepacia* J2315 (98.9% identity), and less homologous (84% identity) to that from *Alcaligenes* sp. 4HAP. The degree of homology was higher in the middle region (residue nos. 74-116) containing the cupin fold than in the N- and C-terminal regions. Cupin is a binding site of metal ions in a variety of proteins, in which three His residues and a Glu residue are possible metal ligands.[30,31] In DAD, they appeared to be His77, His79, His115, and Glu96 or Glu109 (residue number based on the amino acid sequence of DAD from *Burkholderia* sp. AZ11), because of their complete conservation in DAD from all bacterial strains examined. *Burkholderia* sp. AZ11 and *B. cenocepacia* J2315 have GTG as the initiation codon, whereas other bacterial strains have ATG.

Although the enzyme activity (0.086 ± 0.004 μmol/min/mg) of the crude extract from *E. coli* JM109 harboring pUDP1.3 was about 2 times higher than that from *Burkholderia* sp. AZ11 grown on DHAP, the extent of gene expression of *dad* was not sufficient. The initiation codon of *dad* of *Burkholderia* sp. AZ11 is GTG, which occurs less frequently than ATG in *E. coli*.[32] Hence the initiation codon was converted from GTG to ATG by site-directed mutagenesis. The resulting plasmid, designated pUDP1.3m, brought about high-level expression of DAD in recombinant *E. coli*. The specific activity (0.330 ± 0.008 μmol/min/mg) of

the crude extract was about 8 times higher than that of *Burkholderia* sp. AZ11.

Purification of recombinant DAD

The recombinant enzyme was briefly purified from the crude extract of *E. coli* JM109 harboring pUDP1.3m. Table 1B summarizes the purification procedures. It shows 17-fold purification with 28% recovery of the enzyme. The specific activity (5.49 μmol/min/mg) of the purified enzyme was about 2 times higher than that (2.85 μmol/min/mg) of the recombinant enzyme as prepared by Hopper and Kaderbhai.[15] The activity was also higher than that of the enzyme purified from *Burkholderia* sp. AZ11 (Table 1A), suggesting that the latter enzyme preparation was partially inactivated during the purification procedure. The purified enzyme was stable for at least 1 month when stored at −25 °C. Repeated freezing and thawing caused a loss of activity.

On SDS–PAGE, the enzyme showed a single protein band corresponding to M_r of 23 ± 1 kDa (Fig. 1B). Its M_r was estimated to be 90 ± 3 kDa by gel filtration on Superdex 200 (data not shown), suggesting that it is a tetramer. The N-terminal amino acid residue of the purified enzyme was identified as Val with dansyl chloride, indicating that the N-terminal Met residue was removed. Thus the recombinant enzyme was virtually identical to the native enzyme in amino acid sequence. The non-heme iron content of the purified enzyme was estimated to be 1.69 ± 0.04 mol/mol of enzyme by atomic absorption spectrometry, and 1.63 ± 0.04 mol/mol of enzyme by Fish's colorimetric method, while the copper content was less than 0.01 mol/mol of enzyme. The enzyme had no significant amount of inorganic sulfide (less than 0.03 mol/mol of enzyme).

Absorption spectrum of DAD

The absorption spectrum of the enzyme was measured with diluted enzyme solution, since the dark-gray enzyme protein precipitated from a concentrated enzyme solution. The enzyme showed a simple UV spectrum with maximum at 280 nm and a shoulder at 290 nm (Fig. 4). The A_{280}/A_{260} ratio was 1.68. It showed a broad visible spectrum, with two maxima at 350 nm and 560 nm, possibly due to Fe^{3+} bound to the enzyme protein. The molar extinction coefficients at 280, 350, and 560 nm were calculated to be 115, 5.8, and 1.4 $mM^{-1}\,cm^{-1}$ respectively, based on a M_r of 90 kDa. No significant absorption indicating the presence of heme and flavins was detected. The spectral feature in the visible region was similar to that of Fe^{3+}-containing catechol 1,2-dioxygenase (EC 1.13.11.1), which has a red color and shows a shoulder at about 320 nm and a broad peak at around 440 nm.[33,34] The repeated freezing and thawing inactivated the enzyme with a decrease in absorption at longer than 350 nm, probably owing to a loss of iron.

To determine the spectral change on binding with DHAP, the spectra of the enzyme and DHAP were measured separately, and were subtracted from the spectrum obtained by incubation of the enzyme with DHAP under N_2 (Fig. 5A). The absorption at around 400 nm increased, possibly due to binding of DHAP to Fe^{3+} in the enzyme. A similar experiment was carried out in air, and the concentrations of DHAP and 4-

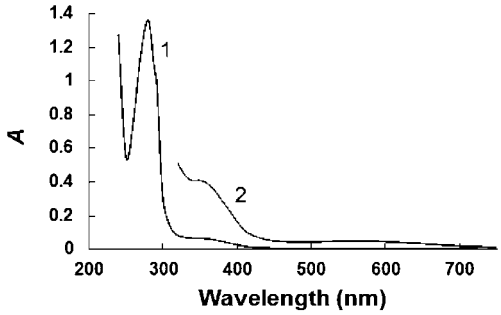

Fig. 4. Absorption Spectrum of Purified DAD.
The concentrations of the enzyme were 1.07 mg/mL (curve 1) and 6.40 mg/mL (curve 2) in 50 mM PPB (pH 7.0).

hydroxybenzoate during the reaction were monitored (Fig. 5B and C). On mixing the enzyme with DHAP, ΔA_{400} increased rapidly, remained at the increased level, and then decreased with exhaustion of DHAP. Most of the dissolved O_2 in the reaction mixture was rapidly consumed immediately after the start of the reaction, concomitantly with the consumption of DHAP and the formation of 4-hydroxybenzoate. Thus the dissolution rate of O_2 into the mixture controlled the enzyme reaction, and the reaction progressed slowly under the limited O_2 concentration until DHAP was exhausted. These results suggest that the enzyme-DHAP complex reacts with O_2.

Substrate specificity

O_2 consumption activity toward various compounds (each 0.35 mM) was measured at 25 °C in air-saturated 50 mM PPB (pH 7.0). Among the various acetophenone derivatives examined, only 2-hydroxyacetophenone (2-hydroxy-1-phenylethanone) showed weak enzyme activity (4.4 ± 0.4% as compared with DHAP). The reaction product was extracted and identified as benzoate by HPLC and silica gel TLC. No significant O_2 consumption was observed for the following compounds: 2-(4-hydroxyphenyl)ethanol, 4-hydroxyphenylacetone, 2-phenylethanol, 4′-hydroxypropiophenone, acetophenone and its derivatives including 4′-hydroxy-, 3′-hydroxy-, 2′-hydroxy-, 4′-methoxy-, 4′-amino-, 4′-chloro-, and 4′-fluoroacetophenone.

Kinetic constants

Enzyme activity was measured spectrophotometrically at various DHAP concentrations (1.25 μM–14.3 mM) with an air-saturated mixture (0.25 mM O_2). The enzyme showed a hyperbolic saturation curve with weak substrate inhibition at concentrations higher than 0.03 mM (data not shown). The apparent K_m for DHAP and the apparent V_{max} were estimated by double reciprocal plot to be 1.60 ± 0.03 μM and 6.28 ± 0.06 μmol/min/mg respectively.

Effects of pH, temperature, and inhibitors

The effect of pH on enzyme activity was examined by measuring O_2 consumption in various buffers. Potassium phosphate and 2-morpholinoethanesulfonic acid (MES) buffers were effective, and the enzyme showed a maximum activity at pH 6–7 (data not shown). It was moderately thermostable, and retained about 80% of its activity after incubation at 70 °C for 20 min (data

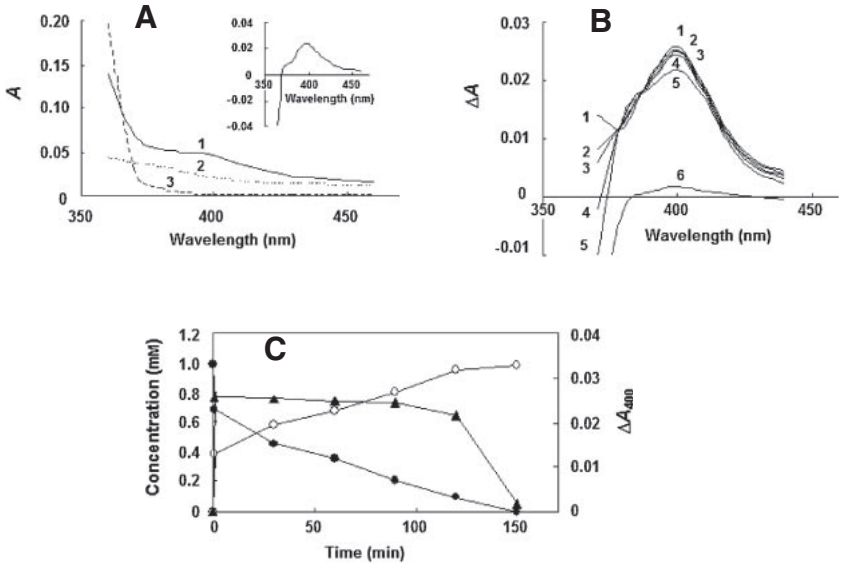

Fig. 5. Spectral Changes in DAD in the Enzyme Reaction.

All spectra were recorded at 600 nm/min. A, Spectral change in DAD under anaerobic conditions. The spectrum under N_2 was measured with a Thunberg-type cuvette. The enzyme (2.35 mg) was incubated at 25 °C with 1 mM DHAP in 2.8 mL of 50 mM PPB (pH 7.0). The absorption spectra of the enzyme (curve 2), DHAP (curve 3), and the enzyme plus DHAP (curve 1) were measured separately against the buffer as reference. The inset shows a difference spectrum, which was calculated by subtracting curves 2 and 3 from curve 1. B, Spectral change in DAD under aerobic conditions. The enzyme was incubated with DHAP under the same conditions as in A, except that an air-saturated reaction mixture was used and mixed moderately. The difference spectra were calculated as above. Curves 1–6 were measured at 0.5, 30, 60, 90, 120, and 150 min respectively after the start of the reaction. C, Time course of the enzyme reaction. During the reaction in B, samples (30 µL) were withdrawn at the indicated times, and mixed with 0.27 mL of 10 mM HCl to stop the reaction. The mixture was neutralized with NaOH, deproteinized by centrifugation, and analyzed by HPLC to determine the concentrations of DHAP (●) and 4-hydroxybenzoate (○). The values of ΔA_{400} (▲) obtained in B are also shown.

Table 2. Effects of Various Reagents on Enzyme Activity

Reagent (1 mM)	Activity (%)[a]	
	A[b]	B[c]
$CuCl_2$	9	5
$HgCl_2$	13	15
$CoCl_2$	54	73
$NiCl_2$	79	58
$ZnCl_2$	77	60
$MgCl_2$	97	98
$CaCl_2$	98	102
$FeSO_4$	120	92
$FeCl_3$	104	92
EDTA	95	103
2,2′-Dipyridine	87	10
1,10-Phenanthroline	94	53
Tiron	102	61
8-Hydroxyquinoline	98	10
Diethylpyrocarbonate	42	25
p-Chloromercuribenzoate	92	58
2-Mercaptoethanol	113	93

[a]The activity (5.1 µmol/min/mg in A, and 3.3 µmol/min/mg in B) obtained without reagent was taken to be 100%. Values are means of duplicate analysis.
[b]Lubrol-untreated enzyme.
[c]Lubrol-treated enzyme.

not shown). Although the O_2 concentration in the reaction mixture decreased with the rise in temperature, the enzyme activity increased as the temperature increased up to 65 °C (data not shown). $CuCl_2$ and $HgCl_2$ strongly inhibited the enzyme (Table 2). $CoCl_2$, $NiCl_2$, and $ZnCl_2$ weakly inhibited it, while $FeSO_4$ weakly activated it. Although the enzyme prepared from the recombinant *E. coli* was hardly affected by various metal chelators, including EDTA, 2,2′-dipyridine, 1,10-phenanthroline, tiron (disodium 4,5-dihydroxybenzene-1,3-disulfonate), and 8-hydroxyquinoline, the enzyme previously treated with 0.4% Lubrol PX was inhibited by 2,2′-dipyridine and 8-hydroxyquinoline. It is probable that the integrity of the protein structure was partially destroyed by the detergent so as to allow the chelators to react with iron in the enzyme. Diethylpyrocarbonate and *p*-chloromercuribenzoate weakly inhibited the enzyme, while 2-mercaptoethanol activated it slightly. KCN, NaN_3, ascorbate (each 1 mM), and ethanol (10%, v/v) were almost entirely ineffective (data not shown).

Discussion

Alcaligenes sp. 4HAP degrades 4-hydroxyacetophenone *via* DHAP, and the DAD of strain 4HAP is involved in 4-hydroxyacetophenone degradation.[13] On the other hand, the DHAP-degrading bacterium *Burkholderia* sp. AZ11 did not grow on 4-hydroxyacetophenone. Moreover, no functional relationship to 4-hydroxyacetophenone metabolism was found in the genes upstream and downstream of *dad* of strain AZ11 and *B. cenocepacea* J2315, which appear to be closely related. Hence the DAD of strain AZ11 appears to participate in a metabolic pathway other than the 4-hydroxyacetophenone degradation pathway. Some plants, such as *Carthamus* and *Rhodiola*, contain aromatic glycosides with DHAP as an aglycon.[35,36] It is likely that DAD in most soil bacteria is engaged in the degradation of aromatic glycosides derived from plants.

Dad catalyzes oxygenative cleavage of the hydroxyacetyl group of DHAP. Both carbonyl and hydroxy parts in the hydroxyacetyl group are essential for

enzyme activity, since neither 2-phenylethanol nor 4′-hydroxyacetophenone served as substrate. The 4′-hydroxy group of DHAP is also important, but not indispensable for enzyme activity. Since the hydroxyacetyl group can tautomerize to the *cis*-1,2-ethenediol group, the DAD reaction appears to be analogous to the intradiol cleavage of a catechol ring catalyzed by intradiol dioxygenases. Intradiol dioxygenases have a characteristic color due to Fe^{3+} at the active site, while Fe^{2+}-containing extradiol dioxygenases such as catechol 2,3-dioxygenase (EC 1.13.11.2) are colorless.[37] The recombinant DAD purified from *E. coli* has a dark gray color, and shows a similar absorption spectrum to that of catechol 1,2-dioxygenase.[33,34] Therefore, although the electronic state of the iron must be determined more definitely by ESR at 4K, DAD appears to be a Fe^{3+}-containing metalloprotein.

The analogy with catechol 1,2-dioxygenase suggests a possible catalytic mechanism of DAD. The formation of a coordination complex between the enzyme-Fe^{3+} and the *cis*-1,2-ethenediol form of DHAP can be inferred from the difference spectrum of the enzyme-DHAP complex obtained under N_2. Enzyme-bound DHAP might donate 1*e* to Fe^{3+} to form a radical at C1, and O_2 might attack that radical to give a peroxy-radical according to a mechanism similar to that of catechol 1,2-dioxygenase.[38] Subsequently, migration of the 4-hydroxybenzoyl group to form acid anhydride followed by hydrolytic cleavage yields 4-hydroxybenzoate and formate.

The purified DAD had about 1.63–1.69 mol of non-heme iron. The iron might be bound at the inner part of the enzyme molecule, and does not react with metal chelators unless the protein structure is partially perturbed by a detergent such as Lubrol PX (Table 2). Repeated freezing and thawing resulted in a reduction of the specific activity of the enzyme concomitant with a decrease in absorption in the visible region, possibly due to loss of the bound iron. It is plausible to consider that the enzyme loses some Fe^{3+} during enzyme purification and storage. Consistently with this, the purified enzyme was slightly activated by $FeSO_4$ (Table 2). $FeCl_3$ was almost entirely ineffective, suggesting that Fe^{2+} binds to the enzyme protein and is subsequently oxidized to Fe^{3+} to yield an active enzyme. Based on these considerations, the enzyme protein composed of a homotetramer was assumed to have 2 mol of Fe^{3+}. Possibly, a Fe^{3+} ion is located and functions around the interface of two subunits, as found for catechol 1,2-dioxygenase, a dimer containing 1 mol of Fe^{3+} per mol of enzyme.[39] The DAD from *Alcaligenes* sp. 4HAP, whose specific activity is about half of the DAD purified from *E. coli* JM109 carrying pUDP1.3m, has been reported to be colorless and to consist of a homotetramer (M_r = 81.6–87 kDa) containing 1 mol of non-heme iron.[15]

DAD is moderately thermostable. This made easy purification of the recombinant DAD possible (Table 1B). This thermostability and high affinity to DHAP as well as narrow substrate specificity suggest that DAD is applicable in the enzymatic determination of DHAP. The thermostability of DAD might be due to the cupin fold in its structure. The cupin fold is a conserved β-barrel containing four metal-binding ligands.[30,31] Multiple sequence alignment of DAD from various bacterial strains revealed three His residues (His77, 79, and 115) and a Glu residue (Glu96 or 109) as ligands. Prediction of the protein secondary structure by the Chou-Fasman method has suggested that Glu109 is located in a turn structure, not in a β-strand, and that Glu96 is adequate as a ligand for the active-site Fe ion.[40]

In conclusion, we have established an easy procedure for the purification of DAD from *Burkholderia* sp. AZ11, and we found some of the molecular and catalytic properties of DAD. The purified preparation of DAD should be useful in future investigations of its three-dimensional structure and catalytic mechanism.

Acknowledgment

We would like to thank Ms. Keiko Takase of the Institute of Waste Water Treatment at Gifu University for atomic absorption analysis.

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