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Purification and Characterization of the Oxygenase Component of Biphenyl 2,3-Dioxygenase from *Pseudomonas* sp. Strain LB400

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The iron-sulfur protein of biphenyl 2,3-dioxygenase (ISP_{BPH}) was purified from *Pseudomonas* sp. strain LB400. The protein is composed of a 1:1 ratio of a large (α) subunit with an estimated molecular weight of 53,300 and a small (β) subunit with an estimated molecular weight of 27,300. The native molecular weight was 209,000, indicating that the protein adopts an $\alpha_3\beta_3$ native conformation. Measurements of iron and acid-labile sulfide gave 2 mol of each per mol of αβ heterodimer. The absorbance spectrum showed peaks at 325 and 450 nm with a broad shoulder at 550 nm. The spectrum was bleached upon reduction of the protein with NADPH in the presence of catalytic amounts of $ferredoxin_{BPH}$ and $ferredoxin_{BPH}$ oxidoreductase. The electron paramagnetic resonance spectrum of the reduced protein showed three signals at $g_x = 1.74$, $g_y = 1.92$, and $g_z = 2.01$. These properties are characteristic of proteins that contain a Rieske-type [2Fe-2S] center. Biphenyl was oxidized to *cis*-(2*R*,3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene by ISP_{BPH} in the presence of $ferredoxin_{BPH}$, $ferredoxin_{BPH}$ oxidoreductase, NADPH, and ferrous iron. Naphthalene was also oxidized to a *cis*-dihydrodiol, but only 3% was converted to product under the same conditions that gave 92% oxidation of biphenyl. Benzene, toluene, 2,5-dichlorotoluene, carbazole, and dibenzothiophene were not oxidized. ISP_{BPH} is proposed to be the terminal oxygenase component of biphenyl 2,3-dioxygenase where substrate binding and oxidation occur via addition of molecular oxygen and two reducing equivalents.

Bacteria that utilize biphenyl for growth under aerobic conditions oxidize the substrate to *cis*-2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (*cis*-biphenyl 2,3-dihydrodiol) as the first step of the catabolic pathway (21) (Fig. 1). The 2*R*,3*S* configuration of the hydroxyl groups, first reported for the dihydrodiol produced by cells of *Beijerinckia* sp. strain B8/36 (48), has recently been confirmed for whole cells of *Pseudomonas* sp. strain LB400 (38). The reaction in strain LB400 is catalyzed by biphenyl 2,3-dioxygenase, a multicomponent enzyme system that adds both atoms of molecular oxygen at the 2,3-position of one of the aromatic rings (27). Preliminary studies suggest that component A is an NAD(P)H:ferredoxin oxidoreductase ($reductase_{BPH}$) and component B is an electron transport protein ($ferredoxin_{BPH}$). Both proteins are required to reduce the artificial electron acceptor cytochrome *c* with NAD(P)H as a source of electrons, suggesting that they form a short electron transport chain that supplies electrons to component C (the iron-sulfur protein of biphenyl 2,3-dioxygenase [ISP_{BPH}]), the putative oxygenase, which contains the active site.

Most biphenyl-oxidizing bacteria that have been isolated from areas contaminated with polychlorinated biphenyls will also oxidize some chlorinated biphenyl congeners (CBs) containing a few chlorine substituents (7). Oxidation usually occurs at the 2,3-position of the least chlorinated ring (5, 18). Biphenyl-grown cells and cell extracts of strain LB400 oxidize a broad range of CBs, including some with six chlorine substituents (7, 10, 15, 20). However, strain LB400 has been shown to incorporate oxygen at the 3,4-position of 2,5,2′,5′-tetrachlorobiphenyl (35), indicating that the chlorine substituents might block oxidative attack at the 2,3-position (7). This observation indicated either that one dioxygenase in strain LB400 incorporates oxygen at the 2,3- and the 3,4-positions or that a second dioxygenase is present that oxidizes the 3,4-position of congeners blocked by chlorine substituents at the 2,3-position (6). Recently, we reported that the biphenyl 2,3-dioxygenase from strain LB400 catalyzes both reactions (26). Here, we describe the purification and characterization of ISP_{BPH}, the catalytic oxygenase component of biphenyl 2,3-dioxygenase, from strain LB400.

(A preliminary account of this study has been presented [25].)

MATERIALS AND METHODS

Organism and growth conditions. Strain LB400 was isolated from a polychlorinated biphenyl-contaminated site (10) and was provided by Herman L. Finkbeiner, Research and Development Center, General Electric Company, Schenectady, N.Y. The organism was cultured in a basal salts medium with biphenyl as the carbon source, as previously described (27).

Preparation of cell extract. Cells (25 g [wet weight]) were suspended in 50 ml of buffer composed of 50 mM bis(2-hydroxyethyl)imino-Tris (pH 7.0) containing 5% (vol/vol) each ethanol and glycerol (BEG buffer). The suspension was amended with the following (final concentration): dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (0.2 mM), and DNase I and RNase A (0.01 mg/ml each [Sigma Chemical Co., St. Louis, Mo.]). The cells were broken by passage through a French pressure cell and centrifuged at 145,000 × *g* at 4°C for 1 h to remove cell debris and membranes.

Protein purification. Cell extract (1.97 g of protein) was applied to a column (5 cm by 16 cm) packed with Q-Sepharose Fast Flow (Pharmacia LKB Biotechnology, Piscataway, N.J.) and equilibrated with BEG buffer. Protein was eluted with a linear salt gradient to 400 mM KCl (total gradient volume, 1.72 liters). Fractions containing ISP_{BPH} were pooled and concentrated by ultrafiltration with a 100-kDa-cutoff membrane filter (Amicon, Danvers, Mass.). The concentrated protein solution was adjusted to 1.7 M ammonium sulfate and centrifuged at 15,800 × *g*. The supernatant was applied to a phenyl-Sepharose column (2.6 by 10 cm; Pharmacia) equilibrated with 1.7 M ammonium sulfate in BEG buffer. Bound protein was eluted with a decreasing salt gradient from 1.7 to 0 M ammonium sulfate (gradient volume, 1 liter). Fractions containing ISP_{BPH} were concentrated and exchanged into 10 mM potassium phosphate buffer (pH 7.0) via ultrafiltration as described above. The concentrated protein solution was applied to a hydroxyapatite column (2.6 by 34 cm; Bio-Rad Laboratories,

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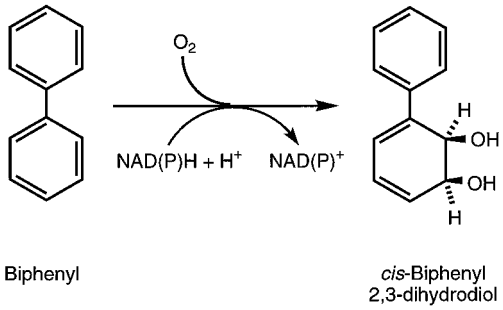

FIG. 1. Reaction catalyzed by biphenyl 2,3-dioxygenase.

Hercules, Calif.) equilibrated with 10 mM potassium phosphate buffer. The column was washed with 0.16 liters of 100 mM phosphate buffer. ISP_{BPH} was then eluted with 250 mM phosphate. Fractions containing ISP_{BPH} were concentrated and equilibrated with BEG buffer by ultrafiltration, as described above, and stored at −70°C.

Fractions from the Q-Sepharose column step that contained $ferredoxin_{BPH}$ were concentrated and equilibrated with BEG buffer on a 10-kDa-cutoff membrane filter and used in assays for biphenyl 2,3-dioxygenase activity during purification of ISP_{BPH}. $Ferredoxin_{BPH}$ was further purified on an S300 gel filtration column (2.6 by 67 cm; Pharmacia) prior to use in determinations of the substrate specificity of biphenyl 2,3-dioxygenase.

Fractions from the Q-Sepharose column step that contained $reductase_{BPH}$ were concentrated with a 30-kDa-cutoff membrane filter and used without further purification. Fractions containing $reductase_{BPH}$ and ISP_{BPH} previously showed some overlap at this step when the buffer pH was 6.0 (27); however, baseline separation was achieved with BEG buffer (pH 7.0).

Enzyme assays. Biphenyl 2,3-dioxygenase activity was determined by measuring the amount of *cis*-[^{14}C]biphenyl 2,3-dihydrodiol formed after incubation of ISP_{BPH}, partially purified $reductase_{BPH}$, and $ferredoxin_{BPH}$ with [^{14}C]biphenyl (Sigma), NADPH, and ferrous ammonium sulfate, as previously described (27). The assay was optimized for buffer type, concentration, and pH; incubation time; and the concentrations of biphenyl, NADPH, and ferrous ammonium sulfate. The activity of ISP_{BPH} in cell extract and following each purification step was determined by the same procedure. In this case, 5-μl portions of extract or each column fraction were incubated with partially purified $reductase_{BPH}$ (88 μg) and partially purified $ferredoxin_{BPH}$ (171 μg). One unit of activity equals the amount of protein that produces 1 μmol of product per min.

Electrophoresis. Protein purity and subunit molecular weights were determined by electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels (12% total monomer) under reducing conditions (SDS-PAGE) by the method of Laemmli (31). Migration distance and relative intensity of Coomassie blue R250-stained protein bands were measured with a model 1313 gel scanner/UA-5 absorbance detector (Isco, Inc., Lincoln, Nebr.). Protein standards used for estimation of subunit molecular masses were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45 kDa) for the large (α) subunit of ISP_{BPH} and ovalbumin, carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa) for the small (β) subunit.

Molecular mass and Stokes' radius. The native molecular mass and Stokes' radius of ISP_{BPH} were determined at two different salt concentrations, using a Superose 12 column (1.6 by 50 cm; Pharmacia) equilibrated with buffer containing 100 or 500 mM KCl. The column was calibrated with thyroglobulin (724 kDa, 85 Å [8.5 nm]), ferritin (440 kDa, 61 Å [6.1 nm]), catalase (186 kDa, 52.2 Å [5.22 nm]), aldolase (158 kDa, 4.81 Å [0.48 nm]), bovine serum albumin (67 kDa, 35.5 Å [3.55 nm]), ovalbumin (43 kDa, 30.5 Å [3.05 nm]), chymotrypsinogen A (25 kDa, 20.9 Å [2.09 nm]), and RNase A (13.7 kDa, 16.4 Å [1.64 nm]).

Protein concentrations. Protein concentrations were determined by the method of Bradford (11) with bovine serum albumin as the standard.

N-terminal amino acid sequencing. The α and β subunits of ISP_{BPH} were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. The N-terminal amino acid sequences were then determined by Edman degradation and analysis on an automated sequencer (Applied Biosystems, Foster City, Calif.).

UV-visible absorbance spectroscopy. The absorbance spectrum of ISP_{BPH} was recorded, as isolated and after reduction with NADPH, in the presence of catalytic quantities of $reductase_{BPH}$ and $ferredoxin_{BPH}$ under a N_2 atmosphere.

EPR spectroscopy. Electron paramagnetic resonance (EPR) spectra of ISP_{BPH} were obtained at 77 K with purified protein, as isolated and after reduction with sodium dithionite. Twenty-five spectra were recorded and averaged with a Bruker model ESP 300 spectrometer with the following settings: 5-mW microwave power, 3,650-G centerfield, 1,000-G scan range, 9.29-GHZ microwave modulation frequency, 42-s sweep time, and 6.3×10^5 receiver gain.

Iron and acid-labile sulfide. Iron and acid-labile sulfide were determined by the methods of Zabinski et al. (47) and Beinert (8), respectively.

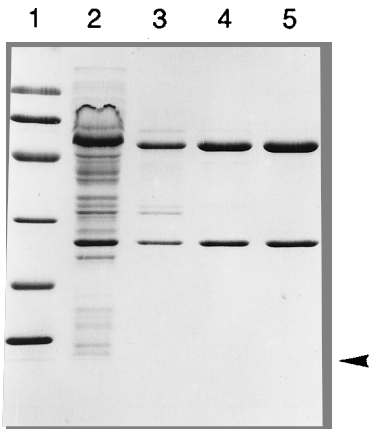

FIG. 2. SDS-PAGE analysis of samples taken during the purification of ISP_{BPH}. Lanes: 1, molecular weight markers; 2, cell extract (15 μg of protein); 3, Q-Sepharose column fractions containing ISP_{BPH} (3.0 μg of protein); 4, phenyl-Sepharose column fractions containing ISP_{BPH} (3.4 μg of protein); 5, hydroxyapatite column fractions containing ISP_{BPH} (3.0 μg of protein). The arrowhead denotes the dye front.

Substrate specificity. The substrate specificity of biphenyl 2,3-dioxygenase was examined by incubation of 645 μg of partially purified $reductase_{BPH}$, 25.5 μg of $ferredoxin_{BPH}$, and 520 μg of pure ISP_{BPH} in 1-ml reaction mixtures containing 0.1% Triton X-100, 0.4 mM ferrous ammonium sulfate, 0.5 mM substrate, 5 mM NADPH, and 50 mM 2-(*N*-morpholino)ethanesulfonate buffer (pH 6.0). The reactions were carried out in 7.4-ml glass vials closed with foil-lined caps and incubated at 30°C with shaking at 250 rpm. Control reaction mixtures contained ISP_{BPH} alone or $reductase_{BPH}$ plus $ferredoxin_{BPH}$ but otherwise were identical to the experimental reaction mixtures described above. After 1 h, the reaction mixtures were saturated with NaCl and extracted twice with 2 ml of ethyl acetate. The combined extracts were dried with anhydrous sodium sulfate, and the solvent was removed under a stream of N_2. Biphenyl, benzene, toluene, 2,5-dichlorotoluene, naphthalene, carbazole, and dibenzothiophene were tested as substrates.

Gas chromatography-mass spectrometry. The extracted residue from half of each substrate transformation reaction was derivatized with 100 μg of *n*-butylboronic acid and analyzed by gas chromatography-mass spectrometry as previously described (26).

HPLC. The extracted residue from the second half of each substrate transformation reaction was dissolved in 100 μl of methanol, and 10 μl was analyzed by reverse-phase high-pressure liquid chromatography (HPLC) as previously described (26). Products eluting from the column were detected at 254 and 210 nm with a Waters model 990-MS photodiode array detector which recorded the 200- to 400-nm spectrum of each compound. Authentic *cis*-biphenyl 2,3-dihydrodiol and *cis*-naphthalene 1,2-dihydrodiol were also analyzed for comparison of retention times and spectra with those of the unknowns. The absolute stereochemistry of *cis*-biphenyl 2,3-dihydrodiol produced by ISP_{BPH} was established by comparison of the retention time with those of authentic (+) and (−) enantiomers on a Chiralcel OB column (4.6 mm by 25 cm; Daicel Chemical Industries, Ltd., Los Angeles, Calif.) with isopropanol-hexane (10:90) as the mobile phase at a flow rate of 0.75 ml/min.

RESULTS

Isolation and characterization of ISP_{BPH}. ISP_{BPH} was purified to apparent homogeneity from the soluble fraction of crude cell extract of strain LB400 that had been grown with biphenyl as the carbon and energy source (Fig. 2). The purified protein consists of a large (α) subunit and a small (β) subunit that are highly expressed in the soluble fraction of cell extract. The yield of pure protein and dioxygenase activity for the final purification step were 118 mg and 18%, respectively (Table 1). The latter value is based on activity of cell extract that was assayed by addition of $reductase_{BPH}$ and $ferredoxin_{BPH}$ to the reaction mixture (see Materials and Methods). The specific activity of cell extract without addition of the electron transport components was sixfold lower, an indication that their concentrations in unamended cell extract are limiting. The

TABLE 1. Purification of ISP_{BPH} from strain LB400

Step	Amt of protein (mg)	Activity[a]		Yield (%)	Purification (fold)
		mU	mU/mg		
Cell extract	1,970	60,700	30.8	100	1
Q-Sepharose	480	19,000	39.6	31	1.3
Phenyl-Sepharose	160	5,440	34.0	9	1.1
Hydroxyapatite	118	10,800	91.5	18	3.0

[a] 1 U = 1 μmol/min.

specific activity of the cell extract is also dependent on the total protein concentration in the reaction mixture (27). As the measurements of activity are dependent on protein concentration and the relative amounts of the individual protein components present, the values reported in Table 1 represent only the trends in the progress of the purification procedure. Hydrophobic interaction chromatography with the phenyl-Sepharose column enhanced purity as judged by SDS-PAGE (Fig. 2); however, this step may have denatured a portion of ISP_{BPH}, or residual ammonium sulfate in the preparation may have inhibited activity in the assays.

The estimated molecular weight of the α subunit was 53,000 ± 1,830 (mean ± 1 standard deviation, $n = 3$), which compares favorably with the value of 51,500 predicted from the deduced amino acid sequence of the *bphA* gene (14). The estimated molecular weight of the β subunit, however, was 27,300 ± 1,070, which is notably higher than that predicted from the *bphE* gene sequence (14). Similar results were obtained when the iron-sulfur proteins of toluene dioxygenase (ISP_{TOL}) from *Pseudomonas putida* F1 and naphthalene dioxygenase (ISP_{NAP}) from *Pseudomonas* sp. strain 9816-4 were electrophoresed alongside ISP_{BPH}. This result suggests that the electrophoretic migration rates of the β subunits of the three dioxygenases were retarded relative to the standard proteins used to produce the molecular weight calibration curve and that the values obtained may be overestimated by this method. A scan of the lane containing pure ISP_{BPH} gave a value of 1.9 for the ratio of the integrated peak areas that corresponded to the α and β protein bands. Therefore, the two subunits are most probably present in a 1:1 ratio. Gel filtration chromatography gave a molecular weight of 209,000 and a Stokes' radius of 47 Å (4.7 nm). The data indicate that the native conformation of ISP_{BPH} is $\alpha_3\beta_3$. The N-terminal amino acid sequence of the α subunit is SSAIKEVQGA-, and that of the β subunit is TNPSPH FFKTF-. These sequences are identical to those predicted from the deduced amino acid sequences of the *bphA* and *bphE* genes (14), with the exception that methionine was not detected as the first residue of either subunit.

The absorbance spectrum of oxidized ISP_{BPH} showed a broad peak at 450 nm, $\varepsilon = 10.1\ mM^{-1}\ cm^{-1}$ (based on $\alpha\beta$ = 73,600 Da [14]), with a shoulder around 550 nm and a larger peak at 325 nm (Fig. 3). The spectrum was partially bleached upon reduction with NADPH in the presence of catalytic amounts of $reductase_{BPH}$ and $ferredoxin_{BPH}$. The EPR spectrum showed no significant signals for the oxidized protein, but following reduction with sodium dithionite, three signals (g_x = 1.74, g_y = 1.92, and g_z = 2.01) with an average g value of 1.89 were found (Fig. 4). The content of iron and acid-labile sulfide was 2.1 and 2.3 mol, respectively, per mol of $\alpha\beta$ heterodimer. These data indicate the presence of a Rieske-type [2Fe-2S] redox center (16).

Omission of ferrous ammonium sulfate from assays for dioxygenase activity reduced the specific activity of purified ISP_{BPH} by 7.4-fold compared with only a slight reduction previously reported for cell extract (27). Ferrous iron, other than iron contained in the iron-sulfur center, may be a dissociable cofactor that is required for activity but lost during protein purification.

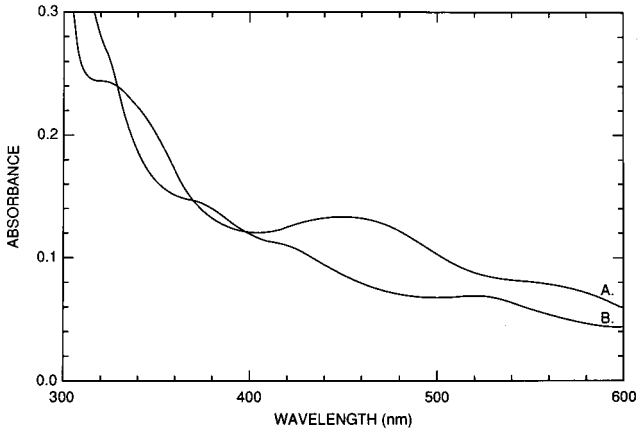

FIG. 3. Absorption spectra of ISP_{BPH} (2.08 mg of protein per ml in a 1-ml total volume). (A) Oxidized; (B) reduced with NADPH in the presence of catalytic amounts (50 μg each) of $reductase_{BPH}$ and $ferredoxin_{BPH}$.

Substrate specificity and oxidation products. ISP_{BPH} oxidized biphenyl to *cis*-biphenyl 2,3-dihydrodiol in the presence of $reductase_{BPH}$, $ferredoxin_{BPH}$, NADPH, and ferrous ammonium sulfate, as previously reported (26). One product peak was detected when the extracted residue from the biphenyl transformation was injected onto a chiral HPLC column capable of resolving the (+) and (−) enantiomers of *cis*-biphenyl 2,3-dihydrodiol. The retention time was identical to that of (+)-*cis*-biphenyl (2*R*,3*S*)-dihydrodiol produced by *Beijerinckia* sp. strain B8/36, showing that the configuration of the hydroxyl groups is 2*R*, 3*S* (48).

Naphthalene was a poor substrate, with only 3% of the substrate being oxidized after 1 h of incubation while 92% of biphenyl was oxidized under the same conditions. The *n*-bu-

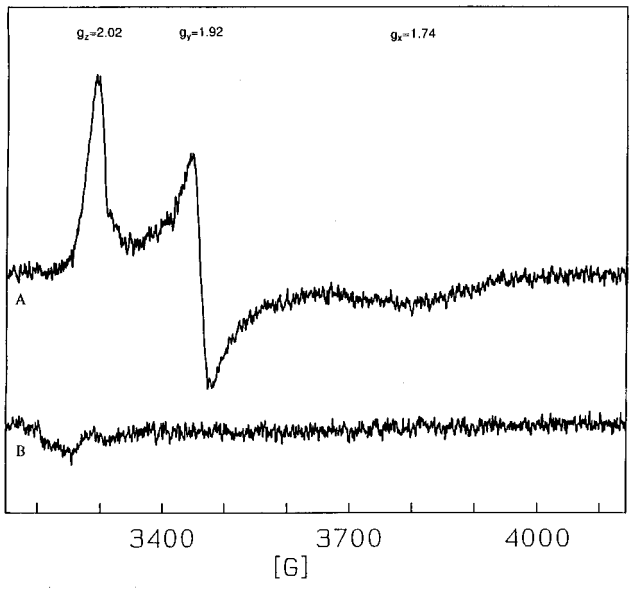

FIG. 4. EPR spectra of ISP_{BPH} (1.2 mg of protein; 7.8 mg/ml). (A) Reduced with sodium dithionite; (B) oxidized.

tylboronate derivative of the product derived from naphthalene gave a gas-chromatographic retention time (19.9 min) and mass spectrum (M^+, 228 *m/z*) that matched those of the *n*-butylboronate derivative of *cis*-naphthalene 1,2-dihydrodiol produced by *Pseudomonas* sp. strain 9816 (30). The HPLC retention time (5.6 min) and absorbance spectrum (λ_{max} = 264 nm) of the enzymatic product also matched those given by *cis*-naphthalene 1,2-dihydrodiol formed by strain 9816.

No products were detected when benzene, toluene, 2,5-dichlorotoluene, carbazole, or dibenzothiophene was the substrate.

DISCUSSION

Biphenyl 2,3-dioxygenase is a member of a growing number of known bacterial enzymes that oxidize aromatic compounds by addition of both atoms of molecular oxygen to the ring. These enzyme systems are multicomponent proteins that contain redox centers consisting of flavins and iron-sulfur clusters arranged as short electron transport chains that transfer reducing equivalents from NAD(P)H to the active site of the dioxygenase component. Products of these reactions are *cis*-dihydrodiols which have both hydroxyl groups oriented on the same side of the ring.

Two-component dioxygenase systems, composed of a reductase and an oxygenase, are most often associated with oxidation of aromatic acids such as benzoate (45), phthalate (4), 2-halobenzoate (17), 4-chlorophenylacetate (33), and 4-sulfobenzoate (32). The reductase components are flavoproteins containing flavin adenine dinucleotide or flavin mononucleotide and a plant-type [2Fe-2S] center, coordinated by four conserved cysteines (3), which transfer electrons from NADH to the oxygenase component. The oxygenase contains a Rieske-type [2Fe-2S] center coordinated by two cysteines and two histidines (23). The subunit composition of the oxygenase component is variable among the different two-component systems. Some oxygenases contain only an α protein of approximately 50,000 Da arranged as dimers (32), trimers (33), or tetramers (4). Other oxygenases contain a second protein subunit with unknown function of about 20,000 Da and adopt an $\alpha_3\beta_3$ configuration (17, 45).

Biphenyl 2,3-dioxygenase is a three-component enzyme system that is similar to dioxygenases that oxidize toluene (46) and benzene (2). *o*-Halobenzoate 1,2-dioxygenase from *P. aeruginosa* 142 is a similar three-component system that oxidizes aromatic acids rather than hydrocarbons (39). These enzyme systems consist of a flavoprotein reductase, a ferredoxin containing a Rieske-type [2Fe-2S], center and an ISP which also contains a Rieske-type [2Fe-2S] center. The requirement of $reductase_{BPH}$ and $ferredoxin_{BPH}$ for reduction of ISP_{BPH} and for dioxygenase activity is consistent with the assignment of their function as electron transport components (27). The electron transport chains of bacterial dioxygenases have been classified on the basis of the number, location, and type of iron-sulfur centers they contain (3). The electron transport chain of biphenyl 2,3-dioxygenase is likely to be classified with those of toluene and benzene dioxygenases (class IIB). Pyrazon dioxygenase (40) and dibenzofuran 4,4a-dioxygenase (12) have a class IIA electron transport chain which contains a ferredoxin component with a plant-type [2Fe-2S] center. $Reductase_{BPH}$ (14, 43), $reductase_{TOL}$ (50), and $reductase_{BNZ}$ (29) lack four conserved cysteine residues found in $reductase_{NAP}$ (41) that are thought to coordinate a plant-type [2Fe-2S] center present in the class III electron transport chain of naphthalene dioxygenase (37). This classification scheme is supported by comparison of the deduced amino acid sequences of the ISP components (36). Thus, the arrangement of the three components of biphenyl 2,3-dioxygenase (Fig. 5) is proposed to be the same as that of the toluene (22), benzene (2), and possibly *o*-halobenzoate (39) dioxygenases.

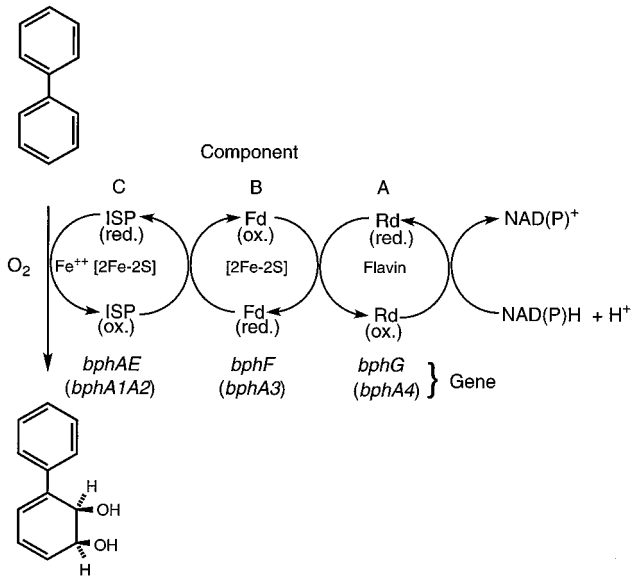

FIG. 5. Proposed arrangement of the protein components of biphenyl 2,3-dioxygenase. Abbreviations: Fd, $ferredoxin_{BPH}$; Rd, $reductase_{BPH}$; red., reduced; ox., oxidized. A, B, and C are designations given to the three components following resolution by anion-exchange column chromatography (27).

The absorbance and EPR spectra of ISP_{BPH} are characteristic of those of proteins containing a Rieske-type [2Fe-2S] iron-sulfur center (34). Partial bleaching of the absorbance spectrum and appearance of EPR signals upon reduction with NADPH and dithionite, respectively, indicate reduction of one of the antiferromagnetically coupled high-spin ferric atoms to the ferrous oxidation state (34). The spectral evidence for the presence of a Rieske-type iron-sulfur center is supported by the finding of conserved cysteine and histidine residues in the deduced amino acid sequence of the large subunit of ISP_{BPH} (1, 14, 43) that are thought to be ligands of the center (37). Analyses for iron and acid-labile sulfide of purified ISP_{BPH} confirmed the presence of 2 mol of each per mol of αβ heterodimer.

A mononuclear iron center, present in the ISP of bacterial dioxygenases, has been hypothesized to be the electron acceptor of the reduced iron-sulfur center during catalysis (9, 19), although it is labile and often lost during purification of various ISPs. Iron in excess of that contained in the iron-sulfur center was not detected for purified ISP_{BPH}, but addition of exogenous ferrous iron was required for maximal activity. Therefore, mononuclear iron may be a component of ISP_{BPH} that was lost during purification. Conserved histidine and tyrosine residues in the amino acid sequence of the large subunit of ISPs may coordinate the mononuclear iron of these proteins (1, 20, 37).

The N-terminal amino acid sequences of the α and β protein subunits of ISP_{BPH} were identical to those predicted from the nucleotide sequences of the *bphA* and *bphE* genes from strain LB400 (14) and the *bphA1* and *bphA2* genes from *P. pseudoalcaligenes* KF707 (43). The molecular weight determined by gel filtration chromatography of the protein from strain LB400 indicates that the native conformation may be $\alpha_3\beta_3$. Both αβ and $\alpha_2\beta_2$ conformations were detected by native PAGE analysis of ISP_{BPH} from strain KF707 expressed in *Escherichia coli* (28), while native PAGE analysis of ISP_{BPH} purified from

strain LB400 showed only one band with an estimated molecular weight that was consistent with the results obtained by gel filtration chromatography (24). The ISP components of toluene (42), naphthalene (13), and benzene (2) dioxygenases are composed of an α subunit and a β subunit of similar size to those of ISP_{BPH}. However, these proteins adopt an $\alpha_2\beta_2$ native conformation. The significance of the native conformation is not apparent for these enzymes, because the number and nature of the substrate binding sites are unknown.

Biphenyl 2,3-dioxygenase showed a narrow specificity for its substrate, because naphthalene and biphenyl were the only aromatic hydrocarbons, of those tested, that were oxidized by the enzyme. Two aromatic rings may be a requirement for binding of the substrate to the enzyme. The biphenyl dioxygenase of strain KF707 expressed in *E. coli* also showed no activity toward toluene or benzene (28), but Tan and Cheong (44) have reported indirect evidence for low levels of activity toward benzene.

The *cis*-biphenyl 2,3-dihydrodiol produced by ISP_{BPH} of strain LB400 was enantiomerically pure, indicating that the oxygenation reaction is highly specific with biphenyl as the substrate. However, the enzyme shows a remarkable tolerance for chlorine substituents on both of the biphenyl rings, and the regiospecificity for the position of oxygenation of certain CB substrates is less specific (26). This effect was shown by the formation of more than one *cis*-dihydrodiol product for CBs with chlorine substituents at the 2- or 2,5-ring positions. Electronic or steric effects of particular chlorine substitution patterns are probably important determinants of the suitability of a particular CB as a substrate and for activation of the position of oxygenation of the substrate. Toluene dioxygenase, on the other hand, oxidizes a broader range of aromatic substrates including biphenyl, benzene, and naphthalene (22, 51), yet the enzyme tolerates only two chlorine substituents on biphenyl and they must be confined to one ring (49). Structural features of dioxygenases that determine substrate specificity are poorly understood, but some evidence has been obtained from site-directed mutagenesis and construction of hybrid ISPs. Erickson and Mondello (15) showed that the amino acid sequence of a small region of the large subunit of ISP_{BPH} affects the ability of the enzyme to oxidize CBs such as 4,4′-dichlorobiphenyl, which has a double-*para*-substitution pattern. Hirose et al. (28) showed that the β subunit influenced the native conformation of ISP and that the α and β subunits influenced substrate specificity, while Tan and Cheong (44) have suggested a greater role for the α subunit.

The availability of large amounts of pure ISP_{BPH} from strain LB400 will promote mechanistic and structural studies of the enzyme that may elucidate the basis for PCB congener specificity. Comparison of different classes of biphenyl 2,3-dioxygenases that attack CBs with different substitution patterns (5) should also enhance our understanding of the degradation of this important class of environmental pollutants.

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REFERENCES

1. **Asturias, J. A., E. Diaz, and K. N. Timmis.** 1995. The evolutionary relationship of biphenyl dioxygenase from Gram-positive *Rhodococcus globerulus* P6 to multicomponent dioxygenases from Gram-negative bacteria. Gene **156:** 11–18.
2. **Axcell, B. C., and P. J. Geary.** 1975. Purification and some properties of a soluble benzene-oxidizing system from a strain of *Pseudomonas*. Biochem. J. **146:**173–183.
3. **Batie, C. J., D. P. Ballou, and C. C. Correll.** 1991. Phthalate dioxygenase reductase and related flavin-iron-sulfur containing electron transferases, p. 543–556. *In* F. Muller (ed.), Chemistry and biochemistry of flavoenzymes. CRC Press, Inc., Boca Raton, Fla.
4. **Batie, C. J., E. LaHaie, and D. P. Ballou.** 1987. Purification and characterization of phthalate oxygenase and phthalate oxygenase reductase from *Pseudomonas cepacia*. J. Biol. Chem. **262:**1510–1518.
5. **Bedard, D. L., and M. L. Haberl.** 1990. Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. **20:**87–102.
6. **Bedard, D. L., M. L. Haberl, R. J. May, and M. J. Brennan.** 1987. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1103–1112.
7. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.
8. **Beinert, H.** 1983. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. Anal. Biochem. **131:**373–378.
9. **Bill, E., F.-H. Bernhardt, and A. X. Trautwein.** 1981. Mossbauer studies on the active Fe. . .[2Fe-2S] site of putidamonooxin, its electron transport and dioxygen activation mechanism. Eur. J. Biochem. **121:**39–46.
10. **Bopp, L. H.** 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J. Ind. Microbiol. **1:**23–29.
11. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
12. **Bunz, P. V., and A. M. Cook.** 1993. Dibenzofuran 4,4a-dioxygenase from *Sphingomonas* sp. strain RW1: angular dioxygenation by a three-component enzyme system. J. Bacteriol. **175:**6467–6475.
13. **Ensley, B. D., and D. T. Gibson.** 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. J. Bacteriol. **155:**505–511.
14. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174:**2903–2912.
15. **Erickson, B. D., and F. J. Mondello.** 1993. Enhanced biodegradation of polychlorinated biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl. Environ. Microbiol. **59:**3858–3862.
16. **Fee, J. A., K. L. Findling, T. Yoshida, R. Hille, G. E. Tarr, D. O. Hearshen, W. R. Dunham, E. P. Day, T. A. Kent, and E. Munck.** 1984. Purification and characterization of the Rieske iron-sulfur protein from *Thermus thermophilus*. J. Biol. Chem. **259:**124–133.
17. **Fetzner, S., R. Muller, and F. Lingens.** 1992. Purification and some properties of 2-halobenzoate 1,2-dioxygenase, a two-component enzyme system from *Pseudomonas cepacia* 2CBS. J. Bacteriol. **174:**279–290.
18. **Furukawa, K., N. Tomizuka, and A. Kamibayashi.** 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. **38:**301–310.
19. **Gassner, G. T., D. P. Ballou, G. A. Landrum, and J. W. Whittaker.** 1994. Magnetic circular dichroism studies on the mononuclear ferrous active site of phthalate dioxygenase from *Pseudomonas cepacia* show a change of ligation state on substrate binding. Biochemistry **32:**4820–4825.
20. **Gibson, D. T., D. L. Cruden, J. D. Haddock, G. J. Zylstra, and J. M. Brand.** 1993. Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **175:**4561–4564.
21. **Gibson, D. T., R. L. Roberts, M. C. Wells, and V. M. Kobal.** 1973. Oxidation of biphenyl by a *Beijerinckia* species. Biochem. Biophys. Res. Commun. **50:**211–219.
22. **Gibson, D. T., G. J. Zylstra, and S. Chauhan.** 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1, p. 121–132. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
23. **Gurbiel, R. J., C. J. Batie, M. Sivaraja, A. E. True, J. A. Fee, B. M. Hoffman, and D. P. Ballou.** 1989. Electron-nuclear double resonance spectroscopy of ^{15}N-enriched phthalate dioxygenase from *Pseudomonas cepacia* proves that two histidines are coordinated to the [2Fe-2S] Rieske-type clusters. Biochemistry **28:**4861–4871.
24. **Haddock, J. D., and D. T. Gibson.** Unpublished data.
25. **Haddock, J. D., J. Horton, and D. T. Gibson.** 1994. Purification and characterization of the terminal oxygenase component of biphenyl dioxygenase of *Pseudomonas* sp. strain LB400, abstr. K-184, p. 308. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
26. **Haddock, J. D., J. R. Horton, and D. T. Gibson.** 1995. Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase

from *Pseudomonas* sp. strain LB400. J. Bacteriol. **177:**20–26.
27. **Haddock, J. D., L. M. Nadim, and D. T. Gibson.** 1993. Oxidation of biphenyl by a multicomponent enzyme system from *Pseudomonas* sp. strain LB400. J. Bacteriol. **175:**395–400.
28. **Hirose, J., A. Suyama, S. Hayashida, and K. Furukawa.** 1994. Construction of hybrid biphenyl (*bph*) and toluene (*tod*) genes for functional analysis of aromatic ring dioxygenases. Gene **138:**27–33.
29. **Irie, S., S. Doi, T. Yorifuji, M. Takagi, and K. Yano.** 1987. Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*. J. Bacteriol. **169:**5174–5179.
30. **Jeffrey, A. M., H. J. C. Yeh, D. M. Jerina, T. R. Patel, J. F. Davey, and D. T. Gibson.** 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. Biochemistry **14:**575–584.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
32. **Locher, H. H., T. Leisinger, and A. M. Cook.** 1991. 4-Sulphobenzoate 3,4-dioxygenase: purification and properties of a desulphonative two-component enzyme system from *Comamonas testosteroni* T-2. Biochem. J. **274:**833–842.
33. **Markus, A., D. Krekel, and F. Lingens.** 1986. Purification and some properties of component A of the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* species strain CBS. J. Biol. Chem. **261:**12883–12888.
34. **Mason, J. R., and R. Cammack.** 1992. The electron-transport proteins of hydroxylating bacterial dioxygenases. Annu. Rev. Microbiol. **46:**277–305.
35. **Nadim, L. M., M. J. Schocken, F. K. Higson, D. T. Gibson, D. L. Bedard, L. H. Bopp, and F. J. Mondello.** 1987. Bacterial oxidation of polychlorinated biphenyls, p. 395–402. *In* Proceedings of the 13th Annual Research Symposium on Land Disposal, Remedial Action, Incineration, and Treatment of Hazardous Waste. U.S. Environmental Protection Agency publication EPA/600/9-87/015. U.S. Environmental Protection Agency, Cincinnati.
36. **Nakatsu, C. H., N. A. Straus, and R. C. Wyndham.** 1995. The nucleotide sequence of the Tn5271 3-chlorobenzoate 3,4-dioxygenase genes (*cbaAB*) unites the class IA oxygenases in a single lineage. Microbiology **141:**485–495.
37. **Neidle, E. L., C. Hartnett, L. N. Ornston, A. Bairoch, M. Rekik, and S. Harayama.** 1991. Nucleotide sequences of the *Acinetobacter calcoaceticus benABC* genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. J. Bacteriol. **173:**5385–5395.
38. **Resnick, S.** Personal communication.
39. **Romanov, V., and R. P. Hausinger.** 1994. *Pseudomonas aeruginosa* 142 uses a three-component *ortho*-halobenzoate 1,2-dioxygenase for metabolism of 2,4-dichloro- and 2-chlorobenzoate. J. Bacteriol. **176:**3368–3374.
40. **Sauber, K., C. Frohner, G. Rosenberg, J. Eberspacher, and F. Lingens.** 1977. Purification and properties of Pyrazon dioxygenase from Pyrazon-degrading bacteria. Eur. J. Biochem. **74:**89–97.
41. **Simon, M. J., T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W.-C. Suen, D. L. Cruden, D. T. Gibson, and G. J. Zylstra.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene **127:**31–37.
42. **Subramanian, V., T.-N. Liu, W.-K. Yeh, and D. T. Gibson.** 1979. Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography. Biochem. Biophys. Res. Commun. **91:**1131–1139.
43. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.
44. **Tan, H.-M., and C.-M. Cheong.** 1994. Substitution of the ISP α subunit of biphenyl dioxygenase from *Pseudomonas* results in a modification of the enzyme activity. Biochem. Biophys. Res. Commun. **204:**912–917.
45. **Yamaguchi, M., and H. Fujisawa.** 1982. Subunit structure of oxygenase component in benzoate-1,2-dioxygenase system from *Pseudomonas arvilla* C-1. J. Biol. Chem. **257:**12497–12502.
46. **Yeh, W.-K., D. T. Gibson, and T.-N. Liu.** 1977. Toluene dioxygenase: a multicomponent enzyme system. Biochem. Biophys. Res. Commun. **78:**401–410.
47. **Zabinski, R., E. Munck, P. M. Champion, and J. M. Wood.** 1972. Kinetic and Mossbauer studies on the mechanism of protocatechuic acid 4,5-oxygenase. Biochemistry **11:**3212–3219.
48. **Ziffer, H., K. Kabuto, D. T. Gibson, V. M. Kobal, and D. M. Jerina.** 1977. The absolute stereochemistry of several *cis*-dihydrodiols microbially produced from substituted benzenes. Tetrahedron **33:**2491–2496.
49. **Zylstra, G. J., S. Chauhan, and D. T. Gibson.** 1990. Degradation of chlorinated biphenyls by *Escherichia coli* containing cloned genes of the *Pseudomonas putida* F1 toluene catabolic pathway, p. 290–302. *In* Proceedings of the 16th Annual Hazardous Waste Research Symposium: Remedial Action, Treatment, and Disposal of Hazardous Waste. U.S. Environmental Protection Agency publication EPA/600/9-90/037. U.S. Environmental Protection Agency, Cincinnati.
50. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940–14946.
51. **Zylstra, G. J., and D. T. Gibson.** 1991. Aromatic hydrocarbon degradation: a molecular approach. Genet. Eng. **13:**183–203.