Cloning, Sequencing, and Expression of the *Pseudomonas putida* Protocatechuate 3,4-Dioxygenase Genes

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The genes that encode the α and β subunits of protocatechuate 3,4-dioxygenase (3,4-PCD [EC 1.13.11.3]) were cloned from a *Pseudomonas putida* (formerly *P. aeruginosa*) (ATCC 23975) genomic library prepared in λ phage. Plaques were screened by hybridization with degenerate oligonucleotides designed using known amino acid sequences. A 1.5-kb *Sma*I fragment from a 15-kb primary clone was subcloned, sequenced, and shown to contain two successive open reading frames, designated *pcaH* and *pcaG*, corresponding to the β and α subunits, respectively, of 3,4-PCD. The amino acid sequences deduced from *pcaHG* matched the chemically determined sequence of 3,4-PCD in all except three positions. Cloning of *pcaHG* into broad-host-range expression vector pKMY319 allowed high levels of expression in *P. putida* strains, as well as in *Proteus mirabilis* after specific induction of the plasmid-encoded *nahG* promoter with salicylate. The recombinant enzyme was purified and crystallized from *P. mirabilis*, which lacks an endogenous 3,4-PCD. The physical, spectroscopic, and kinetic properties of the recombinant enzyme were indistinguishable from those of the wild-type enzyme. Moreover, the same transient enzyme intermediates were formed during the catalytic cycle. These studies establish the methodology which will allow mechanistic investigations to be pursued through site-directed mutagenesis of *P. putida* 3,4-PCD, the only aromatic ring-cleaving dioxygenase for which the three-dimensional structure is known.

The catabolic pathways for bacterial degradation of aromatic compounds converge, in most instances, on a small group of single-ring aromatic compounds. This group includes protocatechuate, catechol, gentisate, and a few other, similar compounds (9, 11, 12, 22, 34). The aromatic ring of these compounds is opened during reactions catalyzed by dioxygenase enzymes in which both atoms of oxygen from O_2 are incorporated into the substrate. These enzymes usually contain nonheme iron stabilized in either the Fe(II) or Fe(III) oxidation state (34, 37, 43, 44). All of the known Fe(III)-containing dioxygenases that function in these pathways utilize catecholic substrates and specifically catalyze reactions that result in ring cleavage between the vicinal hydroxyl functions to yield dicarboxylic acid products (5, 18, 31, 51). Protocatechuate 3,4-dioxygenase (3,4-PCD [EC 1.13.1.3]) was among the first of the Fe(III) dioxygenases to be recognized (18, 51) and has served, along with pyrocatechase (31), as one of the prototypical enzymes for this large class. 3,4-PCD is part of the β -ketoadipate pathway in *Pseudomonas putida* (8, 40, 52) (Fig. 1). This pathway catalyzes the conversion of protocatechuate (PCA) to β -ketoadipyl coenzyme A and subsequently to citric acid cycle intermediates.

3,4-PCD has been isolated from many widely divergent bacteria (6, 13, 18, 34, 56), and its elaboration has been used as a taxonomic characteristic in classifying bacterial strains (53). All of the well-characterized 3,4-PCDs are composed of equimolar amounts of two nonidentical subunits, termed α and β , organized as $\alpha\beta$ protomers. The number of $\alpha\beta$ protomers present in the enzymes from different bacteria is quite variable, with a known range of 3 to 12 (34). Soon after this general quaternary structure was recognized (33, 60), each of the subunits of *P. putida* 3,4-PCD (classified as *P.*

aeruginosa at the time) was chemically sequenced (26, 27, 29, 30). Subsequently, we reported the crystal structure of this enzyme (38). This is the only structure of a mononuclear nonheme iron-containing dioxygenase known. On the basis of this crystal structure, the enzyme from *P. putida* is known to have an $[\alpha\beta\text{Fe(III)}]_{12}$ quaternary structure (38, 39) in which the active site is formed at the interface of the subunits within the protomer unit.

A wide variety of spectroscopic studies have also been applied to 3,4-PCD (reviewed in references 34, 43, and 44). These studies allowed independent evaluation of the iron ligation and coordination geometry, which was later confirmed by the crystal structure. Also, these studies, together with kinetic studies, led to the proposal of a novel mechanism for this enzyme class in which chelation of the active site Fe(III) by the substrate engenders activation of the catecholic ring, thereby allowing it to react directly with dioxygen (34, 41, 43, 45, 55). Together, the structural, spectroscopic, kinetic, and mechanistic studies of 3,4-PCD have firmly established mononuclear nonheme iron-containing enzymes as a unique class which is now known to include many types of enzymes other than dioxygenases (34, 42-44).

The structural, spectroscopic, and mechanistic studies of 3,4-PCD have advanced to the stage at which site-directed mutagenesis and random mutagenesis are required to test and extend the current understanding of this prototypical intradiol dioxygenase. Although the genes for 3,4-PCD have been cloned and expressed from both *P. cepacia* (61, 62) and *Acinetobacter calcoaceticus* (24), their use in mutational studies would be limited by the amount and type of physical and mechanistic data available for the 3,4-PCDs encoded by these genes. In addition, the recombinant enzymes from these sources were not overexpressed or extensively characterized. In contrast, the largest and most complete body of protein chemical, kinetic, spectroscopic, and mechanistically related data has been obtained for the *P. putida* enzyme

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FIG. 1. Metabolism of 4-HBA and PCA via the β -ketoadipate pathway in *P. putida*. CoA, coenzyme A.

(34, 43, 44), and these data will form the basis for evaluation of the effects of mutations. Moreover, only in the case of the *P. putida* enzyme can the X-ray crystal structures of mutants be obtained readily by molecular replacement techniques based on the structure of the native enzyme. In this report, the methods used to clone, sequence, and overexpress the *pcaHG* genes in an expression system that will facilitate future mutagenesis studies are described. We demonstrate for the first time that recombinant 3,4-PCD overexpressed in a foreign host is identical to 3,4-PCD isolated from *P. putida*.

MATERIALS AND METHODS

Bacterial strains. The source of genomic DNA was *P. putida* ATCC 23975. *Escherichia coli* P2392 has the genotype e14 (*mcrA*) *hsdR514 supE44 supF58D(lacIZY)6 galK2 galT22 metB1 trpR55* (P2 lysogen) (50). *E. coli* JM101 has the genotype *D(lac-proAB) supE thi* (F' *traD36 proAB⁺ lacI^qDM15*) (36). *E. coli* RR1 has the genotype F⁻ *mcrB mrr hsdS20(r_B⁻ m_B⁻) leuB6 recA hisB ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^r) supE44* (14). *E. coli* ECY5058 is strain HB101 (*hsd20 recA13 proA2 leuB6 thi-1*) (3) carrying pRK2013 (16). *P. putida* KT2440 (r⁻ m⁺) is a spontaneous restriction-negative derivative of strain mt-2 and was a gift from Michael Bagdasarian, Michigan State University (1). *Proteus mirabilis* (clinical isolate) was a gift from Beulah Gray, University of Minnesota. *P. putida* F1 was a gift from David Gibson, University of Iowa (21). *A. calcoaceticus* ADP1 was a gift from Nicholas Ornston, Yale University (28).

Culture conditions. *P. putida* ATCC 23975 was grown at 30°C in a medium containing 4-hydroxybenzoate (4-HBA) as the sole carbon source (18) or at 30°C in Luria broth (LB; 47). *E. coli*, *A. calcoaceticus*, and *P. mirabilis* were grown at 37°C in LB. *P. putida* KT2440 and *P. putida* F1 were grown at 30°C in LB. Antibiotics were used in selection media at the following concentrations: ampicillin, 150 μ g/ml; tetracycline, 10 μ g/ml for *E. coli*, 10 to 25 μ g/ml for *A. calcoaceticus*, 50 μ g/ml for *P. putida*, and 75 μ g/ml for *P. mirabilis*; kanamycin, 50 μ g/ml. Lactose phenotypes were screened on LB plates containing 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) per ml and 20 mM isopropyl- β -D-thiogalactopyranoside (IPTG).

DNA modification. Restriction endonucleases and modifying enzymes were supplied by GIBCO BRL, New England BioLabs, and Promega. All restriction digests and DNA modifications were carried out as recommended by the supplier. Linkers were supplied by New England BioLabs.

Oligonucleotide synthesis and labeling. All oligonucleotides were synthesized and purified by National Biosciences Inc., Plymouth, Minn. Probes ID (GA⁵GG⁵GA⁵AC⁵GT⁵CTCTCGACTTC) and IIB (GA⁵ATGAACAACGC⁵AACCC⁵CA

TGGA⁵TGC) proved to be the most suitable for cloning. Each consisted of oligonucleotide pools representing 32- and 16-fold degeneracies, respectively.

The oligonucleotides were 5' end labeled with ³²P (47). Typically, 150 μ Ci of [γ -³²P]ATP (NEN Products, Boston, Mass.) was used to label each oligonucleotide. The labeled oligonucleotides were purified on Nacs 0.52 Prepac columns as described by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.).

DNA preparation. *P. putida* genomic DNA was prepared (47) from saturated cultures in which 4-HBA was the sole carbon source, and it was purified by CsCl-ethidium bromide density gradient centrifugation. Plasmid DNA was isolated and purified by standard methods (47).

DNA hybridization. *P. putida* genomic DNA fragments were electrophoresed and transferred to GeneScreen Plus nylon membrane (NEN Products) (47). Prehybridizations were performed at room temperature for 30 min, while hybridizations were allowed to shake at room temperature in a water bath overnight. The membranes were washed for 15 min at room temperature in each of the following solutions: 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M Na citrate)–0.1% sodium dodecyl sulfate (SDS), 0.5 $\times$ SSC–0.1% SDS, and 0.1 $\times$ SSC–0.1% SDS. Membranes probed with IIB were additionally washed as described above in 0.1 $\times$ SSC–0.1% SDS at 45°C. Those probed with ID were similarly washed at 46°C. Labeled membranes were subjected to autoradiography at –80°C.

Low-stringency hybridization with *pcaHG* as the probe was performed as described above, except that the hybridization solution contained 10% dextran sulfate. Duplex DNA was labeled by the random priming method (15). The 1.6-kb *Sma*I fragment from pRWF100 (which contains *pcaHG*) was gel purified and labeled for use as a gene probe. Typically, 10 ng of DNA was labeled and used in each hybridization. The labeled probe was boiled for 10 min before addition to the hybridization solution. Membranes were washed for 15 min in 2 $\times$ SSC–0.1% SDS at room temperature and then again in fresh 2 $\times$ SSC–0.1% SDS for an additional 30 min.

Phage λ library. A *P. putida* λ phage genomic library was prepared by Stratagene Cloning Systems. Lawns of 150 to 300 plaques were obtained by infecting *E. coli* P2392 with the recombinant λ phage library as described by the manufacturer. Plaques were lifted onto GeneScreen Plus colony-plaque hybridization nylon membranes (NEN Products). Plaque lifts were prepared for hybridization as described by the manufacturer, while prehybridizations, hybridizations, and autoradiographies were done as described earlier for probes ID and IIB.

Recombinant λ phage DNA was amplified as described in *Titering, Amplifying and Screening Libraries*, supplied by Stratagene, with the following modifications. Phage adsorption was accomplished by incubation of *E. coli* P2392 and phage for 30 min in a 38°C water bath. Instead of plating the phage-adsorbed cells, we used them to inoculate 37 ml of NZCM (0.01% NZamine A [Difco], 0.005% NaCl, 0.002% MgSO₄, 0.1% Casamino Acids [Difco]) in a 250-ml Erlenmeyer flask. The culture was incubated at 38°C with shaking for 8 to 12 h or until noticeable clearing occurred. Phage DNA was isolated (47) and further purified by CsCl-ethidium bromide density gradient centrifugation.

Plasmid constructions. Plasmid pT7/T3 α -18 was purchased from GIBCO BRL and used as a general cloning vector, as well as a source of single-stranded DNA for sequencing. Plasmid pRWF100 was constructed by cloning a 7.6-kb *Eco*RI-*Bam*HI fragment from λ phage (*pcaHG*) into pT7/

T3 α -18. Cloning of a 1.6-kb *Sma*I fragment containing *pcaHG* from pRWF100 into pT7/T3 α -18 generated pRWF102. Plasmid pRWF103 is the same as pRWF102, except that the *Sma*I fragment is cloned in the opposite orientation. The latter two constructs were used to sequence both strands of *pcaHG*.

Plasmid pMMB66EH, a *Pseudomonas* expression vector, was a gift from Michael Bagdasarian (20). The *pcaHG* gene cluster was cloned into this expression vector in the following way. First, plasmid pRWF121 was generated from pRWF102 by digestion with *Kpn*I and *Spe*I, blunting with mung bean nuclease, and religation. This resulted in removal of a 150-bp segment to leave *pcaHG* with 100 bp of the 5'-flanking sequence. The final construct, pRWF111, was created by cloning a 1.4-kb *Eco*RI-*Bam*HI fragment, containing the entire *pcaHG* insert from pRWF121, into pMMB66EH.

Plasmid pKMY319, a broad-host-range expression vector, was a gift from Kwang-Mu Yen, Amgen Corp. (58). Cloning of the *pcaHG* gene cluster into this vector involved the following plasmids and steps. Plasmid pNH18a was purchased from Promega Corp. and used as a cloning vehicle. Plasmid pRWF110 was constructed by isolating the 1.6-kb *Sma*I fragment from pRWF100 and ligating *Xho*I linkers to its ends. The product was then digested with *Spe*I and *Xho*I and cloned into pNH18a. A hybrid oligonucleotide identical to the 21 bp immediately upstream of *pcaHG* was prepared by heating a 1:1 molar ratio of the complementary oligonucleotides (CGATTCCAAGAGAGGAAACCGGCATG) and (TAAGGTTCTCTCCTTTGGCC) at 80°C for 2 min and allowing the mixture to cool at 23°C. The duplex DNA product contains *Cla*I and *Sph*I overhangs, which allowed it to be easily cloned into pRWF110. Removal of the 100-bp *Cla*I-*Sph*I fragment from pRWF110, followed by its replacement with the hybrid oligonucleotide, resulted in pRWF115, which carries *pcaHG* without any upstream sequences, except the putative *pcaH* ribosomal binding site. Cloning of the 1.3-kb *Cla*I-*Xho*I fragment from pRWF115 into pKMY319 resulted in expression vector pRWF113.

DNA sequencing. The genes that encode 3,4-PCD were sequenced by the dideoxy method (48) with a Sequenase 2.0 kit (USB) and [³⁵S]dATP (NEN Products) as specified by USB. Single-stranded DNAs used as templates in sequencing reactions were prepared from *E. coli* JM101, harboring pRWF102 or pRWF103, by using helper phage M13KO7 (Bio-Rad, Richmond, Calif.) (47). Compressions were eliminated either by using dITP with Sequenase 2.0 or by using 7-deaza dGTP with a Taquence 2.0 kit (USB). The sequence data obtained were analyzed by using the IntelliGenetics Suite (IntelliGenetics Inc., Mountain View, Calif.).

Transformation and mating. Transformation of *E. coli* strains was accomplished by the CaCl₂ method (23, 47). Transfer of expression plasmids from *E. coli* to *P. putida* and *P. mirabilis* were performed with *E. coli* ECY5058 as the mediator (59). Transconjugants were selected on M9 (47) plates supplemented with 0.2% glucose and 50 μ g of tetracycline per ml for *P. putida* and *P. mirabilis*. *A. calcoaceticus* matings were plated at 37°C, while transconjugants were selected on Hutners minimal medium (10) supplemented with succinate at 10 mM and tetracycline at 10 μ g/ml.

Expression. Expression of 3,4-PCD in the various strains was performed in 500 ml of LB in 1-liter Erlenmeyer flasks. A 0.5-ml aliquot of a saturated culture composed of a given strain carrying pRWF113 was used to inoculate the 500-ml culture. The growth medium for expression included tetracycline, ferrous ammonium sulfate at 50 mg/liter, and the

inducer, sodium salicylate, at 0.35 mM for all strains except *P. mirabilis*, which required 75 mM sodium salicylate. Cells were grown until saturation and harvested by centrifugation. Pellets were resuspended in a volume of 50 mM Tris-HCl (pH 8.5) that was twice the wet weight of the cells. Cells were disrupted on ice with a Branson Sonic Power Sonifier 350 cell disrupter. Expression of 3,4-PCD in *P. putida* KT2440 carrying pRWF111 was done in 500 ml of 4-HBA medium. Cultures were harvested, and cells were disrupted as described above.

Large-scale expression of 3,4-PCD from *P. mirabilis* was achieved in 15-liter carboys of "terrific broth" (47) containing 50 mg of ferrous ammonium sulfate per liter, 1% glycerol, 75 μ g of tetracycline per ml, and 75 mM sodium salicylate. A 1-ml saturated culture of *P. mirabilis* carrying pRWF113 was used to inoculate 1 liter of the supplemented terrific broth. The culture was grown to saturation with shaking at 37°C. A 200-ml volume of the saturated culture was used to inoculate each carboy, which was then vigorously aerated by passing air through a sterile filter. Cultures were harvested at an A₅₅₀ of 1.2 to 2.0 by first reducing the volume of the cultures to 2 to 6 liters on an Amicon DC30P Ultrafiltration system equipped with an MP01 hollow filter cartridge. Bacteria were pelleted by centrifugation at 4°C, frozen on dry ice, and stored at -80°C.

Purification of recombinant 3,4-PCD. Purification of the recombinant enzyme was done as previously described (46), with the following modifications. The final temperature of the heat step was 55°C, and microcrystals were obtained between 0.18 and 0.22 g of ammonium sulfate per ml. The microcrystallization process was repeated twice.

Enzyme assays and protein characterization. Enzymatic activity was assayed by monitoring oxygen consumption with an oxygen-sensitive electrode (57). Protein concentration was determined by the method of Bradford with bovine serum albumin as the standard (4) or by measuring the A₂₈₀ with the value of 1.32 ml cm⁻¹ mg⁻¹ at pH 8.5 (18). Determination of iron content per mole of enzyme was performed by first precisely determining the total protein by quantitative amino acid analysis at the Microchemical Facility at the University of Minnesota. The total iron content of an aliquot of the same sample was then determined by inductively coupled plasma emission spectroscopy with an Applied Research Laboratories QA137 spectrometer at the Soil Sciences Center, University of Minnesota.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed by standard techniques (32). About 20 μ g of protein was preincubated in 40 μ l of loading buffer (8.0 M urea, 1.0 M dithiothreitol, 5% SDS, bromophenol blue) at 100°C for 10 min. Gels were stained with Coomassie blue (50% methanol, 10% acetic acid, 0.07% Coomassie blue R-250) and destained with a solution of 30% methanol and 10% acetic acid.

Steady-state kinetics. 3,4-PCD activity was assayed by using an O₂ electrode as described above. The reaction mixture contained 1.5 ml of 50 mM Tris-HCl (pH 7.5) and 20, 40, or 50 μ l of various concentrations (60 μ M to 3 mM) of PCA. The reaction was initiated by injecting 10 μ l of enzyme (stock = 0.375 mg/ml) into the chamber, and the initial velocity was recorded. The *K_m* and *V_{max}* were determined by nonlinear regression fitting by using the program KFIT, developed by Neil C. Millar (King's College, London, United Kingdom).

Optical measurements. Optical spectra were recorded at 4°C in 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM β -mercaptoethanol by using a Hewlett-Packard UV-visible-light diode array spectrophotometer. The enzyme (9.37

mg/ml, 0.5 ml) was made anaerobic by several cycles of evacuation and argon flushing and then transferred to an argon-flushed, serum-stoppered cuvette. After the spectrum was recorded, a 10- μ l aliquot of an anaerobic 500 mM solution of the relatively slow alternate substrate dihydroxyphenylpropionate was transferred to the cuvette to produce the enzyme-substrate (ES) complex. An ES-O₂ complex (probably best described as a transient enzyme product complex) was formed by bubbling pure O₂ into the ES complex for 30 s at 4°C. After a short incubation period, the spectrum was recorded.

Electron paramagnetic resonance (EPR) measurements. EPR spectra were recorded with a Varian E-109 X-band instrument equipped with an Oxford ESR-910 liquid helium cryostat. Data were digitally recorded and analyzed as previously described (17, 56).

Nucleotide sequence accession number. The nucleotide and amino acid sequences reported here (see Fig. 3) have been assigned GenBank accession number L14836.

RESULTS AND DISCUSSION

Cloning and identification of *pcaHG*. The genes that encode the α (*pcaG*) and β (*pcaH*) subunits of 3,4-PCD were isolated from a genomic library constructed in phage λ . The genes were cloned by plaque hybridization with degenerate oligonucleotides prepared on the basis of known amino acid sequences (26, 27, 29, 30). The most specific probes, ID and IIB (see Materials and Methods), were chosen on the basis of the relatively low codon degeneracy deduced from amino acid sequences near the carboxy termini of the α and β subunits, respectively. Duplicate nylon membrane lifts of plaques that arose from infection of *E. coli* with the genomic library were screened by hybridization with probes ID and IIB. One plaque in a primary screen of 1,200 was found to hybridize with both probes. Secondary and tertiary screens of this plaque strongly suggested that it contained the structural genes of 3,4-PCD. Restriction mapping and Southern hybridization were used to localize the genes on a 7.6-kb *EcoRI*-*Bam*HI fragment. Low-level expression of 3,4-PCD activity in *E. coli* driven by the *lac* promoter of pRWF100 suggested that both structural genes were located on this subclone (data not shown). Probes ID and IIB were used as primers in sequencing reactions involving this subclone to verify that it contained at least part of *pcaH* and all of *pcaG*. Subcloning, restriction mapping, and sequencing of the 1.6-kb *Sma*I fragment confirmed the presence and location of *pcaH* and *pcaG* (Fig. 2).

Nucleotide sequence of *pcaHG*. Both strands of the 1.6-kb *Sma*I subclone were sequenced with successive oligonucleotides by following the strategy shown in Fig. 2. The entire sequence of the *Sma*I subclone is shown in Fig. 3. Translation of two successive open reading frames on this fragment revealed polypeptide sequences nearly identical to the 3,4-PCD sequence derived from chemical amino acid sequencing (26, 27, 29, 30). The only differences involved Asp-216 β , which was identified as an Asn residue, and Asn-59 α and Asn-76 α , which were determined to be Asp residues. At the time the amino acid sequence of the α subunit was determined, it was noted that the repetitive yields for the two residues in question were significantly lower than for the remainder of the sequence (29). This is often indicative of cyclization of the Asn side chain with the peptide backbone in Asn-Gly sequences (see, for example, reference 2), as is the case for both Asn-59 α and Asn-76 α . Spontaneous hydrolysis of this cyclic imide would leave Asp in place of Asn.

FIG. 2. Restriction map of the *EcoRI*-*Bam*HI subclone from λ (*pcaHG*). Also shown are the structural genes, *pcaHG*, and the DNA sequencing strategy. Each arrow represents an overlapping sequence determined with internal synthetic oligonucleotides. Restriction enzyme site abbreviations: Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; Kp, *Kpn*I; Nc, *Nco*I; Ps, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Spe*I.

The apparent misidentification of Asp-216 β cannot be accounted for in this way. Independent determination of the amino acid sequence of the β subunit showed Asp in position 216, in agreement with our DNA sequence (25).

The order of transcription is similar to that of the homologous genes cloned from other sources in that the α subunit

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CCCCGCATGACCACTTGTGCTGCTGCTGCTTTCGCCATTCATGTTGCTCTCCGACCGTGAACAAAGAACAAAGATTAGTCACGGCAG 100
CGTCAGGTGTGAGTCAAGTGTTCATCATTAACCAATTGGTTATGGAATAGTGGTGTTCATTAATTCATGAGCGCGACCTGCCGGAACGTAAAG 200
TCCGCTGACGACGCTGACGCTTCACTAATTCGAAGAGAGAACCGGATCCGCGCCAGGACAAACGCGCTGCTGATCCGCTGATCCGCACTGGCA 300
      N P A Q D N S R F V I R D R M H
CCCCAAAGCCTTACGCTGACTACAAAGCTGCTATTCGCGCTGCGCGCGGACGCACTGTGTCAGCATTCACAGTGTGATCAGCAAGCACTGGTCCG 400
      P K A L T P D Y L T S I A R S P R Q A L V S I P O S I S E T T G P
AATTTTCCACCTGGGCTTGGCGCCACGACCATGACTGCTGTAACCTCAACACGGTGGCTGCAATGGCGAGCGCATCATGTGCGCGCC 500
      N F S H L G F G A H D H L L N F N H G L P I G E R I I V A G R
GCGTGTGACGACGATGCGCAAGCTGTGCGCAACCCCTGCGGATGTGCAAGATGTGCAAGCCACCGCGTGGCTACCGGCAAGAACGACCGTTACT 600
      V V D Q T G K P V P N T L V E M Q A N A G G R Y R H K N O R Y L
GGCACCGTGGACCGCACTTGTGCTGCTGCGCGCTGCTGACCGACGACGCGCTACAGCTCCGACCATCAAGCGCGCGCTGACCGCTGG 700
      A P L D P N F G G V G R C L T D S D G Y Y S F R T I K P G P Y P M
CGCAACCGCGCGACGACGCTGCGCGCGCGCGACATCCACTTCGCGATCAGCGCGCGCTGATTGCGACCAAGETGATCCAGCTGTTGATTTGAGGGTG 800
      R N G P N D M R P A H I H F G I S G P S I A T K L I T O L Y F E G D
ACCGCTGATCCGATGTGCGCGCTGCTCAAGTGTGCGCAACCTGAAGTGTACAGAGTGTGATCCGCAAGTGTGATGAACAAAGCGCAACCGAT 900
      P L I P N C P I V K S I A R P E A V Q L I A K L D M N N A N P H
GGACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1000
      C L A Y R F D I V L R G Q R K T H F E N C E N D N P I E L L P
GGAACCCCTTGCAGACCGCGCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1100
      E T P S Q T A G P Y V H I G L A E A G N P T R D E I M N R L
GCCAAGCGGACGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 1200
      A K P D A P G E H I L L L G Q V Y D G G H L V R O S F L E V M Q A
CGACGCAATGCGAGTATCAGATGCTACAACGCTGCGAGACGCTTCAACAGCTTGGCGCGACCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 1300
      D A G E Y Q D A Y N L E N A F N S F G R T A T T F D A G E M T L
TGCACAGGTGCAAGCGCGGTGTGTGCAACATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1400
      H T V K P G V V N N A A G V P N A P H I N I S L F A R G I N I H L
GCACACGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1500
      H T R L Y F D D E A Q A N A K C P V L N L I E O P Q R R E T L I A
AAGCGTTGCAAGTGTGGAAGAACCGCGTATCGTTTCCATCCGATTCAGCGGGAAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 1600
      K R C E V D G K T A Y R F D I R I O G E G E T V F F D F E N D

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FIG. 3. Nucleotide sequence of the *Sma*I subclone containing *pcaHG*. The underlined sequences upstream of the start codons are the putative ribosomal binding sites. Two possible *Pseudomonas* promoters are indicated by lines over the relevant bases. Amino acids which differ from the sequence derived chemically are boxed.

gene follows the β subunit gene (Fig. 3) (24, 62). The putative Shine-Dalgarno sequence (49) of *pcaG* (Fig. 3) includes the last two bases of the stop codon for *pcaH*. The putative Shine-Dalgarno sequences are identical in sequence, as well as in distance from their respective translational start sites. Analysis of the region upstream of the open reading frames revealed that it may contain two possible *Pseudomonas* promoters, which are indicated in Fig. 3. The one nearest *pcaH* is 75% identical to a proposed consensus sequence for *P. putida* promoters (35), while the other, located between base pairs 150 and 163, is 66% identical.

Expression studies. We originally attempted to express the recombinant enzyme in *E. coli* JM101. The *Sma*I subclone, containing *pcaHG* driven by the *lac* promoter, when induced with IPTG, resulted in a specific activity of 0.02 U/mg. The uninduced specific activity was <0.002 U/mg, the minimum detectable rate. Thus, expression was achieved, albeit at a low level compared with wild-type expression in *P. putida* grown in 4-HBA medium. Other *E. coli* expression systems, such as *tac* and T7, also failed to yield high levels of expression. The level of expression from these systems was not affected by inclusion of 250, 100, or 0 bp of the *pcaHG* upstream sequence. This failure to express at high levels in *E. coli* could result from the high 62% G+C content of the genes, compared with 50% for *E. coli* genes. Alternatively, the pattern of codon usage differs significantly between *E. coli* and *Pseudomonas* sp., with the latter preferring codons with the strongest predicted codon-anticodon interaction (54). This pattern could result in codon bias, which may limit expression at the level of translation.

Since high levels of expression of recombinant 3,4-PCD were required for characterization, expression systems that function in *P. putida* were investigated. Expression of the recombinant enzyme in *P. putida* was performed under growth conditions in which the endogenous enzyme is not expressed (i.e., on rich carbon sources in the absence of 4-HBA and PCA). Initial attempts with broad-host-range expression vector pMMB66EH (20), which utilizes the *E. coli* *tac* promoter, were unsuccessful. Induction of the *tac* promoter in pRWF111, which contains *pcaHG* including 100 bp of the upstream sequence, with 5 mM IPTG resulted in levels of expression similar to that observed in *E. coli* with the *tac* or T7 promoter. However, use of an expression vector that incorporates a *Pseudomonas* promoter allowed high levels of expression. This expression vector, pKMY319 (58), features the strong *nahG* promoter, which is induced by salicylate and is associated with the *Pseudomonas* naphthalene degradation pathway. Construct pRWF113 (Fig. 4), analogous to pRWF111 containing *pcaHG* but lacking all of the 100-bp upstream sequence except for the Shine-Dalgarno sequence, gave a specific activity of 0.41 U/mg in *P. putida* ATCC 23975 when it was induced with salicylate. No activity was detected in the absence of the inducer or in bacteria induced without the plasmid present.

High levels of 3,4-PCD expression from pRWF113 were achieved in a number of other pseudomonads, as well as in a *P. mirabilis* strain which does not contain an endogenous 3,4-PCD (see below) (Table 1). The highest levels of expression were obtained in pseudomonads grown in LB containing 0.35 mM sodium salicylate. These levels were similar to that measured when *P. putida* KT2440 and F1 alone were grown in a medium containing 4-HBA as the sole carbon source. In contrast, these same levels are 10-fold lower than that obtained from *P. putida* ATCC 23975 when grown on 4-HBA (18). Levels of expression approaching that seen in most pseudomonads were measured in *P. mirabilis*, although

FIG. 4. Schematic of broad-host-range expression construct pRWF113, containing *pcaHG* flanked only by its ribosomal binding site. Induction of the *nahG* promoter with salicylate produced recombinant 3,4-PCD in amounts easily purified. Restriction enzyme site abbreviations: Bs, *BsrEII*; C, *ClaI*; Sa, *SalI*; Sp, *SphI*; Xh, *XhoI*.

the concentration of inducer required was 200-fold greater than for the pseudomonads. Expression of functional 3,4-PCD in a foreign host such as *P. mirabilis* suggests that the iron ligated to the protein may insert spontaneously without the need for modifying enzymes. It is not known, however, whether iron ligation initiates or follows protein folding.

Other bacterial strains that express an endogenous 3,4-PCD failed to express the recombinant enzyme. For example, *A. calcoaceticus* was able to maintain the expression vector, but no measurable 3,4-PCD activity was observed in the presence or absence of the inducer, sodium salicylate (Table 1).

Ideally, expression of a recombinant enzyme should be performed in a host that does not produce the same enzyme or an isozyme. Unfortunately, all of the pseudomonads shown to express recombinant 3,4-PCD, grow in media containing 4-HBA as the sole source of carbon and possess an endogenous 3,4-PCD that is physically indistinguishable from the enzyme encoded by *pcaHG*. *P. mirabilis*, on the other hand, does not grow in a medium containing 4-HBA as the sole source of carbon, and the presence of 4-HBA or PCA does not induce measurable 3,4-PCD activity. In a

TABLE 1. Expression of the *pcaHG* gene cluster of *P. putida* from plasmid vector pKMY319

Bacterial strain ^a	3,4-PCD sp act ^b (μmol/min/mg)		
	Vector only ^c	Uninduced	Induced
<i>Pseudomonas putida</i> KT2440	<0.002 ^d	<0.002	0.35
<i>Pseudomonas putida</i> F1	<0.002	<0.002	0.41
<i>Pseudomonas putida</i> ATCC 23975	<0.002	<0.002	0.41
<i>Proteus mirabilis</i> ^e	<0.002	<0.002	0.15
<i>Acinetobacter calcoaceticus</i> ADP1	<0.002	<0.002	<0.002

^a All of the bacterial strains contained recombinant plasmid pRWF113, which is pKMY319 carrying the *pcaHG* gene cluster from *P. putida* ATCC 23975. All cultures were grown in LB to an optical density at 550 nm of 2 to 4 in the presence or absence of 0.35 mM salicylate.

^b Determined as described in Materials and Methods.

^c In the presence or absence of salicylate under the conditions described in Materials and Methods.

^d Activity was below the detection limit under the assay conditions described in Materials and Methods.

^e This strain was induced in the presence of 75 mM salicylate.

further effort to demonstrate that *P. mirabilis* contains no endogenous 3,4-PCD, genomic DNA from *P. mirabilis* was subjected to low-stringency Southern hybridization with the 1.6-kb *Sma*I (*pcaHG*) fragment (Fig. 2) as a probe. As anticipated, *P. mirabilis* did not contain DNA sequences that cross-hybridize with *pcaHG* under conditions of low stringency (data not shown).

Purification and characterization of recombinant 3,4-PCD. Recombinant 3,4-PCD was overexpressed in *P. mirabilis*. Salicylate, the specific inducer of expression construct pRWF113, is toxic to *P. mirabilis* at concentrations exceeding 75 mM. Therefore, growth conditions in which a balance was created between biomass and specific activity were defined. *P. mirabilis* grew well when the concentration of salicylate was less than 75 mM. The biomass increased from 2 g/liter at 75 mM salicylate to 2.5 g/liter, at 35 mM salicylate, but greater than half of the 3,4-PCD specific activity was lost. Thus, 75 mM salicylate was chosen as the best inducer concentration for growth and expression.

Purification of the recombinant enzyme from *P. mirabilis* was identical, in most aspects, to the purification of 3,4-PCD from *P. putida* (46). This procedure is very efficient, consisting of a series of selective bulk precipitations followed by successive crystallizations. The recombinant enzyme required somewhat more time to crystallize than the enzyme crystallized from *P. putida*. However, such procedures are very sensitive to the amount and nature of the other proteins present, and thus it is not surprising that somewhat different results were obtained with extracts from *P. mirabilis*. The size, shape, and color of the crystals of 3,4-PCD derived from *P. mirabilis* and *P. putida* were indistinguishable. The overall purification procedure for the recombinant enzyme resulted in 400-fold purification and 40 to 50% recovery. Typically, 15 to 20 mg of pure enzyme was obtained from 100 g of cells.

Physical characterization of the recombinant enzyme revealed no differences between it and the wild-type enzyme. The fact that the enzyme crystallizes in the same form as the wild type suggests that it has the same overall structure and surface features. SDS-PAGE showed the same pattern for the wild-type and recombinant enzymes (Fig. 5) and revealed no evidence of heterogeneity. The bands at 22,300 and 26,600 correspond to the α and β subunits of 3,4-PCD, respectively. The band at 48,900 corresponds to non-denatured $\alpha\beta$ dimers and is a consequence of the extremely high affinity between these two subunits (60). Table 2 lists a variety of physical and kinetic parameters measured for the recombinant enzyme, as well as the corresponding literature-derived values of the wild-type enzyme. A specific activity of 57 U/mg compares well with that obtained by Fujisawa and Hayaishi (18) and in our past studies (46). The measured K_m , turnover number, λ_{max} , and extinction coefficient are nearly the same as those previously reported for the wild-type enzyme. Iron and total protein analysis revealed 8.4 Fe atoms per mol of enzyme, which is also very close to the literature-derived value. It is interesting that the iron contents are equally low in the wild-type and recombinant enzymes. The crystal structure shows that there are 12 identical sites for iron ligation in the fully assembled enzyme; however, in no case has more than 6 to 8 Fe(III) atoms per mol been reported for the enzyme as isolated. Thus, it is possible that despite the structural equivalence of the individual sites in the crystal, the quaternary structure in solution leads to static or dynamic inequivalence.

Spectroscopic analysis also indicated that the recombinant enzyme is identical to the wild-type enzyme. Figure 6 shows

FIG. 5. SDS-9 to 18% PAGE of purified recombinant 3,4-PCD. Lanes: 1, wild-type 3,4-PCD purified from *P. putida*; 2, recombinant 3,4-PCD purified from *P. mirabilis*. Molecular weights estimated from standards are indicated at the left.

optical spectra of the purified recombinant enzyme in various states. The enzyme as isolated showed the same characteristic red chromophore (λ_{max} , 450 nm) as the wild-type enzyme (18). This spectrum is due to charge transfer to the Fe(III) from its two tyrosine ligands (42), showing that the proper iron ligation had been established. Formation of the anaerobic enzyme-substrate complex with dihydroxyphenylpropionate, an alternate substrate of 3,4-PCD, resulted in the expected red shift of the λ_{max} of the optical spectrum and an increased extinction coefficient. Addition of O_2 caused a transient further red shift in the λ_{max} , to 520 nm, and a decrease in the extinction coefficient characteristic of an enzyme product complex termed ES- O_2 (7, 19). After several minutes, the O_2 was exhausted by turnover and the spectrum of the anaerobic ES complex was reestablished. The time scales of formation for these complexes were the same as those observed during turnover of dihydroxyphenylpropionate by the wild-type enzyme under the same conditions (19). This demonstrates that not only are the kinetics of turnover the same but the easily trapped transient intermediates in the catalytic cycle are apparently also identical.

The high-spin ($S = 5/2$) Fe(III) in the active site of 3,4-PCD has a characteristic EPR spectrum which is dominated by a narrow signal at $g = 4.28$ arising from a transition within the first excited-state Kramers doublet of the spin

TABLE 2. Physical characteristics of wild-type and recombinant 3,4-PCD

3,4-PCD	λ_{max} (nm)	ϵ^a	Mol of Fe/mol of enzyme	K_m for PCA (μ M)	TON ^b
Wild type ^c	50	3.20	6.0	30	70
Recombinant ^d	450	2.86	8.4	29	68

^a Extinction coefficient at 450 nm (per centimeter per millimolar per Fe³⁺ molecule).

^b TON, turnover number (per second per site at 25°C).

^c References 18 and 34.

^d Expressed in *P. mirabilis* via pRWF113.

FIG. 6. Optical spectra of recombinant 3,4-PCD. All spectra were recorded at 4°C in 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM β -mercaptoethanol. —, enzyme (10 μ M) as isolated and made anaerobic; ----, enzyme plus the alternate substrate dihydroxyphenylpropionate (10 mM); - · - · -, ES complex recorded immediately after bubbling with O_2 .

system. A weaker signal from the lowest-energy doublet is also observed at $g \approx 9.6$. Small changes in the environment of the Fe(III) are known to cause dramatic changes in the line width and/or position of these signals (41, 46, 55). Figure 7 shows the EPR spectrum of the recombinant enzyme as isolated. Both the resonance positions and relative intensities of the EPR features are typical of 3,4-PCDs we have studied previously (46, 56).

Taken together, the physical and spectroscopic characterizations show for the first time that recombinant 3,4-PCD is indistinguishable from the wild-type enzyme found in *P. putida*. We have developed an expression system which will allow reliable purification of milligram quantities of recombinant 3,4-PCD for study. Although we could obtain somewhat more enzyme by using *P. putida* as the host, the use of *P. mirabilis* eliminates the possibility of a wild-type 3,4-PCD background in future site-directed mutational studies, as well as the danger of recombinational repair of a given mutant allele by the wild-type gene present on the chromosome. With this system now well characterized, we are ready to begin directed mutational studies in combination with crystallographic and chemical studies from which we hope to gain a better understanding of the catalytic mechanism of this prototypical dioxygenase.

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FIG. 7. EPR spectrum of recombinant 3,4-PCD purified from *P. mirabilis*. The spectrum was recorded at the X band (9.2 GHz) under the following conditions: temperature, 10 K; modulation amplitude, 10 G; microwave power, 0.2 mW. The inset at $g = 9.67$ was recorded at 2 K to enhance the ground state signal. The g values and gain multipliers (after Curie law corrections) are shown. Under the conditions used, the wild-type enzyme would exhibit a spectrum with a ratio of $g = 4.3$ to 9.6 signal intensities of 230 as observed here for the recombinant enzyme. Adventitiously bound iron, impurities, and modified iron environments generally appear as "wings" on the $g = 4.3$ signal; the physical basis for this is well understood (46, 56). The minor amounts of such signals seen here may account for the slightly higher iron content and consequently lower extinction coefficient of the recombinant enzyme.

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