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Characterization of Active Recombinant 2,3-Dihydro-2,3-Dihydroxybiphenyl Dehydrogenase from *Comamonas testosteroni* B-356 and Sequence of the Encoding Gene (*bphB*)

M. SYLVESTRE,* Y. HURTUBISE, D. BARRIAULT, J. BERGERON,† AND D. AHMAD

Institut National de la Recherche Scientifique-Santé, Université du Québec, Point-Claire, Québec, Canada, H9R 1G6

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2,3-Dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (B2,3D) catalyzes the second step in the biphenyl degradation pathway. The nucleotide sequence of *Comamonas testosteroni* B-356 *bphB*, which encodes B2,3D, was determined. Structural analysis showed that the dehydrogenases involved in the bacterial degradation of aromatic compounds are related to each other and that their phylogenetic relationships are very similar to the relationships observed for dioxygenases that catalyze the initial reaction in the degradation pathway. The *bphB* sequence was used to produce recombinant active His-tagged B2,3D, which allowed us to describe for the first time some of the main features of a B2,3D. This enzyme requires NAD^+, its optimal pH is 9.5, and its native M_r was found to be 123,000, which makes it a tetramer. These characteristics are very similar to those reported for the related enzyme *cis*-toluene dihydrodiol dehydrogenase. The K_m value and maximum rate of metabolism for 2,3-dihydro-2,3-dihydroxybiphenyl were 73 ± 16 μM and 46 ± 4 nmol min^{-1} μg^{-1}, respectively. Compared with the *cis*-toluene dihydrodiol dehydrogenase, B2,3D appeared to be more substrate specific since it was unable to attack *cis*-1,2-dihydroxy-cyclohexa-3,5-diene.

The sequential enzymatic steps involved in the conversion of biphenyls (BPHs) and polychlorinated biphenyls (PCBs) to the corresponding benzoates and chlorobenzoates have been elucidated for many bacteria (for reviews, see references 1, 14, and 39). This conversion is initiated by BPH 2,3-dioxygenase (BPH dox), which transforms BPH into *cis*-2,3-dihydro-2,3-dihydroxybiphenyl (2,3-DDBPH) (16). Then 2,3-DDBPH-2,3-dehydrogenase (B2,3D) is required to generate the corresponding diol (Fig. 1), which is cleaved by the 2,3-dihydroxybiphenyl-1,2-dioxygenase (B1,2O).

Because BPH dox is believed to be a major determinant of the substrate selectivity patterns of BPH-degrading bacteria (16), efforts have been made recently to characterize this enzyme (15, 16, 21, 22). BPH dox is a three-component system which includes a terminal oxygenase, which is an iron-sulfur protein (ISP_{BPH}) that is made up of an α subunit (M_r, 51,000) and a β subunit (M_r, 22,000), which in *Comamonas testosteroni* B-356 are encoded by *bphA* and *bphE*, respectively; a ferredoxin (FER_{BPH}; M_r, 12,000) encoded by *bphF*; and a ferredoxin reductase (RED_{BPH}; M_r, 43,000) encoded by *bphG*. A sequence analysis of genes showed that on the basis of its BPH dox (38) strain B-356 belongs to a distinct phylogenetic lineage together with *Pseudomonas* sp. strain KKS102 (11, 23). This group is characterized by the fact that the gene encoding RED_{BPH} is located outside the *bph* gene cluster, and the RED_{BPH}s of its members exhibit low levels of amino acid sequence homology with RED_{BPH}s from other sources.

B1,2O, the third enzyme of the BPH catabolic pathway, has also been well characterized both genetically and biochemically. A sequence analysis of genes showed that although all known B1,2Os belong to a single gene family, this enzyme has diverged into several phylogenetic branches, one of which includes *Beijerinkia* sp. strain B1 B1,2O and *Pseudomonas paucimobilis* Q1 B1,2O (24, 40), both of which are related to *Pseudomonas* sp. strain PpG7 *NahC*, the *meta* cleavage dioxygenase involved in the naphthalene biodegradation pathway. Another branch includes *Pseudomonas* sp. strain LB400 (20), *Pseudomonas pseudoalcaligenes* KF707 (13, 41), *Pseudomonas putida* KF715 (19), and *Pseudomonas* sp. strain KKS102 (11) enzymes. B1,2O has been isolated and characterized from *P. pseudoalcaligenes* KF707 (12) and from *Pseudomonas* sp. strain LB400 (10). In both of these cases the enzyme was found to contain eight identical subunits, each of which had a molecular weight of approximately 33,000. The structures of strain LB400 B1,2O and strain KKS102 B1,2O have recently been elucidated (18, 36).

However, the other two enzymes that participate in the BPH-PCB degradation pathway, B2,3D and 2-hydroxy-6-oxo-6-(phenyl/chlorophenyl)hexa-2,4-dienoic acid hydrolase, have been less well characterized. Because the substrate reactivity of bacteria toward selected PCB congeners not only is determined by the substrate selectivity of BPH dox but also is influenced by the flux of metabolite production (37, 39), strain enhancement requires a knowledge of the biochemical features of each enzymatic step needed for PCB degradation.

In a previous paper we reported the cloning of a 6.3-kb *Sma*I-*Sma*I piece of DNA from strain B-356 that carries most of the B-356 *bph* gene cluster (7). In this paper, we describe a sequence analysis of strain B-356 *bphB* from a subclone of this *Sma*I-*Sma*I fragment. Using the His-BIND system, we obtained active purified recombinant His-tagged B2,3D, which allowed us to determine for the first time some of the essential features of this enzyme.

* Corresponding author. Mailing address: INRS-Santé, Université du Québec, 245 Boulevard Hymus, Point-Claire, Québec, Canada, H9R 1G6. Electronic mail address: Michel_Sylvestre@INRS-Sante.UQuebec.ca.

† Present address: Tembec, Inc., Témiscaming, Québec, J0Z 3R0, Canada.

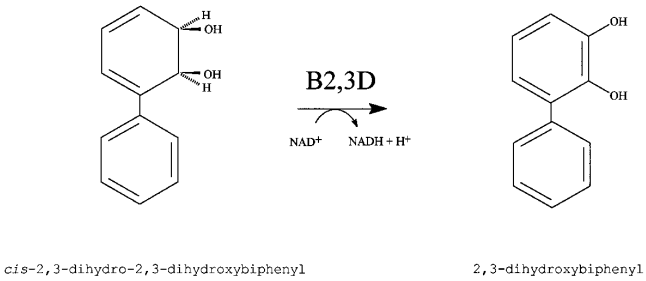

FIG. 1. Reaction catalyzed by B-356 B2,3D.

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 obtained from QIAGEN, Inc., Chatsworth, Calif.) and *C. testosteroni* B-356 (2). The media used were Luria-Bertani broth and H plates (34). Most molecular biology manipulations were done by using protocols described by Sambrook et al. (34). PCRs to amplify *bphB* were performed by using *Pwo* DNA polymerase and procedures outlined previously (21).

Sequencing strategies. The nucleotide sequence of *bphB* in both directions was determined by using subclones in M13mp18 and M13mp19 of a previously described 6.3-kb *Sma*I-*Sma*I fragment (7) which carries most of the strain B-356 *bph* degrading genes. Sequencing was done by the dideoxy chain termination method, using α-^{35}S-labeled dATPαS to label the chains. A Pharmacia T7 sequencing kit or a Gene-ATAQ sequencing kit was used in conjunction with universal or synthetic primers. Oligonucleotide primers were synthesized with a Pharmacia LKB Gene Assembler Plus apparatus.

Production and purification of His-tagged B-356 B2,3D. His-tagged B-356 B2,3D was produced from *E. coli* M15(pREP4) and SG13009(pREP4) by using the QIAGEN expression system. The protocols used for expression and purification of the enzyme were essentially the same as those described for B-356 RED_{BPH} and FER_{BPH} (21). The coding region of B-356 *bphB* was PCR amplified from a cloned DNA fragment. The following oligonucleotides were used for PCR: oligonucleotide I (*Bam*HI) (5′-CGGGATCCGATGGGATTTACTCGC G-3′) and oligonucleotide II (*Kpn*I) (5′-GGGGTACCCCTCAAGCAGCCAAG TG-3′). The construction was such that the His tail added 13 amino acids (MRGSHHHHHHTDP) to the protein in its N-terminal portion. On the basis of the information concerning *Pwo* DNA polymerase provided by the producer (Boehringer Mannheim), we estimated that less than 3% of the PCR-amplified DNA fragments generated in the PCR system described above contain errors. Furthermore, when His-tagged ISP_{BPH} from strain B-356 was produced and purified under conditions identical to those used in this study, it was found that the features of the recombinant enzyme were indistinguishable from those of the parent enzyme purified from the original strain (22). Therefore, although the 848-bp amplified DNA fragment carrying *bphB* was not sequenced, we are confident that the PCR-amplified *bphB* that we used in the present study is an accurate copy of the original gene found in strain B-356.

Protein characterization. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide gel was performed as described by Laemmli (26). The M_r of the His-tagged B2,3D was determined from SDS-PAGE gels by using Promega's mid-range M_r standards. The proteins were

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        10                  30                  50                  70                  90
TTACGATAGA TTGAGGTTGG AGATGAAGCT GACAGGTGAA GTGGCATTGA TTACCGGTGG CGCCTCCGGA CTGGGGCGCG CCTTGGTCGA CCGGTTCGTC
              bphB      M  K  L  T  G  E  V  A  L  I  T  G  G  A  S  G  L  G  R  A  L  V  D  R  F  V
      110                 130                 150                 170                 190
GCCGAGGGAG CACGCGTGGC CGTGCTCGAC AAGTCGGCCG AGCGCCTGCG GGAACTGGAG GTGGCCCATG GAGGGAACGC GGTAGGCGTC GTCGGCGATG
A  E  G  A  R  V  A  V  L  D  K  S  A  E  R  L  R  E  L  E  V  A  H  G  G  N  A  V  G  V  V  G  D  V
      210                 230                 250                 270                 290
TGCGCTCCCT GCAGGATCAG AAGCGCGCCG CCGAGCGCTG TCTTGCTGCA TTCGGAAAGA TCGACACGCT TATTCCTAAT GCGGGCATCT GGGACTATTC
  R  S  L  Q  D  Q  K  R  A  A  E  R  C  L  A  A  F  G  K  I  D  T  L  I  P  N  A  G  I  W  D  Y  S
      310                 330                 350                 370                 390
GACCGCCTTG GCCGATCTGC CGGAAGACAA GATCGATGCG GCATTCGACG ACATTTTTCA TGTCAACGTG AAGGGCTACA TCCACGCCGT CAAGGCCTGC
 T  A  L  A  D  L  P  E  D  K  I  D  A  A  F  D  D  I  F  H  V  N  V  K  G  Y  I  H  A  V  K  A  C
      410                 430                 450                 470                 490
CTGCCAGCCC TGGTGTCCAG CCGCGGCAGT GTTGTCTTCA CGATCTCTAA CGCTGGCTTC TATCCCAACG GCGGCGGCCC GCTCTATACC GCGACCAAGC
L  P  A  L  V  S  S  R  G  S  V  V  F  T  I  S  N  A  G  F  Y  P  N  G  G  G  P  L  Y  T  A  T  K  H
      510                 530                 550                 570                 590
ACGCAGTGGT GGGCCTAGTC CGCCAAATGG CGTTTGAGCT GGCTCCTCAT GTGCGCGTCA ACGGCGTGGC ACCGGGCGGC ATGAACACCG ATCTGCGCGG
  A  V  V  G  L  V  R  Q  M  A  F  E  L  A  P  H  V  R  V  N  G  V  A  P  G  G  M  N  T  D  L  R  G
      610                 630                 650                 670                 690
TCCCTCTTCG CTCGGGCTGA GCGAACAGTC GATCTCGTCG GTGCCGCTCG CCGACATGCT GAAATCGGTG CTGCCGATCG GCCGCATGCC AGCGCTCGAA
 P  S  S  L  G  L  S  E  Q  S  I  S  S  V  P  L  A  D  M  L  K  S  V  L  P  I  G  R  M  P  A  L  E
      710                 730                 750                 770                 790
GAGTATACCG GTGCCTATGT CTTCTTCGCC ACGCGGGGAG ATTCGCTGCC CGCCACCGGC GCCTTACTGA ACTACGACGG CGGCATGGGC GTTCGAGGTT
E  Y  T  G  A  Y  V  F  F  A  T  R  G  D  S  L  P  A  T  G  A  L  L  N  Y  D  G  G  M  G  V  R  G  F
      810                 830                 850
TTTTGACGGC AGCGGGCGGC GCCGACCTGC CCGAGAAGCT GAACATCAAT CGCGAGGGAC AAGAATGA
  L  T  A  A  G  G  A  D  L  P  E  K  L  N  I  N  R  E  G  Q  E  *
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FIG. 2. Nucleotide sequence of B-356 *bphB*.

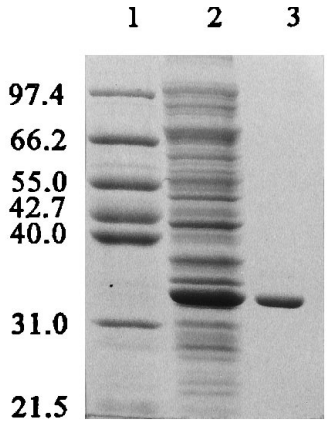

FIG. 3. SDS-PAGE of His-tagged B-356 B2,3D. Lane 1, M_r marker; lane 2, crude preparation (32 μg of protein); lane 3, preparation (0.7 μg of protein) obtained after elution of Ni-nitrilotriacetic acid resin (see Materials and Methods for details).

stained with Coomassie brilliant blue (34). Protein concentrations were estimated by the method of Lowry et al. (27), using bovine serum albumin as the standard. The M_r of the native protein was determined by gel filtration high-performance liquid chromatography (HPLC) with a Perkin-Elmer series 3 chromatograph. A Protein Pak 300SW column (7.8 by 300 mm; Waters) was eluted in the isocratic mode with 100 mM potassium phosphate buffer (pH 7.0) at a rate of 1 ml/min. The UV detector used was a Perkin-Elmer model LC65T detector set at 280 nm. The column was calibrated with catalase (M_r, 232,000), bovine serum albumin (M_r, 67,000), albumin (M_r, 46,000), and RNase A (M_r, 13,700).

Chemicals. BPH was obtained from Aldrich Chemicals, Milwaukee, Wis., and *cis*-1,2-dihydro-cyclohexa-3,5-diene was obtained from Pfaltz & Bauer, Waterbury, Conn. 2,3-DDBPH was produced from BPH by using purified BPH dox obtained from strain B-356 (21). It was then purified by reverse-phase chromatography on an octyldecyl silane Hypersil II (5 μm) column (4 mm by 25 cm). The column was equilibrated with water-methanol-acetonitrile (50:25:25) and then with a 3-min linear gradient to methanol-acetonitrile (80:20). The column was washed for 5 min under the same conditions. 2,3-DDBPH was detected with a Perkin-Elmer model LC95 UV-visible detector set at 306 nm. The peak corresponding to 2,3-DDBPH was collected and extracted with ethyl acetate. The purity of 2,3-DDBPH was assessed by gas chromatography-mass spectrometry. A gas chromatography-mass spectrometry analysis of the trimethylsilyl derivative in which *N*,*O*-bis-trimethylsilyltrifluoroacetamide was used was performed as described previously (21).

The dosage of purified 2,3-DDBPH used was obtained by measuring NAD^+ consumption in the B2,3D reaction with excess NAD^+ and enzyme and by determining the amount of 2-hydroxy-6-oxo-6-(phenyl/chlorophenyl)hexa-2,4-dienoic acid produced from 2,3-DDBPH when purified B1,2O was added to the reaction vial after the B2,3D reaction was completed. The 2-hydroxy-6-oxo-6-(phenyl/chlorophenyl)hexa-2,4-dienoic acid concentration was evaluated spectrophotometrically at 434 nm by using an ε_{434} value of 22,000 M^{-1} cm^{-1} (37). The steps used to obtain purified B1,2O have been described previously (19a). Because the stoichiometry of this dual reaction was such that for each 1 mol of NAD^+ consumed 1 mol of 2-hydroxy-6-oxo-6-(phenyl/chlorophenyl)hexa-2,4-dienoic acid was produced, we are confident that our estimate of the concentration of the 2,3-DDBPH preparation was exact.

B2,3D assay. B2,3D activity was measured at 37°C in 100 mM bicine (pH 9.5). The reaction mixture (total volume, 50 μl) contained 1,000 μM NAD^+, 0.130 μM purified enzyme, and different amounts of 2,3-DDBPH (75 to 1,000 μM) as the substrate. The reactions were initiated by adding the substrate dissolved in acetone and were stopped after 1 min by adding 100 μl of methanol. Each mixture was centrifuged for 30 s, and then 50 μl of the supernatant was injected into an octyldecyl silane Hypersil II HPLC column as described above.

The reaction product was also extracted with ethyl acetate, treated with *N*,*O*-bis-trimethylsilyltrifluoroacetamide, and analyzed by gas chromatography-mass spectrometry as described previously (21). Kinetic parameters, K_m, and the maximum rate of metabolism for 2,3-DDBPH were determined at 37°C by using the standard assay described above.

Nucleotide sequence accession number. The GenBank accession number for the *bphB* sequence is U57451.

RESULTS

DNA sequence of B-356 *bphB*. The sequence of *bphB*, which specifies strain B-356 B2,3D, is shown in Fig. 2. The G+C content of B-356 *bphB* was 62 mol%. The 848-nucleotide gene encoded a polypeptide having a molecular mass of 29.4 kDa. The gene product exhibited 80.2% identity with *Pseudomonas* sp. strain LB400 B2,3D (20) and *P. pseudoalcaligenes* KF707 B2,3D (41). It also exhibited 80.5% identity with the C-terminal portion of *P. putida* KF-715 B2,3D (19) and 75% identity with *Pseudomonas* sp. strain KKS102 B2,3D (11). The levels of homology with rhodococcal B2,3Ds were lower; the levels of homology with *Rhodococcus globerulus* P-6 B2,3D (3) and *Rhodococcus* sp. RHA1 B2,3D (28) were 58.7 and 53.9%, respectively. *bphB* was expressed from pT7-6 in *E. coli*. The polypeptide representing B2,3D was extracted from an SDS-PAGE gel, and the amino acid sequence of the N-terminal portion was determined to confirm that it corresponded to the nucleotide sequence shown in Fig. 2.

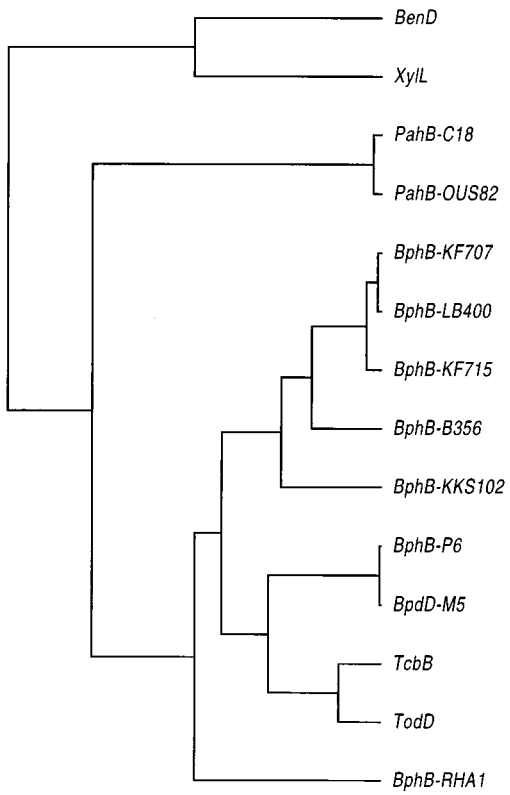

FIG. 4. Dendrogram obtained from alignment of amino acid sequences of gene products by using the PILEUP program of the GCG software. BenD, *Alcaligenes calcoaceticus BenD* (GenBank accession number, M7690) (30); XylL, TOL plasmid *XylL* (M64747) (30); PahB-C18, *Pseudomonas* sp. strain C18 *PahB* (D49343) (9); PahB-OUS82, *P. putida* OUS82 *PahB* (D16629) (42); BphB-KF707, *P. pseudoalcaligenes* KF707 *BphB* (M83673) (40); BphB-LB400, *Pseudomonas* sp. strain LB400 *BphB* (M66122) (20); BphB-KF715, *P. putida* KF715 *BphB* (A35124) (19); BphB-B356, *C. testosteroni* B-356 *BphB* (this study); BphB-KKS102, *Pseudomonas* sp. strain KKS102 *BphB* (D17319) (25); BphB-P6, *R. globerulus* P6 *BphB* (X80041) (3); BpdD-M5, *Rhodococcus* sp. strain M5 *BpdD* (U27591) (44); TcbB, *Pseudomonas* sp. strain P51 *TcbB* (U15298) (43); TodD, *P. putida* F1 *TodD* (J04996) (45); BphB-RHA1, *Rhodococcus* sp. strain RHA1 *BphB* (D32142) (29).

Characterization of His-tagged B2,3D from strain B-356. His-tagged B-356 B2,3-D was expressed in *E. coli* and was purified as described in Materials and Methods. The yield of purified B2,3D was approximately 7 mg/g of cell paste. No contaminating bands were detected on SDS-PAGE gels (Fig. 3). The M_r of the purified enzyme estimated by SDS-PAGE (32,000) was similar to the M_r predicted from the DNA sequence of *bphB* after addition of the 13 amino acid residues of

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BenD          151IHRIPYSACKGGVNALTASLA//    250GSVLPVGGGDQG*
XylL          148IHRVPYGAAKGGVNALTACLA//    246GITLPVAGGDLGCQSCSV
PahB-C18      148GGGSCYIASKHAVLGMVKALA//    245GTVISIDGGMALGRK*
PahB-OUS82    148GGGSCYIASKHAVLGMVKALA//    245GTVISIDGGMALGRK*
BphB-KF707    150GGGPLYTAAKHAIVGLVRELA//    246GALLNYDGGLGVRGFFSGAGG
BphB-LB400    150GGGPLYTAAKHAIVGLVRELA//    246GALLNYDGGLGVRGFFSGAGG
BphB-KF715     57GGGPLYTAAKHAIVGLVRELA//    144GALLNYDGGLGVRGFFSGAGG
BphB-B-356    150GGGPLYTATKHAVVGLVRQMA//    246GALLNYDGGMGVRGFLTAAGG
BphB-KKS102   150GGGPLYTGAKHAVVGMVRELA//    246GALLNHDGGMGVRGFFEATGG
BphB-P6       150GGGALYTGAKHAIVGMVKQLA//    247GSVLNYDGGIGVRGMSEANRG
BphD-M5       150GGGALYTGAKHAIVGMVKQLA//    247GSVLNYDGGIGVRGMSEANRG
TcbB          150GGGVLYTAGKHAVIGLVKQLA//    247GSVLNIDGGMGVRGLFEASLG
TodD          150GGGVLYTAGKHAVIGLIKQLA//    247GSVLNFDGGMGVRGLFEASLG
BphB-RHA1     143GGGPLYTASKHAVVGLIKELA//    240GAIINCDGGMGVRGLAETAGG
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FIG. 5. Alignments of portions of sequences from various dehydrogenases involved in the degradation of aromatic compounds. The abbreviations are the same as those used in Fig. 4. Alignments were obtained by using the PILEUP program of the GCG software.

the His-tagged protein. The M_r of the native protein was estimated to be 123,000 by gel filtration chromatography, suggesting that the protein is a tetramer. The native conformations of the homologous molecules *cis*-toluene dihydrodiol dehydrogenase (33) and *cis*-1,2-dihydroxy-cyclohexa-3,5-diene oxidoreductase (32) were also shown to be tetramers.

The His-tagged B2,3D preparation was very active, and the enzyme activity was stable. When tested in the assay system described above, the enzyme remained fully active for 18 h at 37°C and retained 60% of its activity after incubation for 1 h at 55°C. Storage at −70°C for months did not affect its activity. The optimal pH and temperature were 9.5 and 55°C, respectively, but the enzyme was fairly active at 37°C and pH 7. NAD^+ was essential for B2,3D activity. A similar observation was reported for the *Pseudomonas* sp. strain LB400 enzyme, and it was found that $NADP^+$ could not replace NAD^+ for B2,3D activity (17) in crude extracts. Other NAD^+-dependent dehydrogenases involved in the biodegradation of aromatic compounds have been purified and characterized, and all of them were inactive when NAD^+ was replaced by $NADP^+$ (4, 32).

The K_m value and maximum rate of metabolism for 2,3-DDBPH were 73 ± 16 μM and 46 ± 4 nmol min^{-1} μg^{-1}, respectively, when the dehydrogenase reaction was performed under the conditions described in Materials and Methods.

DISCUSSION

The isolation and purification of individual enzymes required for each catalytic step of PCB degradation have at least two important implications. First, the information obtained from purified enzyme characterization should give a clear picture of the contribution of each enzyme of the pathway to the overall degradation process with regard to individual congeners. Second, knowledge concerning the reaction parameters that influence the enzyme activity and knowledge concerning the structural determinants of specificity are also required to design strategies to improve PCB degradation. In this paper we describe for the first time the purification of a B2,3D by the His-bind purification system. Our data show that like His-tagged FER_{BPH}, RED_{BPH}, and ISP_{BPH} (21, 22), His-tagged B2,3D is also active. Furthermore, the features of His-tagged B2,3D, including the NAD^+ requirement, the optimal pH, and the native molecular weight, are very similar to the features reported previously for the related molecule *cis*-toluene dihydrodiol dehydrogenase (33). The His-bind purification system therefore apparently is an interesting tool for comparing the abilities of B2,3Ds from various bacterial strains to degrade PCB congeners. In contrast to *cis*-toluene dihydrodiol dehydrogenase, which exhibited a broad substrate reactivity, pattern, B-356 B2,3D was not able to transform *cis*-1,2-dihydroxy-cyclohexa-3,5-diene. A similar narrow spectrum of substrate activity was observed with B-356 BPH dox (21). This suggests that the BPH-degrading pathway in gram-negative bacteria has evolved to be specific for the BPH molecule, while the TOL degradation pathway (including TOL dox) can degrade both mono- and bicyclic aromatic compounds.

The aromatic ring-hydroxylating dioxygenases have been subdivided on the basis of the number of components and cofactor requirements (6, 29). The classification of these enzymes on the basis of component arrangement also correlates with the chemical nature of their substrates and with the alignments of the deduced amino acid sequences of their polypeptide components (3, 38). While class I enzymes catalyze hydroxylation of aromatic compounds carrying at least one carboxyl substituent, class II dioxygenases catalyze the hydroxylation of mono- and biaryls, such as toluene, benzene, and BPH, and members of class III attack the multimember aromatic rings in which interlinked rings have two atoms in common, such as polycyclic aromatic hydrocarbons and dibenzothiophenes.

It is noteworthy that the same division is obtained when the PILEUP program of the GCG software is used to compare the amino acid sequences of all known aryl-dihydrodiol dehydrogenases. This program differentiates two main clusters of dihydrogenases (Fig. 4). One cluster includes enzymes involved in the degradation of monoaryls containing a carboxyl group for which biodegradation is initiated by class I dioxygenases. The second cluster is divided into two lineages; one of these lineages includes the dehydrogenases involved in the degradation of compounds for which the initial reaction is catalyzed by class III dioxygenases, and the other lineage comprises the dehydrogenases that participate in pathways involving class II dioxygenases.

Furthermore, when the subgroup that consists of the various B2,3Ds is considered, the PILEUP program gives the same distribution as the distribution obtained for BPH dox molecules (3, 38); gram-negative bacterial B2,3Ds are clustered on one branch, while *R. globerulus* P6 B2,3D is found on a second branch along with the dehydrogenase involved in the toluene degradation pathway (*TodD*). This suggests that at least the first two enzymes of these aromatic compound degradation pathways evolved simultaneously.

The ^{155}YTATKHAVVGL sequence of strain B-356 B2,3D fits into the consensus pattern Y(S,T,A,G, or C)(S,T,A,G,C or V)(S,T,A,G, or C)KX(A or G)(L,I,V,M,A, or G)XX(L,I,V,M, or F) for short-chain alcohol dehydrogenases (8, 31). The alignment in Fig. 5 shows that this region is very similar in dehydrogenases involved in the degradation of polycyclic aromatic hydrocarbons, toluene, and trichlorobenzene and slight-

ly different in dehydrogenases involved in the degradation of carboxyl-substituted aryls (BenD, XylL).

NAD^+ is an essential cofactor for His-tagged B2,3D. The NAD (ADP) binding sites of dehydrogenases are recognized by the consensus sequence GxGxxGxxxG (35), in which the GxGxxG portion is essential for NAD^+ binding to NAD^+-dependent enzymes (35). B2,3D contains two Gly-rich regions. One region located at the N-terminal portion of the polypeptide does not have this typical consensus sequence. However, because this region is highly conserved in all members of the short-chain alcohol dehydrogenase family, including strain KF707 B2,3D, it was thought to be part of the NAD^+ coenzyme binding domain (5). Another region located at the C-terminal portion of the polypeptide, starting at position G^{257}, matches exactly the NAD (ADP) consensus binding site (35). However, when the N-terminal portions of various dihydrodiol dehydrogenases were aligned by using the PILEUP program of the GCG software, no sequence matching this consensus sequence was found in the dehydrogenases involved in the degradation of carboxyl-containing aryls and polycyclic aromatic hydrocarbon type biaryls (Fig. 5). Therefore, the cofactor binding domain on the enzyme is not known at this time.

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