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Heterologous Expression and Characterization of the Purified Oxygenase Component of *Rhodococcus globerulus* P6 Biphenyl Dioxygenase and of Chimeras Derived from It

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In this work, we have purified the His-tagged oxygenase (ht-oxygenase) component of *Rhodococcus globerulus* P6 biphenyl dioxygenase. The α or β subunit of P6 oxygenase was exchanged with the corresponding subunit of *Pseudomonas* sp. strain LB400 or of *Comamonas testosteroni* B-356 to create new chimeras that were purified ht-proteins and designated ht-$\alpha_{P6}\beta_{P6}$, ht-$\alpha_{P6}\beta_{LB400}$, ht-$\alpha_{P6}\beta_{B\text{-}356}$, ht-$\alpha_{LB400}\beta_{P6}$, and ht-$\alpha_{B\text{-}356}\beta_{P6}$. ht-$\alpha_{P6}\beta_{P6}$, ht-$\alpha_{P6}\beta_{LB400}$, ht-$\alpha_{P6}\beta_{B\text{-}356}$ were not expressed active in recombinant *Escherichia coli* cells carrying P6 *bphA1* and *bphA2*, P6 *bphA1* and LB400 *bphE*, or P6 *bphA1* and B-356 *bphE* because the [2Fe-2S] Rieske cluster of P6 oxygenase α subunit was not assembled correctly in these clones. On the other hand ht-$\alpha_{LB400}\beta_{P6}$ and ht-$\alpha_{B\text{-}356}\beta_{P6}$ were produced active in *E. coli*. Furthermore, active purified ht-$\alpha_{P6}\beta_{P6}$, ht-$\alpha_{P6}\beta_{LB400}$, ht-$\alpha_{P6}\beta_{B\text{-}356}$, showing typical spectra for Rieske-type proteins, were obtained from *Pseudomonas putida* KT2440 carrying constructions derived from the new shuttle *E. coli-Pseudomonas* vector pEP31, designed to produce ht-proteins in *Pseudomonas*. Analysis of the substrate selectivity pattern of these purified chimeras toward selected chlorobiphenyls indicate that the catalytic capacity of hybrid enzymes comprised of an α and a β subunit recruited from distinct biphenyl dioxygenases is not determined specifically by either one of the two subunits.

Aryl hydroxylating dioxygenases catalyze the first enzymatic step for most bacterial catabolic pathways involved in the degradation of aromatic compounds (28). They catalyze a dihydroxylation reaction onto vicinal carbons of the aromatic ring. These enzymes can catalyze the hydroxylation of several substrate analogs, which makes them potentially useful for the development of biocatalytic processes to destroy persistent pollutants such as polychlorinated biphenyls (PCBs). Biphenyl dioxygenase (BPH dox) can catalyze the hydroxylation of several PCB congeners, but to extend its capacity to hydroxylate more persistent congeners, new engineered enzymes will need to be developed.

BPH dox has been purified from *Comamonas testosteroni* B-356 (18, 19) and from *Pseudomonas* sp. strain LB400 (8, 13, 14). It comprises three components (8, 13, 14, 18, 19): the terminal oxygenase, an iron-sulfur protein (ISP_{BPH}) made up of an α (M_r = 51,000) and a β (M_r = 22,000) subunit; a ferredoxin (FER_{BPH}; M_r = 12,000); and a ferredoxin reductase (RED_{BPH}; M_r = 43,000). The encoding genes for both strain B-356 and strain LB400 are *bphA* (ISP_{BPH} α subunit), *bphE* (ISP_{BPH} β subunit), *bphF* (FER_{BPH}), and *bphG* (RED_{BPH}) (11, 35). BPH dox hydroxylates vicinal *ortho-meta* carbons of one of the BPH rings to generate 2,3-dihydro-2,3-dihydroxybiphenyl. FER_{BPH} and RED_{BPH} are involved in electron transfer from NADH to ISP_{BPH}, which is directly involved in the catalytic oxygenation of the molecule (19). The enzyme is also found in the genus *Rhodococcus*. The four genes that code for *Rhodococcus globerulus* P6, *Rhodococcus* strain RHA1, and *Rhodococcus* sp. strain M5 BPH dox have been sequenced (2, 27, 37). They are designated *bphA1* (α subunit), *bphA2* (β subunit), *bphA3* (FER_{BPH}), and *bphA4* (RED_{BPH}) in strain P6 (2).

In previous work, rhodococcal BPH dox was poorly expressed in *Escherichia coli* (27, 29). However, the genes encoding rhodococcal BPH dox components were expressed in recombinant *Pseudomonas* (29) and *Rhodococcus* (27). The substrate selectivity patterns of *Pseudomonas putida* KT2442 carrying the genes coding for strain P6 BPH dox were analyzed by testing the catalytic capacity of resting cell suspension on 3,4′-dichlorobiphenyl and 2,2′-dichlorobiphenyl. Data suggested that the enzyme metabolizes preferentially the *meta*-substituted ring over the *para*-substituted and poorly transformed the double-*ortho*-substituted congener 2,2′-dichlorobiphenyl (29). Furthermore, P6 BPH dox was unable to catalyze the hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl used to determine the capacity of the enzyme to catalyze the *meta-para* hydroxylation of the biphenyl molecule (29). Coincidentally, these features are similar to those reported for strain B-356 BPH dox (17). Unlike these two strains, strain LB400 BPH dox shows a preference for 2,2′-dichlorobiphenyl, poorly transforms 3,3′-dichlorobiphenyl, and is able to catalyze the *meta-para* hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl (15, 17, 22, 30).

Although *Pseudomonas pseudoalcaligenes* KF707 and LB400 BPH doxes components show a high level of identity, their substrate selectivity patterns toward chlorobiphenyls are quite distinct. Only few amino acid residues located at the N-terminal portion of the terminal oxygenase α subunit were found to determine the substrate specificity of these enzymes (22, 30). Based on sequence comparison between enzymes of several strains, Mondello et al. (30) have identified four regions of the terminal oxygenase α subunit in which specific sequences were consistently associated with either broad (LB400-type) or narrow (KF707-type) PCB substrate specificity. Based on published data of DNA sequence and substrate specificity (2, 29),

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like B-356 ISP_{BPH}, P6 ISP_{BPH} correlates with the KF707-type strain.

Recently, His-tagged purified chimeras (ht-chimeras) were obtained by exchanging the α and β subunits of LB400 and B-356 BPH dox terminal oxygenases (17). The amino acid residues of the α subunit which were found to determine the substrate selectivity pattern of LB400 and KF707 terminal oxygenase (22, 30) were not found to influence the substrate selectivity pattern of the engineered chimeras (17). Furthermore, the substrate selectivity pattern of ISP_{BPH} chimeras comprised of B-356 α subunit with LB400 β subunit ($\alpha_{B\text{-}356}\beta_{LB400}$) was very similar to that of LB400 BPH dox, which suggests an involvement of the β subunit on the reactivity pattern of the terminal oxygenase toward PCBs. To extend this study, in this work we purified recombinant P6 ht-ISP_{BPH} expressed from *E. coli* and *Pseudomonas* and compared its substrate selectivity pattern toward chlorobiphenyls with that of engineered purified ht-ISP_{BPH} chimeras obtained by exchanging P6 α or β subunit with corresponding peptide of LB400 or B-356 ISP_{BPH}.

MATERIALS AND METHODS

Bacterial strains, culture media, and general protocols. The bacterial strains used in this study were *E. coli* M15[pREP4] and SG13009[pREP4] (both from Qiagen Inc., Chatsworth, Calif.), *E. coli* DH11S (25), *P. putida* KT2440 (4), *C. testosteroni* B-356 (1), *Pseudomonas* sp. strain LB400 (6) (also referred as *Burkholderia* sp. strain LB400 or *Pseudomonas cepacia* LB400 [22]), and *R. globerulus* P6 (2, 3, 12). The media used were Luria-Bertani (LB) broth or solidified with agar (33). Plasmid DNA from *E. coli*, restriction endonuclease reactions, ligations, agarose gel electrophoresis, and transformation of *E. coli* cells were done according to protocols described by Sambrook et al. (33). The transformation of *P. putida* KT2440 was done according to the protocol described by Sambrook et al. (33) for the transformation of *E. coli* except that Ca^{2+} was replaced by Rb^{2+} and the cells were incubated for 2 h at 28°C (instead of 45 min at 37°C) before plating. The transformation rates were approximately 10^2 transformants per μg of DNA added to the ligation reaction medium. PCR was performed with *Pwo* DNA polymerase according to the method recommended by Boehringer Mannheim.

Plasmids. Several vector and plasmids were used in this study. Plasmid pQE31, designed to create His-tagged fused proteins, and pQE51, which is identical to pQE31 except for the lack of the His_6-tagged fused gene, were both from Qiagen. Plasmid pYH31 (17, 20) is also designed to create His-tagged fused proteins but is compatible with ColE1-based plasmids. Plasmid pEP31, obtained during this work (Fig. 1A), is a shuttle vector designed to produce fusion ht-protein in *Pseudomonas* and in *E. coli*. It confers resistance to both tetracycline and ampicillin. For construction of pEP31, pUCP26 (10, 34), which was graciously provided by H. P. Schweizer (Department of Microbiology, Colorado State University, Fort Collins), was treated with *Pvu*I. The 3.2-kb fragment carrying the *rep* region (*ori* [origin of replication] of *Pseudomonas aeruginosa* PAO, which allows the plasmid to replicate in *Pseudomonas*) and tetracycline resistance (pALTER-1) was made blunt ended. Similarly, the 2.5-kb *Pvu*II/*Nde*I fragment of pQE31 carrying the *ori* for replication, amplicillin resistance, and the promoter-operator region plus the His-tagged fusion gene was made blunt ended and then ligated with the 3.2-kb DNA fragment from pUCP26. Likewise, for construction of pEP51, the 2.5-kb *Pvu*II/*Nde*I fragment of pQE51 was ligated to the 3.2-kb *Pvu*I fragment of pUCP26. For construction of the 7.8-kb plasmid pQE51[LB400-*bphFGBC*], a 3.5-kb *Sma*I/*Nde*I fragment from pQE51 was ligated to a 4.3-kb *Bbr*PI/*Nde*I fragment which carries LB400 *bphFGBC* from pAH17 (graciously provided by V. De Lorenzo, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Crentíficas Madrid, Spain). Similarly, for construction of the 8.3-kb plasmid pYH31[LB400-*bphFGBC*], the 4.3-kb *Bbr*PI/*Nde*I fragment from pAH17 was ligated blunt end to the 4-kb plasmid pYH31 digested with *Kpn*I at its unique *Kpn*I site.

Plasmid constructions used to express P6 ht-ISP_{BPH} and ht-ISP_{BPH} chimeras. In this work we have produced purified preparations of P6 ht-ISP_{BPH} (ht-$\alpha_{P6}\beta_{P6}$) and of all four hybrid combinations between the P6 terminal oxygenase α and β subunits and the corresponding subunits of LB400 and B-356 terminal oxygenases, thus producing ht-$\alpha_{P6}\beta_{LB400}$, ht-$\alpha_{LB400}\beta_{P6}$, ht-$\alpha_{P6}\beta_{B\text{-}356}$, and ht-$\alpha_{B\text{-}356}\beta_{P6}$.

The constructs used to express these enzymes are listed in Table 1. The plasmids and strategies used to obtain these constructs are explained below.

Plasmids pYH31[B-356-*bphA*] and pYH31[LB400-*bphA*] were obtained by subcloning the corresponding *Bam*HI/*Kpn*I fragment of pQE31[B-356-*bphA*] and pQE31[LB400-*bphA*] described previously (17). The oligonucleotides used to amplify *bphA1* from strain P6 were based on known nucleotide sequences (2). They were oligonucleotides I ([*Bam*HI] 5′-CGGGATCCGATGACCAATCAATTGG-3′) and II ([*Kpn*I] 5′-GGGGTACCCCTCAGGCCGAGACATC-3′). P6 *bphA1* was cloned as a *Bam*HI/*Kpn*I fragment into corresponding sites of pQE31, pYH31, pEP31, and pEP51 to create pQE31[P6-*bphA1*], pYH31[P6-*bphA1*], pEP31[P6-*bphA1*], and pEP51[P6-*bphA1*].

pQE51[LB400-*bphE*] carrying LB400 *bphE* as a *Kpn*I/*Kpn*I fragment has been described previously (17). B-356 *bphE* was amplified with oligonucleotides III ([*Kpn*I] 5′CCGGGTACCCATGATATCCACTCCCT3′) and IV ([*Kpn*I] 5′GGGGTACCCCTCAAAAGAACACGCT3′), and the 0.6-kb fragment was cloned into the *Kpn*I site of pQE51 to construct pQE51[B-356-*bphE*]. Oligonucleotides V ([*Kpn*I] 5′CGAGGTACCTATGACGGACACCGTCG 3′) and VI ([*Kpn*I] 5′CGACGGTACCCTAGAAGAAGAAGCT 3′) were used to amplify strain P6 *bphA2*. P6 *bphA2* was cloned into *Kpn*I-digested pQE51 to construct pQE51[P6-*bphA2*].

P6 *bphA2* was subcloned from pQE51[P6-*bphA2*] into the unique *Kpn*I site of pQE31[P6-*bphA1*] to create pQE31[P6-*bphA1*/*bphA2*] (Fig. 1B). pQE31[P6-*bphA1*/B-356-*bphE*] was obtained by cloning the *Kpn*I/*Kpn*I B-356 *bphE* fragment into *Kpn*I-treated pQE31[P6-*bphA1*]. pQE31[P6-*bphA1*/LB400-*bphE*] was obtained by cloning the 0.6-kb *Kpn*I/*Kpn*I fragment of pQE51[LB400-*bphE*] (17) into *Kpn*I-digested pQE31[P6-*bphA1*].

For the construction of pEP31[P6-*bphA1*/*bphA2*], P6 *bphA2* was cloned from pQE51[P6-*bphA2*] into the *Kpn*I site of pEP31[P6-*bphA1*]. Another construct was made such that both P6 *bphA1* and P6 *bphA2* were expressed from pEP31 but each was placed immediately downstream of a pQE promoter-operator region (indicated as "p-o" in plasmid designations). pEP31[P6-*bphA1*/p-o/*bphA2*] (Fig. 1B) was constructed in the following way. P6 *bphA2* was amplified by using oligonucleotides VII ([*Bam*HI] 5′CGGGATCCCATGACGGACACCGTCG 3′) with oligonucleotide VI. The *Bam*HI/*Kpn*I fragment was cloned into pQE51. The resulting plasmid was digested with *Xho*I/*Kpn*I to release a 0.6-kb fragment carrying the pQE51 promoter-operator region plus P6 *bphA2*, which was made blunt ended and ligated to the blunt-ended *Kpn*I-digested pEP31[P6-*bphA1*]. Similar strategies were used to construct pEP51[P6-*bphA1*/*bphA2*] and pEP51[P6-*bphA1*/p-o/*bphA2*].

For the construction of pEP31[P6-*bphA1*/B-356-*bphE*] and pEP31[P6-*bphA1*/LB400-*bphE*], the 1.9-kb *Bam*HI/*Hin*dIII fragment of pQE31[P6-*bphA1*/B-356-*bphE*] or pQE31[P6-*bphA1*/LB400-*bphE*] was cloned into appropriately treated pEP31. pEP51[P6-*bphA1*/B-356-*bphE*] and pEP51[P6-*bphA1*/LB400-*bphE*] were constructed in a similar manner.

The various constructions used in this work were designed to produce ht-purified ISP_{BPH} component carrying the His tag on the α subunit. All constructions were such that the His tag added the same 13 amino acids (MRGSHHH HHHTDP) to the protein at the N-terminal portion.

Expression and purification of P6 ISP_{BPH} and its chimeras in *E. coli* and *P. putida* KT2440. The ht-enzymes were expressed in *E. coli* cells according to protocols described previously (18, 19). When the proteins were expressed from pEP31 in *E. coli* cells, the antibiotic concentrations used were 200 μg of ampicillin per ml and 10 μg of tetracycline per ml (tet 10).

Several parameters, such as culture medium, antibiotic concentration, incubation time, temperature, size of the inoculum, concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) as inducer, and cell density at the time of induction, were varied to optimize the expression of ISP_{BPH} from pEP31[*bphA1*/p-o/*bphA2*], pEP31[P6-*bphA1*/LB400-*bphE*], and pEP31[P6-*bphA1*/B-356-*bphE*] in *P. putida* KT2440. The following optimized protocol was retained for this work. Cells from a frozen culture were grown overnight with shaking at 29°C in LB broth containing 20 μg of tetracycline per ml (tet 20). This culture was used to inoculate two to four 1-liter Erlenmeyer flasks each containing 600 ml of LB broth plus tet 20. The cultures were grown at 250 rpm at 29°C until the optical density reached 0.6 at 600 nm. Then 0.5 mM IPTG was added, and the cultures were incubated in the same conditions for 6 h. Cells were then harvested, washed with piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; 50 mM, pH 7.4) buffer containing 5% (wt/vol) ethanol, 10% (wt/vol) glycerol, and 300 mM NaCl, and suspended in 5 volumes of the same buffer. This suspension was sonicated on ice until maximum cell breakage. Further purification steps were identical to those described previously for the purification of ht-protein from *E. coli* cells (19). Under these conditions, approximately 2 mg of purified enzyme was obtained per liter of IPTG-induced culture.

Previously described procedures (19) were used to obtain purified preparations of B-356 ht-FER_{BPH} and B-356 ht-RED_{BPH} from recombinant *E. coli* cells.

Protein characterization. Sodium dodecyl sulfate (SDS)-polyacrylamide gels were developed according to method of Laemmli (24). Proteins were stained with Coomassie brillant blue (33). Protein concentrations were estimated by the method of Lowry (26), using bovine serum albumin as a standard. The concentrations of all ht-ISP_{BPH} preparations were also determined spectrophotometrically, using the ε_{455} value of 8,300 M^{-1} cm^{-1} established for B-356 ht-ISP_{BPH} (18). The preparations of B-356 ht-FER_{BPH} and B-356 ht-RED_{RED} used in this work were also quantified spectrophotometrically as previously described (19). The M_r of the native proteins was determined by high-pressure liquid chromatography (HPLC) as described previously (18).

Monitoring of enzyme activities and identification of metabolites. Enzyme assays for BPH dox were performed as described previously (17). The reaction was initiated by adding 50 nmol of biphenyl or 25 nmol of one of the following chlorobiphenyls: 2,2′-, 3,3′-, or 2,5-dichlorobiphenyl or 2,2′,5,5′-tetrachlorobiphenyl (all from ULTRAScientific, Kingstown, R.I.) (added in 2 μl of acetone). Based on previous data showing that the origin of FER_{BPH} did not influence the

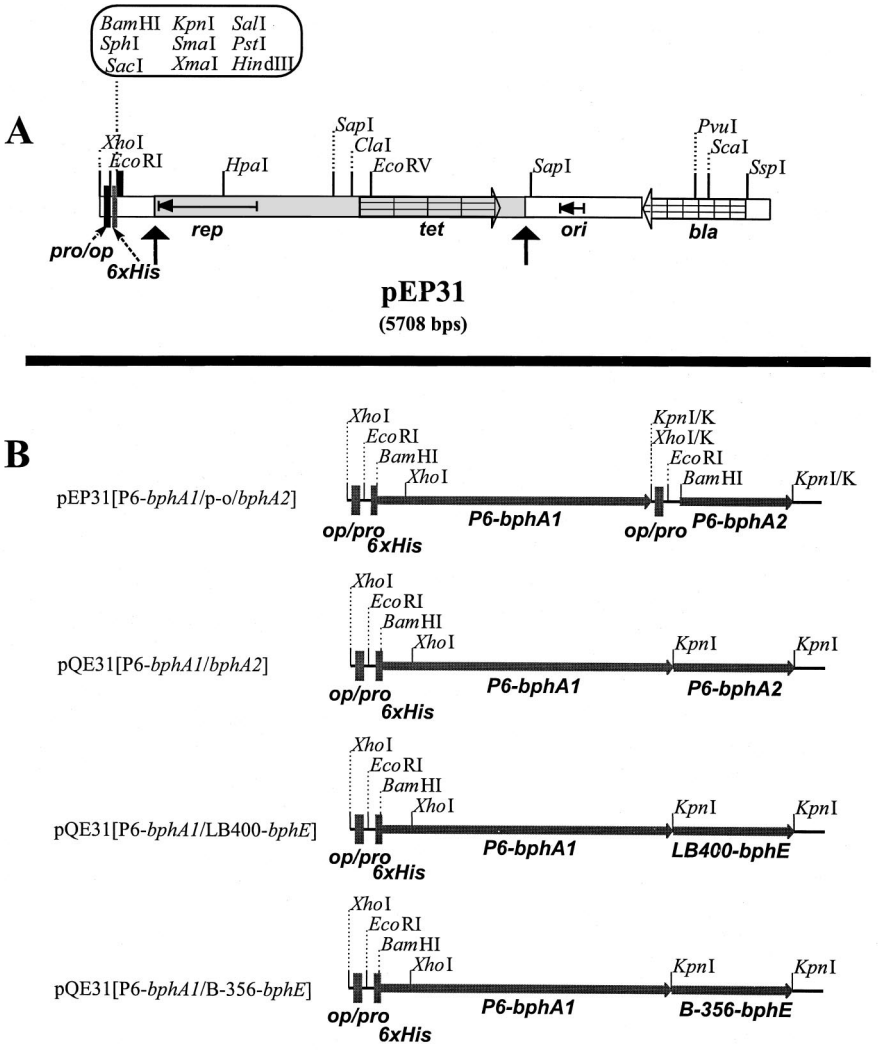

FIG. 1. (A) Linear map of the *E. coli-Pseudomonas* shuttle vector pEP31 designed to produce ht-proteins. Only selected restriction sites are shown. The grey and white areas derive from pUCP26 and pQE31, respectively. *rep*, origin of replication in *Pseudomonas*; *tet*, tetracycline resistance; *bla*, ampicillin resistance; *ori*, ColE1 origin of replication; *pro*/*op*, pQE31 promoter-operator; *6xHis*, the six-histidine-tagged fusion gene. The map of pEP51 is identical to that of pEP31 except for the absence of the six-histidine-tagged fusion gene. (B) Constructs used to produce P6 ht-ISP_{BPH} and chimeras derived from it. All constructs except pEP31[P6-*bphA1*/p-o/*bphA2*] were made in pQE31, and the cloned DNA fragment was transferred to pEP31 when needed. Details of the strategies used to construct these plasmids are given in Materials and Methods. Only the restriction sites important for the cloning strategies are shown. *Kpn*I/K and *Xho*I/K indicate sites that were made blunt ended with the Klenow fragment of DNA polymerase I, resulting in their loss in the final construct.

BPH dox substrate reactivity pattern (17), the reconstituted BPH doxes comprised either P6 ht-ISP_{BPH} or one of the ht-ISP_{BPH} chimeras described above ($\alpha_{P6}\beta_{LB400}$, $\alpha_{LB400}\beta_{P6}$, $\alpha_{P6}\beta_{B\text{-}356}$, or $\alpha_{B\text{-}356}\beta_{P6}$) plus B-356 ht-$FER_{BPH}$ and B-356 ht-RED_{BPH}. The catalytic oxygenation was evaluated by monitoring substrate depletion by HPLC analysis 5 or 10 min after initiation of the reaction, as described previously (17). When 2,2′,5,5′-tetrachlorobiphenyl was the substrate, the catalytic oxygenation was evaluated by monitoring the metabolite production by HPLC using the conditions described previously (5).

A *trans*-complementation assay was also used to verify whether the P6 ISP_{BPH} component was produced active in *E. coli*. *E. coli* DH11S cells carrying pYH31[LB400-*bphFGBC*] were transformed with pEP31[P6-*bphA1*/p-o/*bphA2*] or pQE31[P6-*bphA1*/*bphA2*]. The recombinant cells were inoculated on a nitrocellulose membrane placed onto the surface of an LB agar plate. The culture was incubated overnight at 37°C, and then the nitrocellulose membrane was transferred onto fresh LB agar plates containing 0.5 mM IPTG. The culture was incubated for 1 to 2 h and then sprayed with a solution of biphenyl in ether (5%, wt/vol). Positive colonies were recognized by the production of the yellow *meta*-cleavage metabolite produced from biphenyl. A positive control of *E. coli* DH11S cells carrying pYH31[LB400 *bphFGBC*] along with pQE31[B-356-*bphAE*] was included in the test.

RESULTS

Stability of pEP31 and pEP51 in *E. coli* and *Pseudomonas*. Plasmids pEP31 and pEP51 were maintained for over 50 generations in *E. coli* DH11S grown on tet 10 as well as in *P. putida* KT2440 grown on tet 20. As shown by data in Fig. 2, the cloned DNA fragment carrying P6 *bphA1*/p-o/*bphA2* was stably maintained for over 50 generations both in *E. coli* and in *Pseudomonas* since both subunits of P6-ht-ISP_{BPH} were produced in those recombinants. Same results were obtained with P6-*bphA1*/LB400-*bphE* or P6-*bphA1*/B-356-*bphE*.

Expression in *E. coli* and in *P. putida* KT2440 and purification of P6 ht-ISP_{BPH}. MacKay et al. (29) observed that P6 biphenyl dioxygenase's ISP_{BPH} component was not detected in induced *E. coli* DH5α carrying recombinant P6 *bphA1* and *bphA2* cloned downstream of the *lac* or T7 promoter. They suggested that the lack of P6 ISP_{BPH} component in these cells

TABLE 1. Plasmid constructions used to express and produce purified P6 ht-ISP_{BPH} and its chimeras

ISP_{BPH}	Plasmid construction(s)[a]	Reference(s)
ht-$\alpha_{P6}\beta_{P6}$	pQE31[P6-*bphA1*/*bphA2*]	This work
	pYH31[P6-*bphA1*] and pQE51[P6-*phA2*]	This work
	pEP31[P6-*bphA1*/p-o/*bphA2*]	This work
	pEP51[P6-*bphA1*/p-o/*bphA2*]	This work
ht-$\alpha_{P6}\beta_{LB400}$	pQE31[P6-*bphA1*/LB400-*bphE*]	This work
	pEP31[P6-*bphA1*/LB400-*bphE*]	This work
ht-$\alpha_{P6}\beta_{B-356}$	pQE31[P6-*bphA1*/B-356-*bphE*]	This work
	pEP31[P6-*bphA1*/B-356-*bphE*]	This work
ht-$\alpha_{LB-400}\beta_{P6}$	pYH31[LB400-*bphA*] and pQE51[P6-*bphA2*]	17, this work
ht-$\alpha_{B-356}\beta_{P6}$	pYH31[B-356-*bphA*] and pQE51[P6-*bphA2*]	This work

[a] See text and Fig. 1 for details.

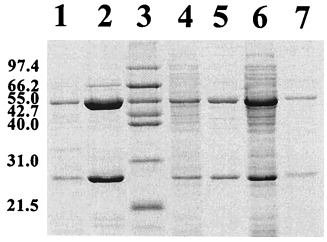

FIG. 2. SDS-PAGE of P6 ht-ISP_{BPH} or P6 ISP_{BPH} produced in *E. coli* or *P. putida* KT2440 carrying pEP31[P6-*bphA1*/p-o/*bphA2*] or pEP51[P6-*bphA1*/p-o/*bphA2*]. Lane 1, crude extract (1.9 μg of protein) of *E. coli* DH11S carrying pYH31[LB400-*bphFGBC*] plus pEP31[P6-*bphA1*/p-o/*bphA2*], where protein induction was achieved as described in Materials and Methods except that the inoculum was a culture grown for 50 generations in LB broth (tet 10); lane 2, purified preparation (1.1 μg of protein) of ht-$\alpha_{P6}\beta_{P6}$ obtained from the extract in lane 1; lane 3, M_r marker; lane 4, crude extract (2.4 μg of protein) of *P. putida* KT2440 carrying pEP31[P6-*bphA1*/p-o/*bphA2*] where the inoculum was grown for 50 generations in LB broth (tet 20); lane 5, purified preparation (0.1 μg of protein) of ht-$\alpha_{P6}\beta_{P6}$ obtained from the extract shown in lane 4; lane 6, crude extract (8 μg of protein) of *P. putida* KT2440 carrying pEP51[P6-*bphA1*/p-o/*bphA2*] grown for 50 generations as in lane 4; lane 7, crude extract (1 μg of protein) of *E. coli* DH11S carrying pYH31[LB400-*bphFGBC*] plus pEP51[P6-*bphA1*/p-o/*bphA2*] grown for 50 generations as in lane 1. Sizes are indicated on the left in kilodaltons.

was due to either inefficient translation of *bphA1A2* or rapid degradation of the gene product (29).

As shown in Fig. 2 (lanes 1 and 2), under our experimental conditions, the P6 ISP_{BPH} α and β subunits were produced in equivalent amounts in *E. coli*. However, the purified protein was inactive. HPLC analysis showed that like other ISP_{BPH}s the native conformation of P6 ISP_{BPH} was predominantly $\alpha_3\beta_3$ (not shown). However, the UV-visible spectrum was not typical of [2Fe-2S] Rieske-type proteins, showing a broad peak at about 455 nm and lacking the peak around 320 nm (Fig. 3). All attempts to produce active recombinant enzyme either by changing the purification conditions or by changing the culture conditions failed. These attempts included variation of the temperature and of the inoculum size at the time of induction, variation of the concentration of IPTG and the addition of ions such as Fe^{2+}, variation of the type of buffer (phosphate, PIPES, or morpholineethanesulfonic acid) used to break the cells or to elute the enzyme from the Ni^{+}-nitrilotriacetic acid column, and purification under anaerobic conditions.

The *trans*-complementation assay described in Materials and Methods was used to verify whether the enzyme was synthesized inactive in *E. coli* cells or if it was inactivated after cell breakage. IPTG-induced *E. coli* DH11S colonies carrying pYH31[LB400-*bphFGBC*] plus pQE31[B-356-*bphAE*] immediately turned yellow after spraying with biphenyl, showing the presence of active BPH dox, whereas cells of *E. coli* DH11S carrying pYH31[LB400-*bphFGBC*] plus pEP31[P6-*bphA1*/p-o/*bphA2*] did not react with biphenyl, showing the lack of active BPH dox (not shown). The fact that *E. coli* DH11S carrying pYH31[LB400-*bphFGBC*] plus pEP51[P6-*bphA1*/p-o/*bphA2*] did not produce any yellow color from biphenyl in this assay (not shown) but expressed proteins corresponding to *bphA1* and *bphA2* gene products (Fig. 2, lane 7) shows that the His tag is not the main reason for the inactivity of P6 ISP_{BPH} in *E. coli*.

Equivalent amounts of α and β subunits were produced in *E. coli* carrying pEP31[P6-*bphA1*/*bphA2*] or pQE31[P6-*bphA1*/*bphA2*] (not shown). However, P6 *bphA2* was poorly expressed from pEP31[P6-*bphA1*/*bphA2*] in *P. putida* KT2440. Nevertheless, Fig. 2 (lane 5) shows that purified P6 ht-ISP_{BPH} was obtained and both genes were expressed in equivalent ratios (Fig. 2, lane 4) in *P. putida* KT2440 carrying pEP31[P6-*bphA1*p-o/*bphA2*], where each gene was controlled by a promoter-operator.

All purified P6 ht-ISP_{BPH} preparations obtained from *P. putida* KT2440 carrying pEP31[P6-*bphA1*/p-o/*bphA2*] were active. These preparations showed typical spectra for [2Fe-2S] Rieske-type proteins with maximal absorption peaks at 320 and 455 nm and a shoulder at around 575 nm (Fig. 3). Therefore, data confirm that recombinant *R. globerulus* P6 ISP_{BPH} is not active in *E. coli* cells (29) but is active when expressed in *Pseudomonas*. Data also show clearly that both subunits of P6 ISP_{BPH} are expressed in *E. coli* but the reconstituted enzyme is inactive. Spectral data show that the Rieske cluster on the α subunit is incorrectly assembled (Fig. 3).

Expression in *E. coli* and purification of ht-$\alpha_{B-356}\beta_{P6}$ and ht-$\alpha_{LB400}\beta_{P6}$. SDS-polyacrylamide gel electrophoresis (PAGE) of purified preparations of ht-$\alpha_{LB-400}\beta_{P6}$ and ht-$\alpha_{B-356}\beta_{P6}$ showed two single peptide bands of M_r corresponding to that of ht-α_{B-356} or ht-α_{LB400} with β_{P6} (Fig. 4, lanes 7 and 8; compared to lane 1 to 3). Both enzymes showed spectral features typical of Rieske-type proteins. When biphenyl was the substrate, ht-$\alpha_{LB-400}\beta_{P6}$ was very poorly active (only traces of the dihydrodiol metabolites were produced from biphenyl), but as shown below, the enzyme was able to transform various chlorobiphenyls efficiently. In this case, however, two of the four purified preparations that we obtained were very poorly active

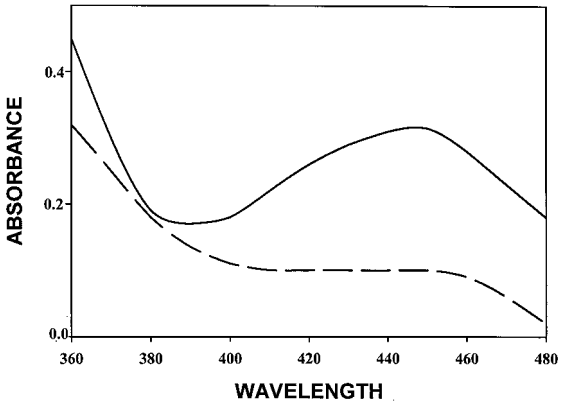

FIG. 3. Absorption spectra of ht-P6 ISP_{BPH} (40 μM) obtained from recombinant *P. putida* KT2440 cells carrying pEP31[P6-*bphA1*/p-o/*bphA2*] (solid line) and obtained from recombinant *E. coli* DH11S cells carrying pEP31[P6-*bphA1*/p-o/*bphA2*] (dashed line).

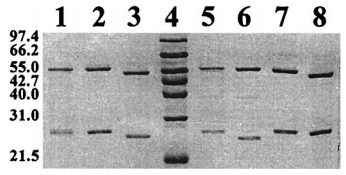

FIG. 4. SDS-PAGE of purified active preparations of ISP_{BPH} chimeras. Lane 1, P6 ht-ISP_{BPH}, 50 pmol; lane 2, LB400 ISP_{BPH}, 80 pmol; lane 3, B-356 ISP_{BPH}, 50 pmol; lane 4, M_r marker; lane 5, ht-$\alpha_{P6}\beta_{LB400}$, 70 pmol; lane 6, ht-$\alpha_{P6}\beta_{B\text{-}356}$, 170 pmol; lane 7, ht-$\alpha_{LB400}\beta_{P6}$, 60 pmol; lane 8, ht-$\alpha_{B\text{-}356}\beta_{P6}$, 50 pmol. P6 ht-$ISP_{BPH}$, ht-$\alpha_{P6}\beta_{LB400}$, and ht-$\alpha_{P6}\beta_{B\text{-}356}$ were produced from *P. putida* KT2440; the other chimeras were produced from *E. coli* M15. Sizes are indicated on the left in kilodaltons.

toward all congeners tested, suggesting that the enzyme becomes easily inactivated during purification. On the other hand, ht-$\alpha_{B\text{-}356}\beta_{P6}$ was stable and catalyzed the hydroxylation of biphenyl at a rate similar to that of ht-$\alpha_{P6}\beta_{P6}$.

Expression in *E. coli* and in *P. putida* and purification of ht-$\alpha_{P6}\beta_{LB400}$ and ht-$\alpha_{P6}\beta_{B\text{-}356}$. Purified preparations of ht-$\alpha_{P6}\beta_{B\text{-}356}$ and ht-$\alpha_{P6}\beta_{LB400}$ chimeras containing equivalent amounts of each subunit were obtained from *E. coli* carrying pQE31[P6-*bphA1*/B-356-*bphE*] and pQE31[P6-*bphA1*/LB400-*bphE*], respectively. However, as for ht-$\alpha_{P6}\beta_{P6}$ (see above), the purified enzymes were not active and the spectral features of the purified enzyme preparations were not typical of Rieske-type proteins (not shown).

On the other hand, purified active ht-$\alpha_{P6}\beta_{B\text{-}356}$ and ht-$\alpha_{P6}\beta_{LB400}$ chimeras were obtained when the enzymes were expressed in *P. putida* KT2440 from pEP31[P6-*bphA1*/B-356-*bphE*] and pEP31[P6-*bphA1*/LB400-*bphE*], respectively. Both ISP_{BPH} chimeras were active when biphenyl was used as the substrate (Table 2). Spectral features were typical of [2Fe-2S] Rieske-type proteins. SDS-PAGE of purified preparations showed two peptide bands of M_r corresponding to that of ht-α_{P6} with either β_{LB400} or $\beta_{B\text{-}356}$ (Fig. 4, lanes 5 and 6). It is noteworthy that LB400 and B-356 α and β subunits migrate differently although their theoretical M_rs are identical (lanes 2 and 3).

Activities of purified ht-P6 ISP_{BPH} and chimeras derived from it toward selected chlorobiphenyls. In a previous investigation (17), purified ht-LB400 BPH dox metabolized 2,2′-dichlorobiphenyl efficiently but 3,3′-dichlorobiphenyl poorly, whereas ht-B-356 BPH dox metabolized 3,3′-dichlorobiphenyl but not 2,2′-dichlorobiphenyl efficiently. Furthermore, unlike ht-LB400 BPH dox, ht-B-356 BPH dox was unable to catalyze the *meta-para* hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl. In another investigation (29), resting cell suspensions of recombinant *P. putida* KT2442 carrying P6 *bphA1A2A3A4* did not metabolize 2,2′-dichlorobiphenyl or 2,2′,5,5′-tetrachlorobiphenyl.

In this work the catalytic activity of purified ht-P6 ISP_{BPH} and of hybrids obtained by exchanging the α and the β subunits of strain P6, LB400, or B-356 ISP_{BPH} toward chlorobiphenyls was determined (Table 2). Data confirm that P6 ISP_{BPH} is unable to hydroxylate 2,2′,5,5′-tetrachlorobiphenyl. However, unlike the resting cell suspension of *P. putida* KT2442 carrying P6 *bphA1A2A3A4* (29), purified P6 ht-ISP_{BPH} metabolized 2,2′-dichlorobiphenyl. 3,3′-Dichlorobiphenyl was metabolized about twice as fast as 2,2′-dichlorobiphenyl by this enzyme (Table 2). This is a feature that distinguishes P6 BPH dox from both LB400 and B-356 BPH doxes, where the rates of metabolism for these two congeners differed markedly (17).

All the preparations of ht-$\alpha_{LB400}\beta_{P6}$ were either inactive or very poorly active when biphenyl was the substrate. However, these preparations could transform 2,2′-dichlorobiphenyl and 2,2′,5,5′-tetrachlorobiphenyl. Table 2 reports the value obtained with the most active preparation. Data show that like LB400 BPH dox, ht-$\alpha_{LB400}\beta_{P6}$ shows a marked preference for 2,2′-dichlorobiphenyl and catalyzes the hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl. However, unlike LB400 BPH dox (13), ht-$\alpha_{LB400}\beta_{P6}$ was unable to oxygenate naphthalene, as demonstrated by HPLC analysis of the reaction product (data not shown).

On the other hand, unlike both parent enzymes, ht-$\alpha_{P6}\beta_{LB400}$ is unable to catalyze the hydroxylation of 2,2′- or 3,3′-dichlorobiphenyl. However, this chimera showed a weak activity on 2,2′,5,5′-tetrachlorobiphenyl. The metabolic capacity of this chimera contrasts with that of ht-$\alpha_{B\text{-}356}\beta_{LB400}$ (17), which shows features very similar to those of LB400 ISP_{BPH}.

Unlike B-356 BPH dox (17), $\alpha_{B\text{-}3566}\beta_{P6}$ metabolizes 2,2′-dichlorobiphenyl rapidly. It is also noteworthy that although the rate of transformation is very low, unlike both parents, $\alpha_{P6}\beta_{B\text{-}356}$ produces small amounts of 3,4-dihydro-3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl from 2,2′,5,5′-tetrachlorobiphenyl. All four chimeras analyzed in this investigation were able to metabolize 2,5-dichlorobiphenyl, in contrast to the data obtained with $\alpha_{LB400}\beta_{B\text{-}356}$ (17). Altogether, these data indicate that the catalytic capacity of hybrid enzymes comprised of an α and a β subunit recruited from distinct BPH dox is not determined by either one of the two subunits. The catalytic capacity is rather unpredictable and depends on the association between the two subunits.

DISCUSSION

In a previous work (18), we had reported that purified monomeric B-356 ht-ISP_{BPH} α subunit produced alone in recombinant *E. coli* cells was able to assemble an intact [2Fe-2S]

TABLE 2. Amount of substrate depleted 5 min after initiation of reaction with various enzymes

Substrate	nmol of substrate depleted or metabolite produced/1.2 nmol of enzyme[a]				
	ht-$\alpha_{P6}\beta_{P6}$ ISP_{BPH}	ht-$\alpha_{P6}\beta_{LB400}$ ISP_{BPH}	ht-$\alpha_{P6}\beta_{B\text{-}356}$ ISP_{BPH}	ht-$\alpha_{LB\text{-}400}\beta_{P6}$ ISP_{BPH}	ht-$\alpha_{B\text{-}356}\beta_{P6}$ ISP_{BPH}
2,2′-Dichlorobiphenyl	8.3	0	9	22.5	10.8
3,3′-Dichlorobiphenyl	14.5	0	2.5	0	6.3
2,5-Dichlorobiphenyl	23.3	6.8	21	15.3	21.8
2,2′,5,5′-Tetrachlorobiphenyl	0	1	1	9	0
Biphenyl	34	17	5	Trace[b]	43

[a] BPH dox reactions were carried out as described in Materials and Methods. The reaction was initiated by adding 25 nmol of substrate except for biphenyl (50 nmol). Values refer to amounts of substrate consumed 5 min after initiation of the reaction when 1.2 nmol of enzyme was present except in the case of 2,2′,5,5′-tetrachlorobiphenyl, values for which refer to nanomoles of 3,4-dihydro-3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl produced per 1.2 nmol of enzyme 10 min after initiation of the reaction.

[b] Trace of 2,3-dihydro-2,3-dihydroxybiphenyl detected by HPLC analysis in the reaction medium.

Rieske cluster, showing that the β subunit is not involved in folding the Rieske center. However, the purified B-356 ht-ISP_{BPH} α subunit could not associate in vitro with the purified β subunit to generate an active $\alpha_3\beta_3$ protein. Conversely, the α subunit combined in cell extract with purified exogenous β subunit to generate an active complex, suggesting the presence in *E. coli* of a cell constituent that interacts with the α subunit to maintain its correct folding in the absence of the β subunit (18). In the present work, we show that rhodococcal ISP_{BPH} subunits produced in *E. coli* cells are assembled into the correct $\alpha_3\beta_3$ configuration (this was also true for the $\alpha_{P6}\beta_{LB400}$ and $\alpha_{P6}\beta_{B\text{-}356}$ chimeras). However, the [2Fe-2S] Rieske cluster of the α_{P6} subunit is incorrectly assembled in *E. coli* but correctly assembled in *Pseudomonas*. Altogether these observations show that the subunit association is independent of the Rieske center assembly and suggest that the maturation of the ISP_{BPH} [2Fe-2S] cluster, like subunit association, may require the involvement of cell constituents such as chaperones.

ht-P6 ISP_{BPH} is produced active in *Pseudomonas* cells, showing the importance of choosing the proper organism to express heterologous proteins. Since aryl hydroxylating dioxygenases are found mostly in gram-negative aerobes such as *Pseudomonas*, *Alcaligenes*, and *Comamonas* and in the gram-positive rhodococci, pEP31 and pEP51 constructed during this work provide potentially useful tools to further investigate the biochemical features of these enzymes.

In a recent investigation with B-356 and LB400 ISP_{BPH}, the catalytic features of $\alpha_{B\text{-}356}\beta_{LB400}$ toward chlorobiphenyls were found to be very similar to those of LB400 BPH dox. On the other hand, with one exception (16), all recent reports (7, 31, 32, 36) indicated that the substrate specificity of the aryl hydroxylating dioxygenase is determined by the terminal oxygenase's α subunit; the β subunit was not found to contribute to this function. The fact that these investigations were done with whole-cell suspensions of recombinant *E. coli* clones carrying the four genes required for dioxygenase activity on plasmids preclude direct comparison with data obtained with *in vitro*-reconstituted purified enzyme. Nevertheless, it was imperative to verify if the behavior of the chimera obtained by exchanging the α and β subunits of LB400 and B-356 ISP_{BPH} was an exception.

Unlike the data obtained with $\alpha_{B\text{-}356}\beta_{LB400}$ (17), none of the four hybrids described in this work showed features identical to those that characterize the parent which provided the β subunit. Interestingly, the catalytic activity toward chlorobiphenyls of $\alpha_{LB400}\beta_{P6}$ was similar to that of LB400 BPH dox. Based on this result, it would be tempting to conclude that the substrate specificity pattern is determined by the α subunit alone and that $\alpha_{B\text{-}356}\beta_{LB400}$ is an exception. However, it is noteworthy that $\alpha_{LB400}\beta_{P6}$ was practically inactive on biphenyl. Furthermore, the catalytic features of the three other chimeras studied in this investigation differed significantly from those of the parent which provided the α subunit. Thus, although 2,2′-dichlorobiphenyl is a very poor substrate for B-356 ISP_{BPH}, $\alpha_{B\text{-}356}\beta_{P6}$ oxygenate this congener faster than 3,3′-dichlorobiphenyl. Furthermore, unlike both parents, $\alpha_{P6}\beta_{LB400}$ was unable to oxygenate 2,2′-dichlorobiphenyl but showed a slight activity toward 2,2′,5,5′-tetrachlorobiphenyl, which was not attacked by P6 ISP_{BPH}. Together these data support the previous conclusion that each new chimera obtained by exchanging the α or β subunit of parent dioxygenases acquires its own new catalytic features that are not determined exclusively by one or the other subunit (16, 17).

Two structural features are essential to obtain an active enzyme. First, the Rieske cluster must be correctly assembled. Second, we have previously reported that ISP_{BPH} must associate into $\alpha_3\beta_3$ heterodimer to be active (18). Based on the present data, it is likely that the three-dimensional structure of the enzyme's catalytic region, as determined by the association between the α and β subunits, will determine the range of substrates that the enzyme can oxygenate. Selected amino acid residues of the α or β subunits are likely, because of their position or charge, to interfere with the enzyme-substrate interaction. However, depending on the final three-dimensional structure of the catalytic region, the amino acid residues that affect the substrate selectivity in one type of α-β arrangement will not inevitably affect the substrate selectivity in other types of α-β arrangements. This conceptual model explains why the amino acid residues of the oxygenase α subunit that were found to affect the substrate specificity of strain LB400 and KF707 dioxygenases (22, 30) had no effect on the enzyme reactivity pattern of $\alpha_{B\text{-}356}\beta_{B\text{-}356}$ or in $\alpha_{B\text{-}356}\beta_{LB400}$ (17). However, structure analysis of the biphenyl dioxygenase oxygenase component will be required to assess this hypothesis. Recently, Kauppi et al. (21) reported the three-dimensional structure of the terminal oxygenase component of the homologous naphthalene dioxygenase. Structure analysis shows a major involvement of the α subunit in enzyme catalytic activity. However, so far, structure analysis has not helped to identify the role of the β subunit in enzyme catalytic activity or specificity.

Nevertheless, if structural features of the β subunit influence the enzyme's specificity, this fact will be of consequence for enzyme-engineering programs designed to create new enhanced enzymes for the degradation of more persistent chlorobiphenyls. Crameri et al. (9) have recently shown that the use of homologous genes to provide functional diversity accelerates the in vitro-directed evolution process based on DNA shuffling. Kumamaru et al. (23) have shuffled strain LB-400 *bphA* with *P. pseudoalcaligenes* KF707 *bphA1* and successfully obtained mutants expressing phenotypes of both parents. However, our data suggest that the development by molecular evolution of mutants able to catalyze the oxygenation of congeners that both parents are unable to oxygenate may also have to take into consideration other portions of the ISP_{BPH} molecule, including perhaps the β subunit.

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