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Purification and Characterization of the *Comamonas testosteroni* B-356 Biphenyl Dioxygenase Components

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In this report, we describe some of the characteristics of the *Comamonas testosteroni* B-356 biphenyl (BPH)-chlorobiphenyl dioxygenase system, which includes the terminal oxygenase, an iron-sulfur protein (ISP_{BPH}) made up of an α subunit (51 kDa) and a β subunit (22 kDa) encoded by *bphA* and *bphE*, respectively; a ferredoxin (FER_{BPH}; 12 kDa) encoded by *bphF*; and a ferredoxin reductase (RED_{BPH}; 43 kDa) encoded by *bphG*. ISP_{BPH} subunits were purified from B-356 cells grown on BPH. Since highly purified FER_{BPH} and RED_{BPH} were difficult to obtain from strain B-356, these two components were purified from recombinant *Escherichia coli* strains by using the His tag purification system. These His-tagged fusion proteins were shown to support BPH 2,3-dioxygenase activity in vitro when added to preparations of ISP_{BPH} in the presence of NADH. FER_{BPH} and RED_{BPH} are thought to pass electrons from NADH to ISP_{BPH}, which then activates molecular oxygen for insertion into the aromatic substrate. The reductase was found to contain approximately 1 mol of flavin adenine dinucleotide per mol of protein and was specific for NADH as an electron donor. The ferredoxin was found to contain a Rieske-type [2Fe-2S] center (ϵ_{460}, 7,455 M^{-1} cm^{-1}) which was readily lost from the protein during purification and storage. In the presence of RED_{BPH} and FER_{BPH}, ISP_{BPH} was able to convert BPH into both 2,3-dihydro-2,3-dihydroxybiphenyl and 3,4-dihydro-3,4-dihydroxybiphenyl. The significance of this observation is discussed.

The enzymatic steps involved in the conversion of biphenyl (BPH) and chlorobiphenyls (PCBs) into, respectively, benzoate and chlorobenzoates have been elucidated in many bacteria (5, 8, 11, 12, 23, 24, 29, 32, 43). The first step (shown in Fig. 1) involved BPH 2,3-dioxygenase (dox), which transforms BPH into 2,3-dihydro-2,3-dihydroxybiphenyl. This enzyme is believed to determine the substrate selectivity of BPH-degrading bacteria (17).

PCB-degrading bacteria have been divided into four groups based on substrate selectivity patterns (4). *Pseudomonas* sp. strain LB400 has a unique feature in being able to degrade *ortho*-substituted PCB congeners preferentially (17). Most other bacteria preferentially transform *meta*- and *para*-substituted congeners. In another study (submitted for publication) based on alignments of gene and gene product sequences, we determined the existence of two separate BPH dox lineages among gram-negative bacteria. *Comamonas testosteroni* B-356 BPH dox belongs to a distinct phylogenetic lineage together with *Pseudomonas* sp. strain KKS102 (11, 24). This group is characterized by the fact that the gene encoding the BPH ferredoxin reductase (RED_{BPH}) is located outside the *bph* gene cluster and is phylogenetically unrelated to all other known bacterial RED_{BPH}-encoding genes.

The two members of the second lineage are strain LB400 (8) and *Pseudomonas pseudoalcaligenes* KF707 (40). The BPH dox of these strains has a distinct substrate selectivity pattern, suggesting that only minor factors determine this characteristic. Indeed, it was found that changes in only four amino acid residues of the strain LB400 BPH iron-sulfur protein (ISP_{BPH}) α subunit were sufficient to increase the capacity of the recombinant strain to degrade *para*-substituted congeners (9).

Since BPH dox is phylogenetically linked to the toluene (TOL) dox (2) whose components have been purified from *P. putida* F1 (36–38, 45), the functions of the gene products involved in BPH dox catalytic activity were deduced on the basis of this similarity (1, 5, 8, 11, 24, 40). Thus, BPH dox comprises four components (Fig. 1), which are ISP_{BPH}, which is made up of an α subunit (51 kDa) and a β subunit (22 kDa) encoded by *bphA* and *bphE*, respectively, in strain B-356; a ferredoxin (FER_{BPH}; 12 kDa) encoded by *bphF*; and a ferredoxin reductase (RED_{BPH}; 43 kDa) encoded by *bphG*. FER_{BPH} and RED_{BPH} are thought to pass electrons from NADH to ISP_{BPH}, which then activates molecular oxygen for insertion into the substrate.

It has been suggested that although the major pathway for PCB degradation proceeds via hydroxylation at the *ortho* and *meta* (C-2 and C-3) positions, a minor pathway found in a few strains involves oxygenation at the *meta* and *para* (C-3 and C-4) positions (28, 30). It has also been postulated that the ability of strain LB400 to degrade *ortho*-substituted congeners preferentialy is linked to its ability to attack these congeners at the C-3 and C-4 positions (9, 30). Recent studies of partially purified LB400 BPH dox components have shown that this enzyme system can indeed catalyze both C-2–C-3 and C-3–C-4 oxygenations (17).

In contrast to strain LB400, strain B-356 preferentially degrades the *meta*- and *para*-substituted PCB congeners (15). It is therefore of interest to examine B-356 BPH dox biochemistry to determine the features of this enzymatic system that might be responsible for the failure of strain B-356 to degrade *ortho*-substituted congeners efficiently. In this report, we describe some of the characteristics of the B-356 BPH dox system. Since highly purified FER_{BPH} and RED_{BPH} were difficult to obtain from strain B-356, we purified these two components

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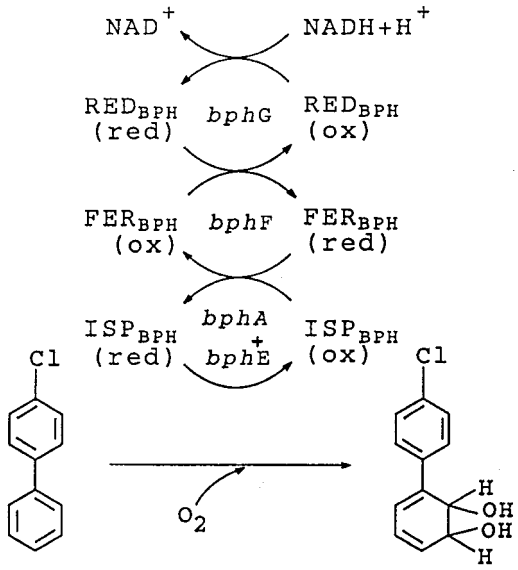

FIG. 1. Reaction catalyzed by BPH dox components.

from engineered *Escherichia coli* strains by using the His tag purification system and found these His-tagged fusion proteins to be functional in the BPH dox system.

MATERIALS AND METHODS

Bacterial strains, culture media, and general protocols. The bacterial strains used in this study were *Escherichia coli* M15(pREP4) and SG13009(pREP4) (both from QIAGEN Inc., Chatsworth, Calif.) and *C. testosteroni* B-356 (20). The media used were Luria-Bertani broth (33), Mueller-Hinton agar (Gibco Diagnostics), nutrient broth, nutrient agar, and minimal medium no. 30 (39) containing 0.05% (wt/vol) BPH.

Most DNA manipulations were done as described by Sambrook et al. (33). *E. coli* cells were transformed as described by Hanahan (21). PCRs to amplify *bphF* were performed with *Taq* DNA polymerase in accordance with procedures outlined previously (5). Amplification of *bphG* was carried out with *Pwo* DNA polymerase as outlined by the supplier, Boehringer Mannheim.

Fractionation of BPH dox components from BPH-induced B-356 cells. The first fractionation step for strain B-356 BPH dox components has already been described (5). Crude extracts of B-356 cells were prepared from 24-h-old cultures grown on minimal medium no. 30-BPH. Harvested cell paste was washed and resuspended in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.3, containing 10% (wt/vol) glycerol. The suspension was sonicated and then cleared by centrifugation. The supernatant was diluted with glass-distilled H_2O containing 10% (wt/vol) glycerol to adjust the buffer concentration to 25 mM.

The crude extract was chromatographed on a Fast-Flow DEAE-Sepharose column as described previously (5). BPH dox components were detected by complementation analysis with crude extracts from various *E. coli* strains carrying parts of the *bph* operon cloned from strain B-356. Fast-Flow DEAE-Sepharose chromatography separated B-356 BPH dox into three components including one fraction (designated fraction D) that contained the yellow RED_{BPH} component, a second fraction (designated fraction E) which had typical Rieske protein spectral characteristics and was shown to contain both the α and β subunits of ISP_{BPH}, and a third fraction containing FER_{BPH}.

Fraction D was partially purified as described previously (5). Fraction E (approximately 100 ml) was brought to 5% (wt/vol) ammonium sulfate, filtered through a 0.22-μm-pore-size membrane to remove particulate material, and then loaded onto a phenyl-Sepharose column (12 by 1.6 cm) equilibrated with 25 mM HEPES buffer, pH 7.3, containing 5% ammonium sulfate and 10% glycerol. The sample was loaded at a flow rate of 1.0 ml/min and then washed with the starting buffer for 30 min. The column was then eluted with the same HEPES buffer lacking ammonium sulfate. A protein peak eluting at approximately 80 ml had Rieske protein spectral characteristics. Fractions from this peak (45 ml) were combined and passed over an Econo-Pac Q cartridge (Bio-Rad Laboratories, Hercules, Calif.) at a flow rate of 1 ml/min. The Rieske-type protein was eluted with 25 mM HEPES buffer, pH 7.3, containing 0.2 M NaCl. The preparation obtained from the Econo-Pac Q cartridge was found to contain both the α and β subunits of the ISP_{BPH} components, and this preparation was used for all of the experiments reported in this study.

Production and purification of His-tagged B-356 FER_{BPH} and RED_{BPH}. His-tagged B-356 FER_{BPH} and RED_{BPH} were produced from *E. coli* M15(pREP4) and SG13009(pREP4). For each protein, the coding region of either B-356 *bphF* or B-356 *bphG* was PCR amplified from a cloned DNA fragment. The oligonucleotides used for PCR were chosen on the basis of the known DNA sequences of these genes (submitted for publication), and they were as follows: oligonucleotide I (*Bam*HI), (5′-CGGGATCCGATGGGATTTACTCGCG-3′) and oligonucleotide II (*Kpn*I) (5′-GGGGTACCCCTCAAGCAGCCAAGTG-3′) to amplify *bphF* and oligonucleotide I (*Bam*HI) (5′-CGGGATCCGATGGGAAACG AGGAGT-3′) and oligonucleotide II (*Kpn*I) (5′-GGGGTACCCCTCAGGCCG ACGCCTG-3′) to amplify *bphG*.

The PCR products were digested with *Bam*HI and *Kpn*I. A 1.2-kb DNA fragment (for *bphG*) and a 0.55-kb DNA fragment (for *bphF*), containing the entire coding sequences, were isolated and cloned into the compatible sites of pQE31. Constructions were such that the His tail added 13 amino acids (MRG SHHHHHHTDP) to the protein at its N-terminal portion.

E. coli strains harboring the appropriate pQE hybrid plasmids were grown for 3 h at 37°C in Luria-Bertani broth containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were then harvested, suspended in 5 volumes of sonication buffer (50 mM Na-phosphate [pH 7.8], 300 mM NaCl, 10% [wt/vol] glycerol) per gram of wet cell paste and sonicated with a Braunsonic ultrasonifier. The lysate (approximately 100 ml) was centrifuged at 20,000 × *g* for 60 min, and the supernatant was mixed with 1 ml of a 50% slurry of an Ni-nitrilotriacetic acid resin suspension in sonication buffer. The mixture was agitated at 4°C for 60 min on a rotatory platform and then poured into an empty column (1 by 11 cm) to load the resin. The resin was then successively washed with 40 ml each of sonication buffer, wash buffer (same as sonication buffer but adjusted to pH 6.0), and wash buffers with 20 and 40 mM imidazole. The His-tagged proteins were finally eluted with wash buffer containing 100 mM imidazole, and the fractions were collected and tested for the presence of the desired protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by complementation assays of BPH dox activity.

Protein characterization. SDS-PAGE on 10, 12, or 15% gels was performed as described by Laemmli (26). The molecular weights of the RED_{BPH}, FER_{BPH}, and ISP_{BPH} α and β subunits were determined from SDS-PAGE gels by using Promega's midrange molecular weight standards. Proteins were stained with Coomassie brillant blue (33).

The iron content of protein was determined with ferrozine by using the Sigma (St. Louis, Mo.) iron determination kit. Acid-labile sulfide was determined by the method of Fogo and Popowsky (10) as modified by King and Morris (25). Protein concentrations were estimated by the method of Lowry (27) or Bradford (6) or with bicinchoninic acid (34) (with or without precipitation of protein with trichloroacetic acid [7]). Standard curves were established by using purified enzyme component preparations that had been dialyzed for 48 h against distilled, demineralized water and lyophilized or by using bovine serum albumin.

Flavin adenine dinucleotide (FAD) or flavin mononucleotide determination was performed by the method of Stuehr et al. (35). Preparations of purified RED_{BPH} (2.25 nmol/ml) were boiled to release the flavin and then centrifuged. High-pressure liquid chromatographic (HPLC) analysis of the supernatant was performed with a Hewlett-Packard series 1050 liquid chromatograph and a Supelcosil LC18 reverse-phase column (150 by 4.6 mm). The column was eluted for 2 min at 1 ml/min with 5 mM ammonium acetate, pH 6.0, containing 7% (vol/vol) methanol, followed by a linear gradient reaching 70% methanol in the same buffer within 18 min. The column was connected to a Hewlett-Packard series 1046A fluorescence detector. The excitation and emission wavelengths were set at 446 and 533 nm. FAD and flavin mononucleotide standards were from Sigma Chemical Co.

The molar extinction coefficient of the oxidized RED_{BPH} was determined by using a number of different preparations. Enzyme solutions with known protein concentrations in the QIAGEN elution buffer were boiled and then centrifuged; spectral analyses of the supernatant solutions were done. Assuming that the visible absorbance of the heat-treated enzyme is entirely due to FAD, molar extinction coefficients were assigned to native reductase on the basis of the amount of FAD released. Spectrophotometric analyses were performed with either a Philips PU8715 or a Hitachi U-2000 recording spectrophotometer.

BPH dox assays. In most cases, enzyme assays were performed at 37°C and the assay buffer used was 100 mM morpholineethanesulfonic acid (MES; pH 5.5). The reaction mixture (200-μl total volume) contained 100 nmol of NADH, 50 nmol of the substrate, 1.2 nmol of $FeSO_4$, and 0.6 nmol of each enzyme component. The reaction was initiated by adding the substrate dissolved in 2 μl of dimethyl sulfoxide or acetone. The enzyme components were added individually as required. Reaction mixtures were incubated for 2 min at 37°C in Eppendorf tubes; the reactions were then stopped by adding 800 μl of ice-cold HEPES buffer (100 mM, pH 8.0). After centrifugation for 30 s in a microcentrifuge, the decrease in A_{340} of the supernatant was used to monitor NADH oxidation. A reaction mixture containing all of the components except the substrate was used as a control. When continuous monitoring of NADH depletion was required, NADH oxidation was evaluated fluorometrically with a Perkin Elmer LS50B fluorimeter. In this case, the excitation wavelength was set at 340 nm and the emitted light was measured at 459 nm.

Oxygenated products of enzymatic reactions were also detected and quantified by HPLC. In this case, at the end of the reaction period, the reaction vial was

TABLE 1. Purification of ISP_{BPH}

Purification step	Total protein (mg)	Activity (U, 10^3)[a]	Sp act (U/mg)	Recovery (%)
Crude extract	2,444	173	71	
Fast-Flow DEAE-Sepharose	65.7	95	1,447	55
Phenyl-Sepharose	21.3	65	3,055	38
Econo-Pac Q	18.6	45	2,412	26

[a] One unit of enzyme activity is defined as 1 μmol of NADH oxidized after 2 min of incubation of the BPH dox enzyme assay mixture as described in the text.

transferred into a boiling water bath for 2 min and then centrifuged in a microcentrifuge for 30 s. Supernatant (10 μl) was injected directly onto a Hewlett-Packard ODS Hypersil II (5 μm) reverse-phase column (4 mm by 25 cm) that had been equilibrated with methanol-water (70%-30%), and the column was eluted with the same solvent. Metabolites were detected with a Perkin-Elmer LC95 UV-visible light detector set at 306 nm (dihydrodihydroxybiphenyl) or 308 nm (dihydrodihydroxy-4′-chlorobiphenyl). Confirmation of the identities of the metabolites eluted from the HPLC column was obtained by gas chromatographic (GC)-mass spectrometric (MS) analysis of ethyl acetate extracts of fractions presumed to contain dihydrodiol derivatives.

RED_{BPH} was quantitated by determining its activity in the ferricyanide and cytochrome *c* reductase assays described by Subramanian et al. (36). FER_{BPH} activity was also determined by using the cytochrome *c* reduction assay in the presence of RED_{BPH}.

The K_m and the maximum rate of metabolism, were estimated at 37°C by using initial reaction velocities. The K_m value for NADH consumption in the ferricyanide assay was obtained by continuous monitoring of NADH fluorescence by using a range of NADH concentrations between 20 and 150 μM in the presence of excess sodium ferricyanide (60 μM) and 230 nM RED_{BPH}. In the cytochrome *c* reduction assay, kinetics assays contained 276 nM RED_{BPH}, 740 nM FER_{BPH}, 20 μM cytochrome *c* (a large excess), and a range of NADH concentrations from 1 to 50 μM. All of the K_ms and maximum rates of metabolism reported were calculated on the basis of the average of at least three separate experiments.

The capacity of the enzyme to catalyze the oxygenation of benzene, toluene, and naphthalene was evaluated in the BPH dox assay (described above); NADH oxidation was monitored spectrofluorometrically.

Identification of reaction products. Reaction products of BPH and 4-chlorobiphenyl (4CBP) were extracted with ethyl acetate at pH 6.0, treated with *N,O*-bis-trimethylsilyl trifluoroacetamide, and analyzed by GC-MS or by GC with an electron capture detector (ECD) (for 4CBP) by using procedures and instruments that have been described previously (28).

Effects of various reagents on enzyme activities. The effects of metal ions on the enzyme assays described above were determined after adding appropriate amounts of $MgCl_2$, $FeCl_3$, $MnCl_2$, $CoCl_2$, $HgCl_2$, $ZnCl_2$, Na_2MoO_4, $CuSO_4$, $FeSO_4$, or $NiSO_4$. Effects of surfactants, solvents, and inhibitors were determined by adding various concentrations of these compounds to the reaction vials before substrate addition. Otherwise, all assays were performed as described above.

Restoration of RED_{BPH} flavin content. To increase the flavin content of RED_{BPH}, the protein was incubated at 4°C for 16 h in 50 mM phosphate buffer, pH 6.0, containing NaCl (300 mM), glycerol (10% wt/vol), and FAD (2 μM). Protein was then precipitated with ammonium sulfate, redissolved in the same buffer lacking FAD, and dialyzed overnight at 4°C. Controls in which protein was subjected to an identical treatment, except that FAD was omitted from the first buffer, were used to compare the activities of restored versus unrestored proteins in ferricyanide, cytochrome *c* reductase, and BPH dox assays.

RESULTS

Purification of ISP_{BPH} subunits. The steps used in the purification of ISP_{BPH} are described in Materials and Methods. Relevant details of the purification procedure are summarized in Table 1. ISP_{BPH} subunits were eluted from the Fast-Flow DEAE-Sepharose column in a single fraction designated E. Subsequent chromatography of fraction E on phenyl-Sepharose gave two major protein peaks, one of which was brownish in color and was found to restore B-356 BPH dox activity when combined with FER_{BPH} and RED_{BPH}. This fraction was concentrated on an Econo-Pac Q cartridge before use in enzyme characterization studies, although this procedure reduced the specific activity of the protein by a small amount (Table 1). Enzyme preparations resulting from this procedure contained predominantly two polypeptides (Fig. 2, lane 2).

Purification of FER_{BPH} and RED_{BPH}. Fraction D from the Fast-Flow DEAE-Sepharose column contained RED_{BPH}, which was partially purified as described previously (5). However, attempts to purify this protein further were unsuccessful. Fraction F from the DEAE column had a spectrum consistent with the presence of a Rieske-type iron-sulfur center and weak FER_{BPH} activity when tested in the BPH dox enzyme assay. However, B-356 FER_{BPH} was very unstable and it was not possible to purify this protein from fraction F further.

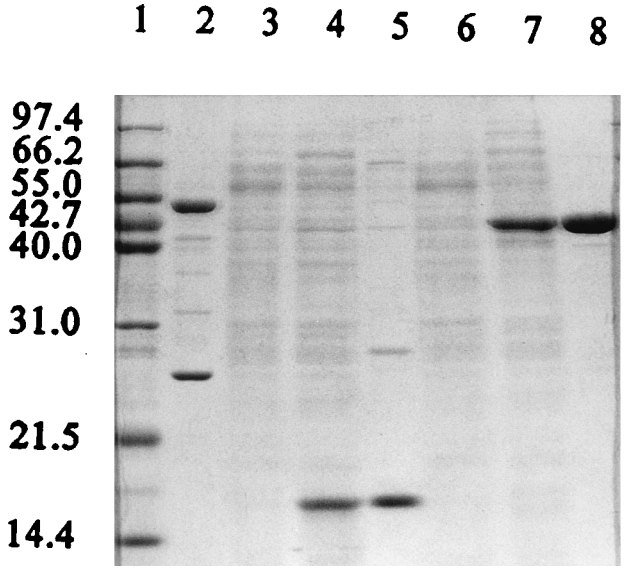

FIG. 2. SDS-PAGE of B-356 ISP_{BPH}, His-tagged B-356 RED_{BPH}, and His-tagged B-356 FER_{BPH} preparations. Lanes: 1, M_r markers; 2, B-356 ISP_{BPH} preparation (3 μg of protein) obtained after the Q-cartridge chromatographic step (Table 1); 3, crude extract (10 μg of protein) from the uninduced strain harboring hybrid plasmid pQE-*bphG*; 4, crude extract (10 μg of protein) after induction; 5, one of the most purified fractions (3 μg of protein) obtained after elution of His-tailed RED_{BPH} from Ni-nitrilotriacetic acid resin; 6, crude extract (6.5 μg of protein) from the uninduced strain harboring hybrid plasmid pQE-*bphF*; 7, crude extract (7.0 μg of protein) after induction; 8, one of the most purified fractions (2.0 μg of protein) obtained after elution of His-tailed FER_{BPH} from Ni-nitrilotriacetic acid resin. Protein bands were revealed by Coomassie blue staining. red, reduction; OX, oxidation. The numbers on the left are molecular masses in kilodaltons.

Nevertheless, use of the QIAGEN His-binding kit made it possible to obtain active purified preparations of both His-tagged RED_{BPH} and FER_{BPH} after expression in *E. coli* (Materials and Methods). The purity of each protein fraction eluted from the QIAGEN column was examined by SDS-PAGE, and only the purest fractions were retained. The yields of purified FER_{BPH} and RED_{BPH} were approximately 1 and 2 mg/g of cell paste (wet weight), respectively.

No contaminating bands were detected on SDS-PAGE gels of the best preparations of His-tagged RED_{BPH} (Fig. 2). The purity of His-tagged FER_{BPH} preparations was also very good, although a minor contaminant coeluted with FER_{BPH} in all fractions (Fig. 2).

Characterization of B-356 ISP_{BPH}. The purified ISP_{BPH} preparation was unable to catalyze the conversion of BPH to 2,3-dihydro-2,3-dihydroxybiphenyl in the absence of His-tagged FER_{BPH} and His-tagged RED_{BPH}. The M_rs of the ISP_{BPH} subunits estimated by SDS-PAGE (56,300 and 25,800) were similar to those predicted from the DNA sequences of *bphA* and *bphE* (51,690 and 21,556, respectively [submitted for publication]). The absorbance spectrum of ISP_{BPH} was typical of a [2Fe-2S] Rieske-type protein, with maxima at 323 and 455 nm and a shoulder at about 575 nm. After reduction with

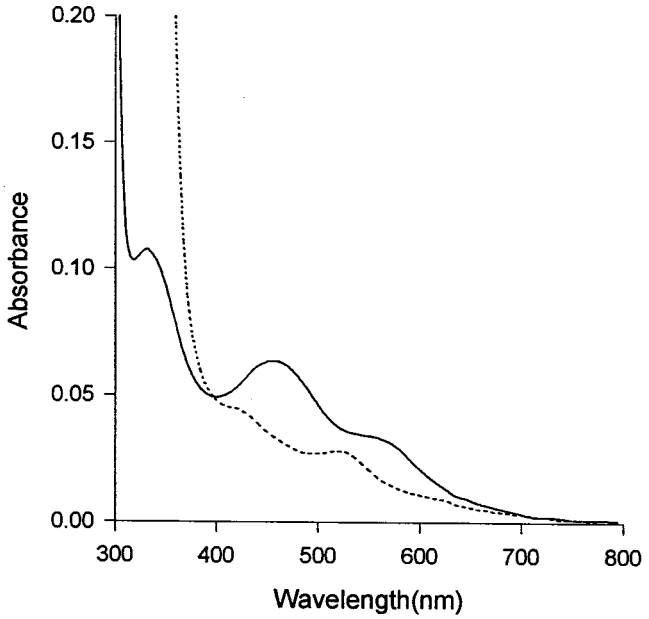

FIG. 3. Absorption spectra of ISP_{BPH}. UV-visible light spectra of oxidized ISP_{BPH} (1.1 mg/ml in 25 mM HEPES buffer, pH 7.4) (solid line) and spectra of the same preparation after aerobic addition of dithionite to reduce the enzyme (dashed line).

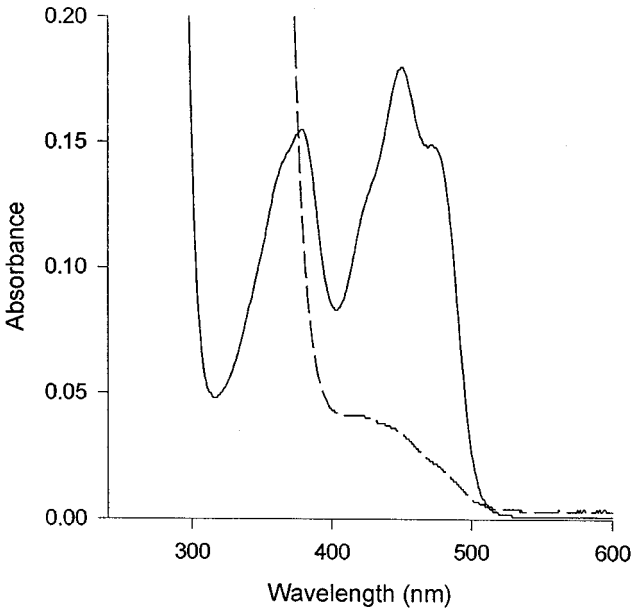

FIG. 4. Absorption spectra of oxidized (solid line) and reduced (dashed line) His-tagged RED_{BPH} (20 μM protein in QIAGEN elution buffer, pH 6.0). The enzyme was reduced by aerobic addition of dithionite.

excess sodium dithionite under aerobic conditions, the spectrum resembled those of other reduced Rieske center-containing proteins (Fig. 3) (19, 36, 38).

The ISP_{BPH} component remained fully active in the BPH dox assay after storage for 48 h at 22°C, 5 days at 4°C, or months at −70°C in 25 mM HEPES buffer (pH 7.3) containing 0.1 M NaCl.

Characterization of His-tagged RED_{BPH}. The purified His-tagged-RED_{BPH} preparation was a bright yellow solution that was able to catalyze the NADH-dependent reduction of ferricyanide and cytochrome *c*. The M_r of this protein was estimated from SDS-PAGE to be 42,700 (the predicted value, based on sequence analysis, was 44,900). Replacement of NADH by NADPH in the cytochrome *c* reduction assay reduced the activity to 15% of the value measured in the presence of NADH.

RED_{BPH} lost less than 10% of its activity in the ferricyanide assay when kept on ice for 24 h in the QIAGEN elution buffer, and 30% of its activity was lost after 48 h. The protein remained active after 5 h at 37°C or 30 min at 45°C.

HPLC analysis identified the RED_{BPH}-associated flavin as FAD. Quantitation of FAD from the HPLC elution peak indicated between 0.7 and 0.8 mol of FAD per mol of protein, depending on the preparation. The flavin content of the protein increased after incubation with FAD (Materials and Methods). Both the FAD-protein ratio and the activity of the fully restored enzyme increased about 25% in comparison with the untreated enzyme.

The visible absorbance spectrum of purified protein was that of a typical flavoprotein with absorbance maxima at 269, 377, and 446 nm and a shoulder at 476 nm (Fig. 4). The ratio of A_{280} to A_{450} ranged between 5.4 and 6.2. Aerobic addition of excess dithionite to the protein preparation bleached the spectrum (Fig. 4). The molar extinction coefficient of the oxidized form of RED_{BPH} at 450 nm, made by comparison of spectra before and after boiling (Materials and Methods), was 11,250 M^{-1} cm^{-1}.

Characterization of His-tagged FER_{BPH}. His-tagged FER_{BPH} preparations were brownish in color. The M_r of the His-tagged protein was calculated from its migration on SDS-PAGE gels to be 15,516 (predicted value, 13,471). The absorption spectrum of the preparation was typical of a Rieske-type iron-sulfur center with maxima at 277, 323, and 455 nm and a shoulder at 575 nm (Fig. 5).

The stability of His-tagged FER_{BPH} at temperatures between 4 and 55°C was determined by using the cytochrome *c* reductase assay and storage at several pHs between 5.5 and 8.0.

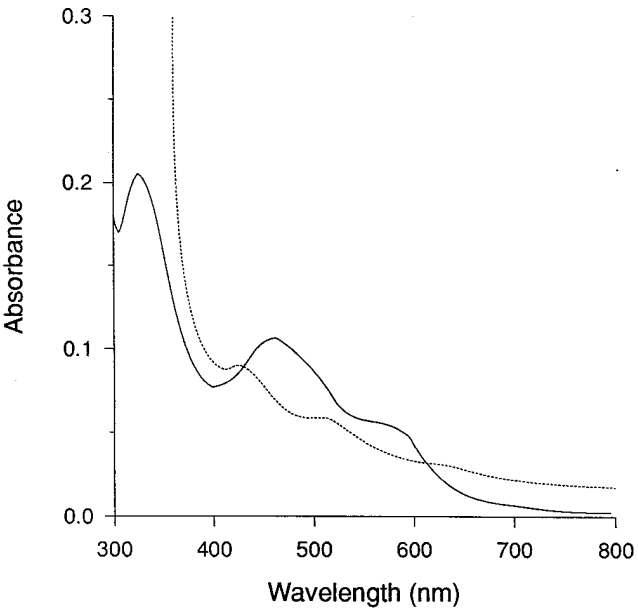

FIG. 5. Absorption spectra of oxidized (solid line) and reduced (dashed line) His-tagged FER_{BPH} (42 μM protein in QIAGEN elution buffer, pH 6.0). The enzyme was reduced by aerobic addition of dithionite.

At 4°C and neutral pH, FER_{BPH} remained fully active for about 5 h, but 35% of the activity was lost after a 24-h incubation period. In contrast to FER_{TOL} (38), the stability of FER_{BPH} was independent of pHs between 5.5 and 8.0. At −70°C, 30% of the activity was lost within 10 weeks. At 22°C, 40% of the activity was lost within 2 h, while at 37°C, 75% of the activity was lost within 2 h.

In contrast to other aryl dox ferredoxins (20, 38) the FER_{BPH} Rieske center is very labile. None of the purified preparations we obtained contained more than 50% of the theoretical iron or sulfur content for a [2Fe-2S] center-containing protein. Protein estimation from the average of the Lowry and bicinchoninic acid protein assays was 41.7 ± 2.3 μM for the preparation that was used to obtain the spectrum shown in Fig. 5. The Fe and inorganic sulfide contents of this protein preparation were estimated to be 0.8 and 0.6 g-atom/mol of protein, respectively. Therefore, only approximately 35% of the protein molecules retained an unaltered [2Fe-2S] Rieske center. On the basis of this estimation, the molar extinction coefficient of FER_{BPH} at 460 nm was calculated to be 7,455 M^{-1} cm^{-1}, which is similar to the ϵ_{460} value determined for FER_{TOL} (38).

Reduction of FER_{BPH} by RED_{BPH} and electron transfer from NADH to ISP_{BPH}. At any FER_{BPH} concentration, the rate of cytochrome *c* reduction increases in direct proportion to the RED_{BPH} concentration, showing a requirement for both components for optimal electron transfer activity. In the ferricyanide and cytochrome *c* reduction assays, the apparent K_m values for NADH were, respectively, 10 and 1 μM for RED_{BPH} under the conditions described in Materials and Methods.

As was shown for RED_{TOL} (37) and RED_{NAH} (19), RED_{BPH} could be replaced by spinach ferredoxin reductase in both cytochrome *c* reduction and BPH dox activity assays when NADPH was provided as the electron donor. However, the assay system containing spinach ferredoxin reductase showed only approximately 10% of the activity observed in the RED_{BPH} assay.

Neither BPH dox nor cytochrome *c* reduction activity was detected when FER_{BPH} was replaced with spinach ferredoxin or clostridial ferredoxin. Moreover, BPH dox was inactive in systems in which both RED_{BPH} and FER_{BPH} were replaced by spinach ferredoxin and ferredoxin reductase. Together, these results indicate that FER_{BPH} can accept electrons from various reductases but RED_{BPH} is more specific in its capacity to transfer electrons from NADH to a ferredoxin.

FAD stimulates electron transfer from RED_{TOL} to ISP_{TOL} (37) when concentrations of RED_{TOL} that support a low level of TOL dox activity are added to the assay. In the case of BPH dox, FAD stimulated NADH consumption in reaction mixtures containing equimolar concentrations of each enzyme component (Table 2). Surprisingly, under the same conditions the amount of dihydrodiol produced, as estimated from the areas under HPLC and GC-ECD peaks, decreased with increased concentrations of FAD (Table 2). When the amount of RED_{BPH} in the assay was lowered to 18 pmol, which is close to the minimal amount required to support BPH dox activity, in the presence of 1 nmol of FAD the amount of dihydrodiol measured by HPLC was approximately 30% of that found in a control reaction carried out in the absence of added FAD. The mechanism by which electron transfer is uncoupled from substrate oxygenation in the presence of excess FAD is not known. Two possibilities are that FAD acts as an electron sink or as a substrate for the oxygenase. However, we cannot exclude noncatalytic conversion of dihydrodiols into metabolites that remained undetected in our system.

TABLE 2. Effect of FAD concentration on NADH oxidation or dihydrodiol production by BPH dox[a]

FAD concn (nmol/ml)	NADH oxidation (%)[b]	Dihydrodiol production (%)[b]
0	100	100
5	142	59
10	267	42
20	350	33
40	417	28

[a] The reaction mixture containing the indicated amount of added FAD was incubated for 2 min at 37°C in 20 μl of 100 mM MES buffer, pH 5.5, containing 1.2 nmol of $FeSO_4$, 0.6 nmol of each enzyme component, 100 nmol of NADH, and 75 nmol of 4CBP. The reaction was stopped by adding 800 μl of ice-cold 100 mM HEPES, pH 8.0.

[b] Relative activity, compared with that of a reaction mixture containing no FAD (100%), when the activity was measured from NADH oxidation or from dihydrodiol production as estimated by the surface area of corresponding GC-ECD peaks.

Effects of inhibitors, metal ions, and surfactants on BPH dox activity. The effects of several compounds recognized as potential inhibitors of electron transfer between RED_{BPH} and FER_{BPH} (3, 37) were evaluated in the cytochrome *c* reduction assay. The results are shown in Table 3. Cytochrome *c* reduction activity was only weakly inhibited, if at all, by both the sulfhydryl-directed inhibitors (iodoacetate and *N*-ethylmaleimide) and the iron chelators (*o*-phenanthroline and EDTA). These results contrast with those obtained for FER_{TOL} under similar conditions (37, 38).

On the other hand, iodoacetate and *N*-ethylmaleimide more strongly inhibit the BPH dox reaction, as determined by the production of dihydrodiols from BPH (Table 3). This result suggests that a cysteine residue of ISP_{BPH} is important for enzyme activity. The inhibition of BPH dox activity by

TABLE 3. Inhibition of cytochrome *c* reduction activity catalyzed by FER_{BPH}-RED_{BPH} and of BPH dox activity catalyzed by all BPH dox components

Inhibitor	% Inhibition[a]	
	Cytochrome *c*	BPH dox
Iodoacetate		
100 mM	0	49
50 mM	0	27
N-Ethylmaleimide		
50 mM	29	35
25 mM	14	—[b]
12.5 mM	0	—
5 mM	—	19
0.5 mM	—	0
EDTA (20 nM)	63	—
o-Phenanthroline		
10 mM	0	—
300 μM	—	93
30 μM	—	68
3 μM	—	50

[a] The activity of the uninhibited enzyme was taken as 0% inhibition in the reaction mixtures described in Materials and Methods for cytochrome *c* reduction and BPH dox (measured in 100 mM MOPS buffer, pH 5.5).

[b] —, not determined.

o-phenanthroline is consistent with the involvement of an iron-binding active center in this reaction, as was suggested by Batie et al. (3) to explain the effect of this compound on the phthalate oxygenase of *P. cepacia*. If mononuclear iron is present on ISP_{BPH}, most of this iron apparently remained bound to the enzyme during purification since the enzyme activity when assayed without supplemental Fe^{2+} was 75% of that observed when an excess of Fe^{2+} was added to the reaction medium.

None of the metal ions tested (Mn^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, or MoO_4^{2+}) stimulated BPH dox activity. Hg^{2+} and Cu^{2+} strongly inhibited the conversion of BPH into dihydrodiol products.

Although BPH dox substrates are poorly soluble in water, both Tween 80 and Triton X-100 (0.1% [wt/vol]) inhibited the activity by about 40%. The detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) had no effect on the enzyme activity. BPH dox activity increased by 30% when the reaction medium contained between 1 and 4% (vol/vol) acetone. This is in agreement with data obtained with other dioxygenase systems (31, 38, 44).

Specificity of the BPH dox reaction and analysis of the reaction products. The substrate specificity of the enzyme was examined to determine the BPH dox activity with toluene, benzene, and naphthalene. NADH oxidation was observed only when benzene was used as the substrate, and the activity was approximately one-third of that observed when BPH was the substrate. Therefore, toluene and naphthalene are not substrates for the enzyme and benzene is either a substrate or a nonsubstrate effector of NADH oxidation.

The reaction products generated from BPH and 4CBP were examined by GC-MS. Figure 6 shows typical spectra obtained from extracts of reaction mixtures in which metabolites 1, 2, and 3 are produced in an approximately 2:3:1 ratio based on the surface area of peaks in GC-MS analysis. Metabolites 1 and 2 showed a parent ion at *m/z* 366 and fragmentation patterns identical to those reported for 4CBP dihydrodiol derivatives. Compound 1 showed a structurally informative doublet at *m/z* 93, 95 $[Me_2Si{=}Cl]^+$ which was more intense than in the compound 2 spectrum. As discussed elsewhere (28), this fragmentation pattern tends to be favored in compounds in which the hydroxyl substituents on the molecule are closer to the chlorine atom. Therefore, compounds 1 and 2 were respectively assigned the structures 2,3-dihydro-2,3-dihydroxy-4′-chlorobiphenyl and 3,4-dihydro-3,4-dihydroxy-4′-chlorobiphenyl. The elution and spectral characteristics of compound 3 were identical to those of 2,3-dihydroxy-4′-chlorobiphenyl, which have been reported previously (28).

Production of both dihydrodiol forms and of the dihydroxy derivatives in similar proportions was obtained over a range of pHs from 5.5 to 7.5 (results not shown), showing that these products were not simply generated by pH-dependent transformations of a single reaction product. Moreover, the same products were obtained whether the substrate was solubilized in dimethyl sulfoxide or in acetone. Therefore, these products appear to be the actual products generated during the catalytic oxygenation of 4CBP by the B-356 BPH dox system.

When BPH was used as the substrate, the reaction products (Fig. 6) showed mass spectral characteristics that were identical to those obtained with 4CBP except for the absence of a chlorine atom. On the basis of the GC peak areas, the concentration of each product increased proportionately with time (results not shown), suggesting that all of the products are generated simultaneously. This observation shows that B-356 BPH dox is able to attack the substrate at the C-3 and C-4 positions to generate 3,4-dihydrodiol derivatives, as is the case for strain LB400 BPH dox (17). However, in contrast to the strain LB-400 enzyme, our purified enzyme preparation generated multiple reaction products from BPH and monochlorobiphenyls.

DISCUSSION

As with other bacterial BPH dox systems that have been described (2, 8, 11, 24, 40), sequence comparisons indicate that B-356 BPH dox appears to be very closely related to TOL dox (submitted for publication). Both enzymes comprise four components including an NADH-dependent flavoprotein, a Rieske-type ferredoxin protein, and a two-subunit iron-sulfur protein containing a Rieske-type [2Fe-2S] center that acts as a terminal oxygenase. Although it proved possible to obtain an active purified ISP_{BPH} component from BPH-grown B-356 cells, FER_{BPH} and RED_{BPH} were very difficult to purify intact. Similar problems were encountered by Haddock et al. (17), who were able to obtain homogeneous ISP_{BPH} from strain LB400 but only partially purified electron transfer components.

In our study, the His tag system proved to be very useful as a means to obtain purified active RED_{BPH} and FER_{BPH}. The ability of these preparations to support BPH dox activity and electron transfer from NADH to ferricyanide or FER_{BPH} suggests that fusion tagging had little effect on the structure of RED_{BPH}. Consistent with this, the visible absorbance spectrum of the most purified preparation of B-356 RED_{BPH} was very similar to the spectrum of His-tailed RED_{BPH} (result not shown). Therefore, we are confident that the properties of His-tagged B-356 RED_{BPH} reported here reflect the properties of the untagged protein.

It is interesting that His-tagged RED_{BPH} retained much of its flavin cofactor during purification, since this cofactor appears to be easily lost during purification of flavoprotein reductases associated with other oxygenases (14, 16, 19, 37). It is likely that the small number of manipulations required to obtain purified His-tagged protein contributed to this success. While highly purified FER_{BPH} was also obtained by using the same strategy, it was not enough to prevent loss of a considerable percentage of the iron-sulfur center of this protein. It is not clear whether this is due simply to the instability of the Rieske center or whether expression in *E. coli* cells contributes to the problem. In this regard, it can be noted that the highly purified vertebrate [2Fe-2S] ferredoxin involved in the transfer of electrons to mitochondrial cytochromes P-450 has been produced with the His tag system (42). The recombinant protein was indistinguishable from that of ferredoxin purified from mammalian cells on the basis of its absorption spectrum, electron paramagnetic resonance spectrum, and activity.

The availability of purified redox-active preparations of His-tagged B-356 FER_{BPH} and RED_{BPH} allowed us to begin characterization of the B-356 BPH dox system. As is the case for TOL dox (37, 38) and benzene dox (13), the *bph*-encoded ferredoxin reductase and the Rieske-type ferredoxin are apparently involved in electron transfer from NADH to the terminal oxygenase. Our study indicates that, as for RED_{TOL} (37), NADH is the electron donor for this enzyme system. Haddock et al. (18) have shown that NADPH, as well as NADH, stimulated BPH dox activity in cell extracts prepared from *Pseudomonas* strain LB400 cells. Nucleotide sequence analysis showed that the B-356 RED_{BPH} primary structure is very distinct from that of strain LB400 RED_{BPH}, while the sequences of the other components are very similar (submitted for publication). Therefore, the flavoprotein reductase in these two strains is a major differentiating feature.

Although strain B-356 BPH dox was shown to belong to a phylogenetic lineage distinct from the one to which those of

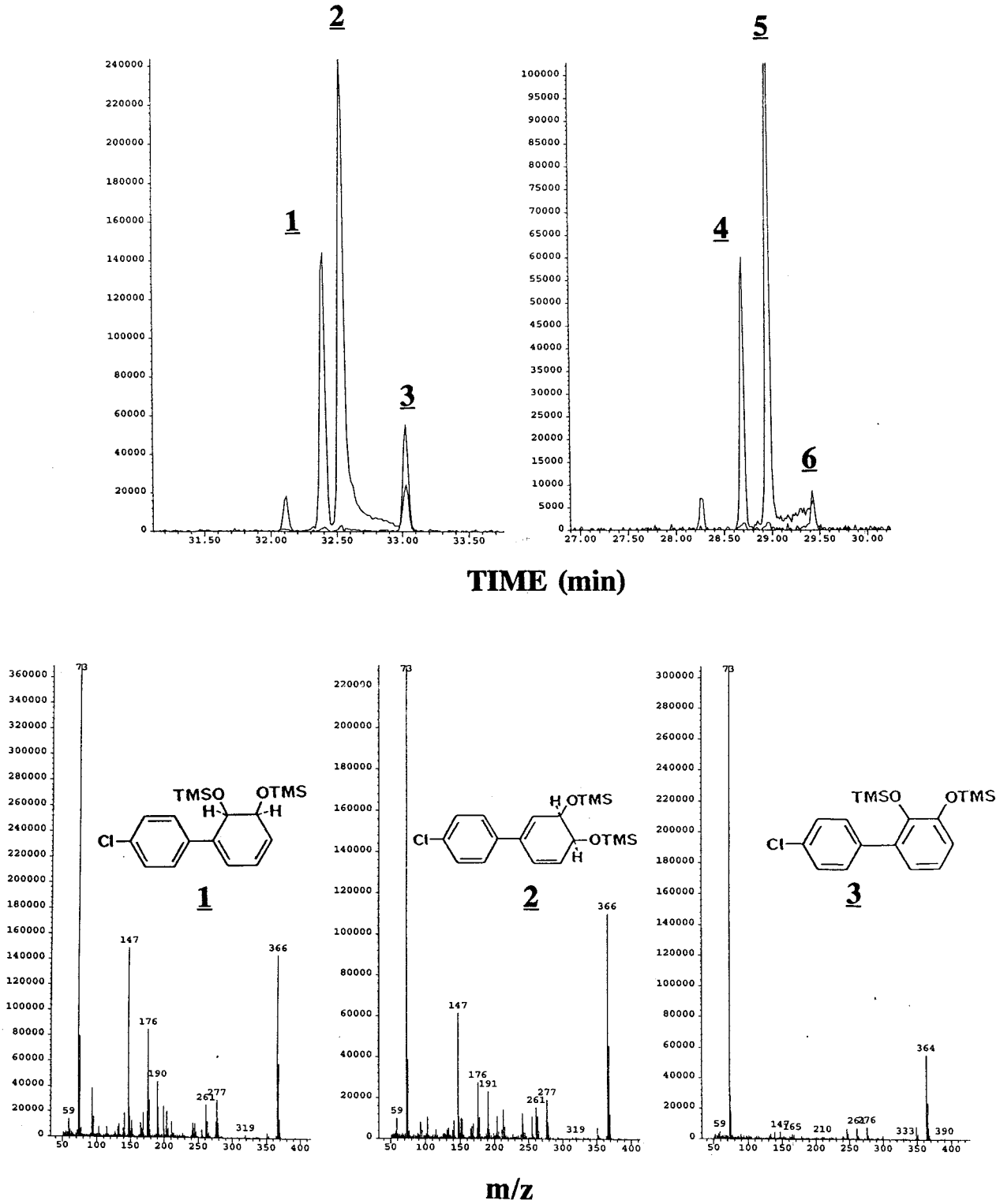

FIG. 6. GC-MS spectra of 4CBP and BPH reaction products of BPH dox. The enzyme reaction was performed under conditions described in Materials and Methods, with either 4CBP or BPH as the substrate. The reaction was initiated by adding the substrate. After 2 min of incubation, the reaction medium was extracted with ethyl acetate, derivatized with *N*,*O*-bis-trimethylsilyl trifluoroacetamide, and analyzed by GC-MS as described in the text. Metabolites 1, 2, and 3 were obtained as reaction products of 4CBP, and their respective MS spectra are shown at the bottom. Metabolites 4, 5, and 6 were reaction products of BPH. Their spectra correspond exactly to those of the 4CBP products minus chlorine (data not shown).

strains LB-400 and KF707 belong, it is noteworthy that the amino acid sequences of the α and β ISP_{BPH} subunits were highly conserved in gram-negative bacteria. For this reason, it was of interest to compare the substrate specificity of strain B-356 BPH dox with that of strain KF707. Thus, as was shown for *P. pseudoalcaligenes* KF707, B-356 BPH dox is unable to catalyze the oxygenation of TOL (22) but is apparently able to attack benzene (41).

An important observation concerning *C. testosteroni* B-356 BPH dox is that it can produce 3,4-dihydrodiol, as well as 2,3-dihydrodiol, derivatives from BPH and 4CBP. In contrast to strain LB400, which appears to produce very small amounts of 3,4-dihydrodiol from BPH (17), strain B-356 produces this compound in fairly large quantities. However, this observation does not necessarily indicate fundamental differences between the mechanisms of action of B-356 BPH dox and LB400 BPH dox. The observed difference could, for example, be attributed to the fact that all of the components of B-356 BPH dox have been purified quite extensively. Other enzyme components, such as the protein with an unknown function encoded by open reading frame 1 of LB400 (8), which is found in all known PCB-degrading strains, may have been present in less purified preparations used in other studies.

The effect of B-356 BPH dox on more highly chlorinated congeners with *ortho* substituents was not considered in this study. However, B-356 cells cannot efficiently degrade these congeners and no metabolites corresponding to C-3–C-4 oxygenation of BPH and 4CBP have ever been detected in cultures of strain B-356 or of recombinant strains carrying BPH dox genes from this strain (unpublished data). Since B-356 BPH dox can catalyze oxygenation in both C-2–C-3 and C-3–C-4 positions, why is B-356 unable to degrade those congeners? Delineation of the factors that impair the degradation of these congeners by B-356 cells will certainly be of great help in designing new enzymes or strains with enhanced degradative ability. Ongoing work in our laboratory aims to answer this question.

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