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Purification and Properties of 2,3-Dihydroxybiphenyl Dioxygenase from Polychlorinated Biphenyl-Degrading *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* Carrying the Cloned *bphC* Gene

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2,3-Dihydroxybiphenyl dioxygenase, involved in biphenyl and polychlorinated biphenyl degradation, was purified from cell extracts of polychlorinated biphenyl-degrading *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas aeruginosa* PAO1161 carrying the cloned *bphC* gene (encoding 2,3-dihydroxybiphenyl dioxygenase). The purified enzyme contained ferrous iron as a prosthetic group. The specific activities decreased with the loss of ferrous iron from the enzyme, and the activity was restored by incubation with ferrous iron in the presence of cysteine. Addition of ferric iron caused the complete inactivation of the enzyme. The molecular weight was estimated to be 250,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single band with a molecular weight of 31,000, indicating that the enzyme consists of eight identical subunits. The enzyme was specific only for 2,3-dihydroxybiphenyl with a K_m value of 87 μM. No significant activity was observed for 3,4-dihydroxybiphenyl, catechol, or 3-methyl- and 4-methylcatechol. The molecular weight, subunit structure, ferrous iron requirement, and NH_2-terminal sequence (starting with serine up to 12 residues) were the same between the two enzymes obtained from KF707 and PAO1161 (*bphC*).

2,3-Dihydroxybiphenyl dioxygenase (2,3-dihydroxybiphenyl:oxygen 1,2-oxidoreductase [23OHBP oxygenase]) has been found in various biphenyl-utilizing bacteria such as *Pseudomonas* spp. (3, 6, 7, 10), *Acinetobacter* sp. (8, 9), and *Alcaligenes* sp. (5). They catabolize biphenyl and polychlorinated biphenyls (PCBs) to benzoic acid and chlorobenzoic acids through the oxidative route (Fig. 1). 23OHBP oxygenase is an extradiol-type dioxygenase and catalyzes the conversion of 2,3-dihydroxybiphenyl (Fig. 1) to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (Fig. 1) with the insertion of two atoms of oxygen. 23OHBP oxygenase from *Pseudomonas cruciviae* S93 has recently been isolated and partially purified (10). We isolated and cloned a gene cluster encoding the catabolism of biphenyl and PCB from the chromosomal DNA of *Pseudomonas pseudoalcaligenes* KF707 (6). The cloned DNA fragment contained at least three structural genes, including *bphA* (encoding biphenyl dioxygenase), *bphB* (encoding dihydrodiol dehydrogenase), and *bphC* (encoding 23OHBP oxygenase). *bphC* has been further subcloned into a broad-host-range plasmid vector pKF330 (pMFB5 as the resultant hybrid plasmid) as described previously (6). In the present paper, we purified and characterized 23OHBP oxygenase from both parent strain KF707 and the recombinant strain *Pseudomonas aeruginosa* PAO1161(pMFB5).

The bacterial strain used was PCB-degrading *P. pseudoalcaligenes* KF707, which was isolated from soil with biphenyl as a sole carbon source as described previously (6). *P. aeruginosa* PAO1161 (*hsdR hsdM leu*)(pMFB5) (6) containing the cloned *bphC* was also used as the enzyme source. *P. pseudoalcaligenes* KF707 was grown with sodium succinate (4 g/liter) in a basal salts medium (pH 7.0) containing (in grams per liter) K_2HPO_4, 4.3; KH_2PO_4, 3.4; $(NH_4)_2SO_4$, 2.0; $MgCl_2$, 0.16; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.0006; $CaCl_2 \cdot 2H_2O$, 0.026; and $Na_2MoO_4 \cdot 2H_2O$, 0.002. Biphenyl was added at a concentration of 0.2 g/liter to induce 23OHBP oxygenase. PAO1161(pMFB5) was grown in LB broth (tryptone, 10 g; yeast extract, 5 g; and NaCl, 10g per liter, pH 7.0)-supplemented streptomycin at a concentration of 200 μg/ml.

Purification of enzyme was done according to the method described for catechol 2,3-dioxygenase (metapyrocatechase) (11). (i) Crude extract. Wet KF707 cells (18.7 g) were suspended in 30 ml of 50 mM Tris hydrochloride buffer, pH 7.5, containing 10% acetone (Tris-acetone buffer) and disrupted with a French pressure cell (Ohtake Co. Ltd., Tokyo, Japan). The supernatant fluid was prepared by centrifugation at 28,000 × *g* for 30 min as crude extract. (ii) Acetone fractionation. DNase I (Sigma Chemical Co., St. Louis, Mo.; 0.5 μg) was added to the crude extract, and the mixture was kept at room temperature for 15 min. To the mixture, 0.8 volume of cold acetone (−20°C) was added, and the resulting precipitate was removed by centrifugation at 10,000 × *g* for 10 min. Another 0.5 volume of cold acetone was added to the supernatant to give a final concentration of 66% acetone. The resulting precipitate was collected by centrifugation and suspended in 20 ml of Tris-acetone buffer. (iii) Chromatography on DEAE-Sephadex A-50. The acetone fraction was applied on a DEAE-Sephadex A-50 column (2.5 by 25 cm) previously equilibrated with Tris-acetone buffer. The column was washed with 500 ml of Tris-acetone buffer containing 1% ammonium sulfate. The enzyme was eluted with a linear gradient between 1 liter of Tris-acetone buffer containing 1% ammonium sulfate (mixing chamber) and 1 liter of the buffer containing 5% ammonium sulfate (reservoir). To the combined active fractions (about 50 ml), 1.7 volumes of cold acetone were added to precipitate the enzyme. The precipitate was collected by centrifugation at 10,000 × *g* for 5 min and dissolved in a small amount of Tris-acetone buffer (about 10 ml). (iv) Gel filtration on Sepharose CL. The DEAE

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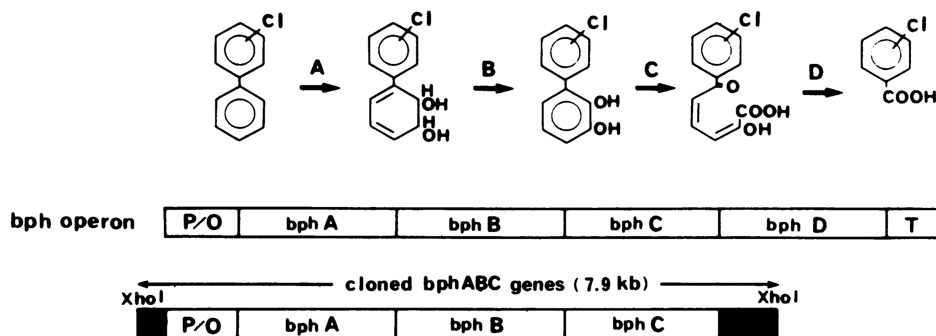

FIG. 1. Catabolic pathway for degradation of biphenyl and chlorobiphenyls and proposed gene organization of the *bphABCD* operon in *P. pseudoalcaligenes* KF707. (Top) Compounds (left to right): biphenyl; 2,3-dihydroxy-4-phenylhexa-2,4-diene (dihydrodiol compound); 2,3-dihydroxybiphenyl; 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage compound); and V, benzoic acid. Enzyme activities: A, biphenyl dioxygenase; B, dihydrodiol dehydrogenase; C, 23OHBP oxygenase; D, *meta*-cleavage compound hydrolase. (Bottom) Symbols: P/O, Putative promoter-operator region; T, putative transcriptional terminator. The cloned *bphABC* (*Xho*I fragment, 7.9 kilobases) is indicated. *bphD* has not yet been cloned.

fraction was subjected to the gel filtration of a Sepharose CL column (2.5 by 90 cm) equilibrated with Tris-acetone buffer. The enzyme was eluted with the same buffer. To the combined active fractions (about 35 ml), 1.7 volumes of cold acetone were added to precipitate the enzyme. The precipitate was collected by centrifugation at 28,000 × g for 10 min and dissolved in a small amount of Tris-acetone buffer (about 1 ml).

23OHBP oxygenase from PAO1161(pMFB5) was also purified from 36 g of wet cells grown in LB broth containing streptomycin (200 μg/ml) by the procedure described above.

Activity of 23OHBP oxygenase was assayed spectrometrically by measuring the increase in the absorbance at 434 nm as described previously (7). The molar extinction coefficient at 434 nm of the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (the *meta*-cleavage compound) at pH 9.0 was determined experimentally to be 22,000 and used for the calculation of the enzyme activity. We defined 1 U of the enzyme as the amount that catalyzed the formation of 1 μmol of the product per min at 25°C. Specific activity was defined as the number of enzyme units per milligram of protein. The protein concentration was measured with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) as indicated by the supplier (1).

Polyacrylamide gel electrophoresis of the native enzyme was done with polyacrylamide gradient gel PAA 4/30 prepared by Pharmacia (Uppsala, Sweden). Polyacrylamide gel electrophoresis of the enzyme treated with 2.5% sodium dodecyl sulfate (SDS) followed by heat treatment (10 min at 10°C) was also performed. The isoelectric point (pI) of 23OHBP oxygenase was determined by automated electrophoresis apparatus (Pharmacia) with Phast Gel isoelectric focusing and pH 4 to 6.5 pI markers. The amino acid sequence of the NH_2-terminal portion of the protein was analyzed by an automated protein sequencer (Applied Biosystems, Inc., Foster City, Calif.; model 470A).

23OHBP oxygenase became stable by the addition of 10% acetone or 10% ethanol to the phosphate buffer. The specific activity of enzyme (units per milligram of protein) in the crude extract from PAO1161 carrying the cloned *bphC* gene was 2.3 times higher than that of wild-type KF707. The DEAE-Sephadex chromatogram of the acetone fraction from *P. pseudoalcaligenes* KF707 cells is presented in Fig. 2. 23OHBP oxygenase activity was observed around fraction 50. Catechol 2,3-dioxygenase activity was also observed around fraction 175, indicating that two different extradiol-type dioxygenases specific for 2,3-dihydroxybiphenyl or catechol exist in KF707 cells. The yield and specific activities of 23OHBP oxygenase purified from *P. aeruginosa* PAO1161(pMFB5) are shown in Table 1. The purified enzymes from KF707 or PAO1161(pMFB5) showed a single band on polyacrylamide gel electrophoresis (Fig. 3).

The enzyme obtained from DEAE-Sephadex chromatography showed absorption at around 412 nm, suggesting the presence of iron in the enzyme molecule. The activity of the enzyme was completely lost by Sepharose CL gel filtration, with the loss of absorption at 412 nm. The preparation could be reactivated by incubation with ferrous iron in the presence of cysteine as previously described, in catechol 2,3-dioxygenase (11, 12). The maximum activity (74.2 U/mg of protein) was gained when ferrous iron was incubated at 10^{-5} M with the purified enzyme. Addition of ferric iron (10^{-5} M in a final concentration) readily inactivated the enzyme.

The molecular weights of native 23OHBP oxygenase from KF707 and PAO1161(pMFB5) were determined by gradient polyacrylamide gel electrophoresis from the relative mobility to the standard proteins (Fig. 3a). Both preparations showed a molecular weight of about 250,000. Polyacrylamide gel electrophoresis of enzymes treated with 2.5% SDS followed by heat treatment (100°C for 10 min) gave a single band (Fig. 3b), and the minimum molecular weight was estimated to be 31,000. These results suggest that the enzyme consists of eight identical subunits.

The 23OHBP oxygenase from KF707 cells was specific only for 2,3-dihydroxybiphenyl. It could not attack 3,4-dihydroxybiphenyl and showed very weak activities for catechol and 3-methyl- and 4-methylcatechols (data not shown).

Since the molar extinction coefficient of the *meta*-cleavage product of 2,3-dihydroxybiphenyl was greatly decreased when the pH was lowered, it was experimentally determined

TABLE 1. Purification of 23OHBP oxygenase from *P. aeruginosa* PAO1161(pMFB5)

Fraction	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)
Crude extract	4,704	1,716	2.7	100
Acetone fraction	2,113	202	10.5	45
DEAE-Sephadex	276	8	34.5	5.8
Sepharose CL	50	0.6	87.2	1.1

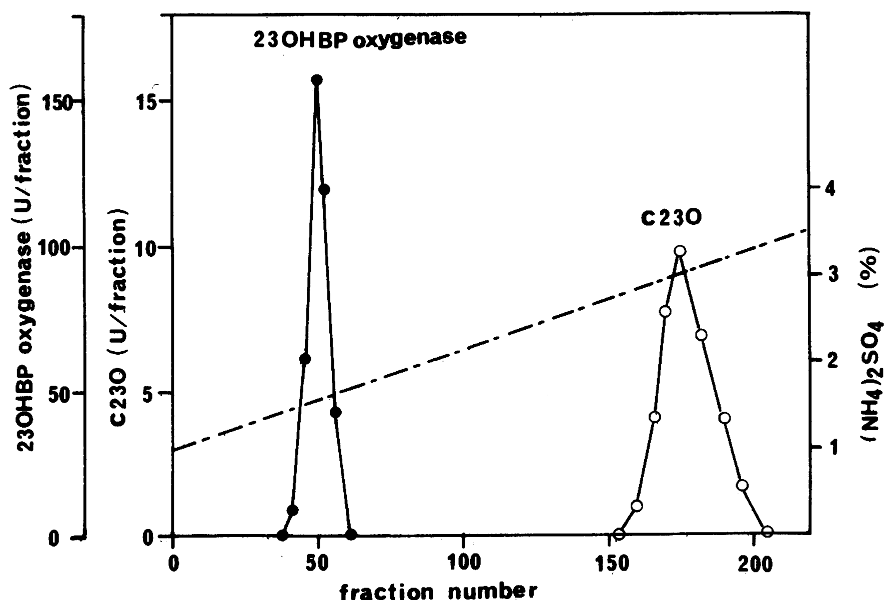

FIG. 2. DEAE–Sephadex A-50 chromatogram of 23OHBP oxygenase and catechol 2,3-dioxygenase (C23O) in *P. pseudoalcaligenes* KF707.

and used for the calculation of the reaction rates. The optimum pH of the enzyme activity was around 9.0. The K_m value for 23OHBP was calculated to be 87 μM. The pI was determined to be 4.5.

Automated Edman degradation of 23OHBP oxygenase for both preparations determined the NH_2-terminal sequence to be Ser-Ile-Arg-Ser-Leu-Gly-Tyr-Met-Gly-Phe-Ala-Val.

In the present paper, we purified and studied some properties of 23OHBP oxygenase involved in biphenyl and PCB catabolism. The enzyme properties from wild-type strain KF707 and from transformant PAO1161(pMFB5) carrying the cloned *bphC* were identical in molecular weight, subunit structure, and requirement of ferrous iron as the prosthetic group. Moreover, in the NH_2-terminal amino acid sequences, up to 12 residues were identical between KF707 and PAO1161(pMFB5). Even though the cloned *bphC* gene in hybrid plasmid pMFB5 is transcribed from the promoter of a kanamycin resistance gene on the vector plasmid, the *bphC* product is not fused with a kanamycin resistance gene product. In a separate communication, we have determined the 23OHBP oxygenase gene (*bphC*) (4). The nucleotide sequence data of the *bphC* gene were in perfect agreement with the NH_2-terminal residues of the purified enzyme. The molecular weight of the subunit deduced from the nucleotide sequence was 33,074, which was close to the M_r value of 31,000 obtained from SDS-polyacrylamide gel electrophoresis in this study.

Catechol 2,3-dioxygenase encoded by the TOL plasmid (pWW0) in *Pseudomonas putida* mt-2 was crystallized and well characterized (14, 15), and the entire DNA sequence has been determined (12). It is composed of four identical subunits of 35,000 molecular weight, and each subunit con-

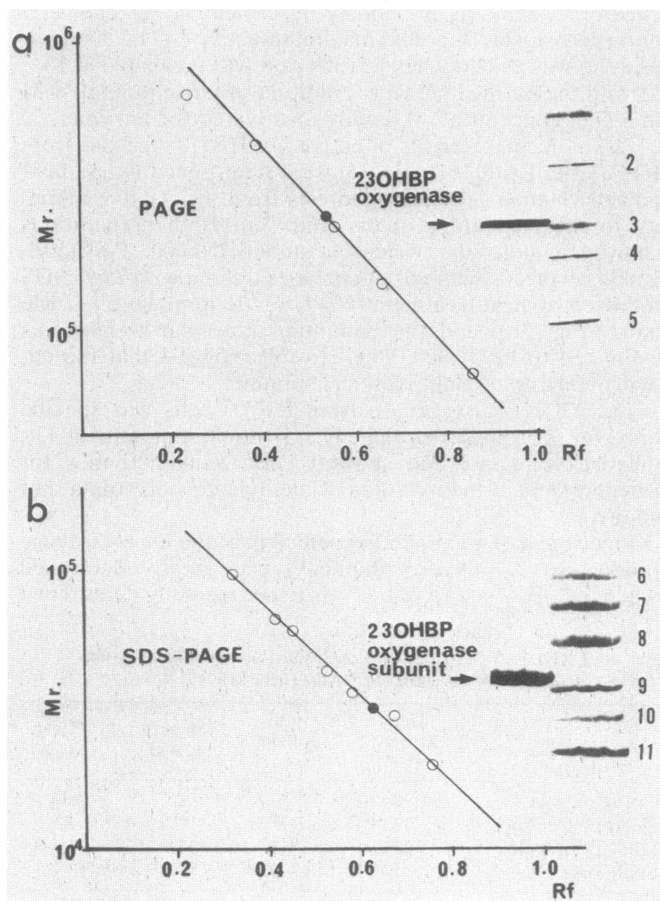

FIG. 3. Polyacrylamide gel electrophoresis (PAGE) (a) and SDS-PAGE (b) of purified 23OHBP oxygenase from *P. aeruginosa* PAO1161(pMFB5). The molecular weights of the native enzyme and the subunit were estimated from the mobility with the standard proteins indicated. The same results were obtained for the purified 23OHBP oxygenase obtained from the parent strain *P. pseudoalcaligenes* KF707. Molecular-weight-standard proteins used: 1, thyroglobulin, 669,000; 2, ferritin, 440,000; 3, catalase, 232,000; 4, lactate dehydrogenase, 140,000; 5 and 7, bovine serum albumin, 67,000; 6, phosphorylase *b*, 94,000; 8, ovalbumin, 43,000; 9, carbonic anhydrase, 30,000; 10, soybean trypsin inhibitor, 20,000; 11, α-lactalbumin, 14,000.

tains 1 g-atom of ferrous iron and forms an active site of the enzyme $[\alpha Fe(II)]_4$ (12). Since the most active preparation of 23OHBP oxygenase in *P. pseudoalcaligenes* KF707 was obtained when 27 g-atoms of iron per mol of enzyme was incubated, it is probable that each subunit contains at least 1 g-atom of iron-forming $[\alpha Fe(II)]_8$.

Intradiol-type dioxygenases have been extensively studied. All of those studied contain the ferric form of iron and are composed of two nonidentical subunits. Protocatechuate 3,4-dioxygenase from *P. aeruginosa* (M_r = 700,000) consists of eight identical protomers, each containing two pairs of two nonidentical subunits, α (M_r = 22,500) and β (M_r = 25,000), forming $[\alpha_2\beta_2Fe(III)]_8$ (16). The same enzyme from *P. putida* (M_r = 200,000) is considered $[\alpha\beta Fe(III)]_4$, with the molecular weight of α and β being 23,000 and 26,500, respectively (2). Pyrocatechase from *Pseudomonas arvilla* C-1 (M_r = 63,000) consists of two subunits, α (M_r = 30,000) and β (M_r = 32,000), and the structure of $[\alpha\beta Fe(III)]$ is proposed (13). It is interesting that extradiol-type dioxygenases, including 23OHBP oxygenase, contain ferrous iron, whereas intradiol-type dioxygenases contain ferric iron as the prosthetic group.

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