2,3-Dihydroxybiphenyl 1,2-dioxygenase, an enzyme of the biphenyl biodegradation pathway that cleaves the first of the aromatic rings, was purified to apparent homogeneity from *Pseudomonas* sp. strain LB400 that had been engineered to hyperexpress the *bphC* gene. The enzyme had a subunit molecular mass of 33.2 kDa as determined by SDS-polyacrylamide electrophoresis. Kinetic studies indicate a $K_M$ of $7 \pm 1 \mu M$ for 2,3-dihydroxybiphenyl. The enzyme is strongly inhibited by substrate ($K_S = 300 \pm 10 \mu M$). Catechol, 3-methylcatechol, and 4-methylcatechol were cleaved less efficiently and showed weaker substrate inhibition. 3,4-Dihydroxybiphenyl was not a substrate for the enzyme. Ammonium sulfate and polyethylene glycol 6000 were used as precipitants to obtain two different crystal forms. Crystals grown from ammonium sulfate and polyethylene glycol 6000 had space groups of $P4_2212$ and $P4_212_2$, respectively. Electron microscopy indicates that the enzyme is an octamer (265 kDa) consisting of subunits arranged in two planar tetramers in a staggered conformation.

Extradiol dioxygenases constitute a class of non-heme ferrous iron-containing enzymes that cleave aromatic compounds adjacent to hydroxyl groups located on dihydroxylated aromatic rings. These enzymes occur in a number of bacterial degradation pathways, including those of toluene, naphthalene, protocatechuate, and biphenyl (1) and dibenzofuran (2, 3). Chemical mutagenesis has shown that the enzyme from the toluene degradation pathway, catechol 2,3-dioxygenase, plays a key role in determining the substrate specificity of the pathway (4). Despite their widespread occurrence and relevance to the degradation of environmentally toxic compounds, extradiol dioxygenases remain relatively poorly characterized, in part due to the instability of the ferrous center and the latter's relative lack of spectroscopic signals. The endogenous iron ligands have yet to be identified, and the mechanism of oxygen activation has yet to be elucidated. No crystal structure of an extradiol dioxygenase has thus far been reported.

2,3-Dihydroxybiphenyl 1,2-dioxygenase is a component of aerobic biphenyl degradation pathways of a number of microorganisms and cleaves 2,3-dihydroxybiphenyl in an extradiol fashion, as shown in Equation 1. Some biphenyl pathways are capable of metabolizing various congeners of PCBs (5), and the sequences of their respective *bph* gene clusters display different degrees of homology (6). *Pseudomonas* sp. strain LB400 displays one of the best capabilities to metabolize congeners of PCBs (7). Characterization of the enzymes of biphenyl degradation pathways is essential to understanding the basis of the substrate specificity of the different pathways as well as in engineering organisms capable of completely mineralizing PCBs. For instance, 3-chlorocatechol, an intermediate that could arise in the degradation of PCBs, is known to be a potent inhibitor of catechol 2,3-dioxygenase (8); and recent evidence indicates that it inhibits 2,3-dihydroxybiphenyl 1,2-dioxygenase of *Achromobacter* sp. strain P6 (9).

We report here the purification and initial characterization of the dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. strain LB400. The ability of the enzyme to degrade different substrate analogues was investigated. Crystals of the enzyme were grown under a number of different conditions. Space groups of these crystals are reported. The quaternary structure of the enzyme was investigated using electron microscopy.

**MATERIALS AND METHODS**

*Strains, Media, and Growth*—2,3-Dihydroxybiphenyl 1,2-dioxygenase was hyperexpressed in the parent strain *Pseudomonas* sp. strain LB400 with pLEBD4, a broad host range expression plasmid containing the structural gene encoding the dioxygenase, as previously described (10).

*Purification*—All procedures were carried out at 4 °C unless otherwise specified. Buffers were made from distilled water purified on a Millipore Milli-Q apparatus to a resistivity of >17 milliohm. Chromatography was performed on a Pharmacia LKB Biotechnology fast protein liquid chromatography system. Buffer A was 10 mM Tris, 10% acetone, pH 7.5. Buffer B contained 10 mM Tris, 1.0 M NaCl, 10% acetone, pH 7.5. Cells from 1 liter of culture were collected by centrifugation in a Sorvall centrifuge and washed with buffer A. The cell pellet was resuspended in 10 ml of buffer A. The cells were disrupted by a single
passage through a French press (Aminco Corp.) at an operating pressure of 20,000 p.s.i. The cell debris was removed by ultracentrifugation at 80,000 rpm for 60 min in a T1270 rotor (Du Pont-New England Nuclear). The clear supernatant fluid was carefully decanted and is referred to as the raw extract.

The raw extract was loaded onto a Mono Q HR10/10 anion-exchange column equilibrated with buffer A. The column was operated at a flow rate of 4.0 ml/min. The enzyme activity was eluted with a linear gradient of 6-2676 buffer B. Fractions of 5 ml were collected. Active fractions were concentrated to 1 ml. Purified 2,3-dihydroxybiphenyl 1,2-dioxygenase >4500 units/ml were combined, dialyzed against buffer A, and concentrated to 1 ml. Purified 2,3-dihydroxybiphenyl 1,2-dioxygenase was stored in buffer A in liquid nitrogen for several months without significant loss of activity.

SDS-polyacrylamide gel electrophoresis was performed on a BioRad Mini-PROTAN II apparatus according to Laemmli (11). Silver staining was performed according to the procedure of Davernal et al. (12). Protein concentrations were determined with a micro bichinonic acid protein assay reagent kit (Fierce Chemical Co.).

**Results**

**Enzyme activity** was measured by following the formation of reaction products using a Beckman DU-70 spectrophotometer equipped with a thermostatted cuvette holder and a Haake circulating water bath. Throughout purification of the enzyme, activity assays were performed in 50 mM phosphate, pH 7.5, with 2.3-p-dihydroxybiphenyl, as previously described (13). In determining the kinetic constants, the measurements were carried out in 50 mM Tris at pH 8.0 and 25.0 ± 0.1 °C. A 100 mM stock solution of 2,3-dihydroxybiphenyl in ethanol was used to make a 1 mM stock solution of the substrate in the assay buffer. Stock solutions and buffers were prepared fresh daily. This stock solution was then diluted with assay buffer to the desired concentration to a total volume of 2.99 ml. The reaction was initiated by the addition of 10 μl of 2,3-dihydroxybiphenyl 1,2-dioxygenase. Kinetic measurements were based on three trials. Data were fit to a substrate inhibition equation (9) using MINSQ (Micromath Inc.), a least-squares fitting routine.

The pH dependence of the molar extinction coefficient of the cleavage product 2-hydroxy-4-oxo-6-phenylhexa-2,4-dienoic acid at 434 nm was determined by preparing a 500-ml solution of the product in 10 mM Tris, pH 8.5; adjusting the pH with microliter quantities of 6 N sodium hydroxide or concentrated hydrochloric acid; and recording both the pH and the absorption spectrum of the solution. Extinction coefficients were calculated based on the published value of 22,000 cm⁻¹ M⁻¹ at pH 9.0 (14).

The pH dependence of the enzymatic activity was determined from pH 7.0 to 8.0 in 50 mM MOPS and from 7.5 to 9.0 in 50 mM Tris. Reaction velocities were determined at a 2,3-dihydroxybiphenyl concentration of 18.5 μM. Relative velocities were calculated using the value obtained at pH 8.0 as the reference. Kinetic measurements with catechol, 3-methylcatechol, and 4-methylcatechol were performed at pH 8.0 in a manner similar to that described for 2,3-dihydroxybiphenyl, except that stock substrate solutions were made up in 50 mM MOPS, pH 8.0. The reactions performed with catechol, 3-methylcatechol, and 4-methylcatechol were monitored at 375, 388, and 382 nm, respectively, using extinction coefficients calculated from Wallis and Chapman (15).

**NH₂-terminal Sequence**—The NH₂-terminal sequence was determined on an Applied Biosystems Model 470A Protein Sequencer.

**Crystallization**—Crystallization was performed using the hanging drop vapor diffusion method (16). Drops of 2-5 μl of 2,3-dihydroxybiphenyl 1,2-dioxygenase were dissolved in 10 mM HEPES, pH 7.2, and air-dried. The electron microscope examinations were done with a Zeiss CEM 902 transmission electron microscope equipped with an integrated electron energy loss spectrometer. Exposures were taken at an electron energy loss of 0 and 115 eV (19).

**RESULTS**

Relevant details of the purification are shown in Table I. The enzyme was estimated to be >95% pure as judged by SDS-polyacrylamide gel electrophoresis followed by silver staining (Fig. 1), which also indicated that the subunit size of 2,3-dihydroxybiphenyl 1,2-dioxygenase is ~33.2 kDa. The amino-terminal amino acid sequence of the protein as determined by Edman degradation is shown in Fig. 2.

Kinetic measurements, performed at substrate concentrations low enough to avoid substrate inhibition using the extinction coefficients presented in Table II, indicate that the pH optimum of the reaction is 8.0. All subsequent kinetic measurements were performed at this pH. A Lineweaver-Burk representation of typical kinetic data is shown in Fig. 3. When 2,3-dihydroxybiphenyl is used as a substrate, the dioxygenase displays strong substrate inhibition. A fit of the data to the equation accounting for substrate inhibition (9) yields a substrate inhibition constant (Kss) of 300 ± 20 μM.

Kinetins performed using either catechol or 3-methylcatechol obey classic Michaelis-Menten kinetics (Table III), with substrate inhibition being observed only at substrate concentrations >0.8 mM. 4-Methylcatechol was cleaved by

![Fig. 1. Silver-stained SDS-polyacrylamide gel of 2,3-dihydroxybiphenyl 1,2-dioxygenase from Pseudomonas sp. strain LB400 showing purified 2,3-dihydroxybiphenyl 1,2-dioxygenase (lane 1) and low molecular mass standards purchased from Bio-Rad (lane 2). The molecular masses (in kilodaltons) of the protein standards are shown on the left.](image-url)

**Fig. 2.** NH₂-terminal amino acid sequence of 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. strain LB400 as determined by Edman degradation.

![Fig. 2.](image-url)
TABLE II
Molar extinction coefficients of meta-cleavage products at the pH values used in this work

Also listed are the relative activities of the 2,3-dihydroxybiphenyl 1,2-dioxygenase for 2,3-dihydroxybiphenyl at the indicated pH values. Values in parentheses represent standard errors of the mean.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wavelength</th>
<th>E</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>mm⁻¹ cm⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td>pH 9.0</td>
<td>434</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>pH 8.5</td>
<td>434</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>pH 8.0</td>
<td>434</td>
<td>17.9</td>
</tr>
<tr>
<td></td>
<td>pH 7.5</td>
<td>434</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>pH 7.0</td>
<td>434</td>
<td>8.0</td>
</tr>
<tr>
<td>Catechol, pH 8.0</td>
<td>375</td>
<td></td>
<td>67.8</td>
</tr>
<tr>
<td>3-Methylcatechol, pH 8.0</td>
<td>388</td>
<td></td>
<td>73.6</td>
</tr>
<tr>
<td>4-Methylcatechol, pH 8.0</td>
<td>382</td>
<td></td>
<td>73.9</td>
</tr>
</tbody>
</table>

FIG. 3. Lineweaver-Burk plot of 2,3-dihydroxybiphenyl cleavage at pH 8.0. The line represents a least-squares best fit of the data to the substrate inhibition equation (see text). The fitted parameters are \( K_M = 6.9 \mu M, \quad K_{so} = 306 \mu M, \) and \( V_{max} = 221 \) nmol/min.

TABLE III
Kinetic constants of 2,3-dihydroxybiphenyl 1,2-dioxygenase with different substrates

Values in parentheses represent standard errors of the mean.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_M ) ( \mu M )</th>
<th>( V_{max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td>7 (2)</td>
<td>1.00</td>
</tr>
<tr>
<td>Catechol</td>
<td>540 (30)</td>
<td>0.08</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>190 (20)</td>
<td>0.05</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4-Dihydroxybiphenyl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Activity too low to be accurately determined.

† No detectable activity.

the enzyme, but too slowly for kinetic parameters to be reliably determined. When 3,4-dihydroxybiphenyl was incubated with the enzyme for 30 min, no change in the absorption spectrum of this compound was observed, indicating that this compound is not cleaved by 2,3-dihydroxybiphenyl 1,2-dioxygenase. These results are summarized in Table III. As the \( V_{max} \) of the reaction is dependent upon the amount of iron that the enzyme contains and atomic absorption spectroscopy measurements indicate that the isolated dioxygenase does not contain its full complement of iron (data not shown), \( V_{max} \) is expressed as a relative value.

Two different crystal forms of 2,3-dihydroxybiphenyl 1,2-dioxygenase were obtained. In 0.9 M ammonium sulfate, 2,3-dihydroxybiphenyl 1,2-dioxygenase grew to a maximum size of \( 1.5 \times 1.5 \times 1.5 \) mm\(^3\) within a few days. They diffract to \( \sim 2.5\) Å resolution, but show a complicated type of disorder. Precession photographs of the \( h0l \) layer show \( 4mmn \) symmetry. All reflections of type \( h + k = 2n + 1 \) are weak and diffuse. \( 0kl \) and \( 1kl \) layers show diffraction patterns with systematic absence of reflections of type \( h + k + l = 2n + 1 \). Indexing the diffraction pattern in space group \( I422 \) by omitting the diffuse reflections is prohibited by the loss of many strong reflections. Therefore, the primitive space group \( P4_22_2 \) with \( a = b = 176.1 \) Å and \( c = 114.0 \) Å has been assigned. This takes into account the diffuse and the well-defined reflections, but leads to a large number of absent or very weak reflections due to the pseudo centering.

In 10% (w/v) polyethylene glycol 6000, 100 mM Tris-HCl, pH 9.0, crystal form II (Fig. 4B) was obtained. These crystals grew, occasionally to a size of \( 0.2 \times 0.2 \times 0.5 \) mm\(^3\), within 3-4 weeks. The space group is \( I222 \) with cell constants \( a = 110.4, \quad b = 112.1, \quad c = 136.5 \) Å. A native data set was collected to 2.9 Å resolution; the R-factor for symmetry-related reflections was 6.0%. Self-rotation functions (20) revealed a local 4-fold axis in parallel to the crystallographic c axis, leading to \( \psi I422 \) symmetry. In space group \( I222 \), the Matthews' parameter value (21) is 3.2 Å\(^3\)/Da assuming two
Initial Characterization of an Extradiol Dioxygenase

Enzyme particles are of uniform appearance in the electron microscope (Fig. 6). In Fig. 6a, the molecules are imaged in the bright-field mode at an electron energy loss of 0 eV and appear as bright shining masses against the greyish stain background. In Fig. 6b, the molecules appear as dark masses due to contrast reversal on imaging at an electron energy loss of 115 eV, specific for uranium. It can be seen from these images that two projections of the molecule predominate. The first of these consists of four distinct protein masses, arranged at the vertices of a square with a dark protein-deficient cavity at the center (Fig. 6, c and f). The edge length of the molecule ranges from 6.5 to 10.1 nm, with an average value of 9.1 ± 0.9 nm (n = 88). These images indicate that four of the subunits of the polymeric dioxygenase are arranged in a planar tetragon. In the second projection, five protein masses arranged in two layers may be discerned. One layer is composed of two distinct protein masses, and the second is composed of three overlapping masses, as indicated by the short lines in Fig. 6d. The latter layer exceeds the corresponding edge length of the former by ~25%. Occasionally, fragments of the molecules resembling dimers can be observed (Fig. 6e). Biochemical studies indicate that the enzyme is a homooctamer of 260 kDa (14). If all eight subunits were posed at the vertices of a cube, then the side and top view projections of the molecule would look identical. From the second projection discussed above, taken to be the side view for convenience of discussion, it is evident that two different layers exist in the polymer. The side view projection could arise if two layers of planar tetragonally arranged subunits are rotated ~45° relative to each other (i.e. they appear staggered from the top view). The spatial relationship of the subunits inferred from the electron microscope images is sketched in the computer models (Fig. 5, f–h).

**DISCUSSION**

We describe the purification of 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas* sp. strain LB400 containing the expression plasmid pLEBD4. This procedure permits the purification of the dioxygenase to >99% homogeneity within 14 h. The determined NH₂-terminal amino acid sequence is identical to the amino acid sequence deduced from the sequence of *bphC*, the structural gene encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase. The molecular mass of the subunit as determined by SDS-polyacrylamide gel electrophoresis corresponds well with that predicted from the DNA sequence (298 amino acids, 32.5 kDa). Electron microscopy of the purified 2,3-dihydroxybiphenyl 1,2-dioxygenase indicates that the enzyme is an octamer in which the subunits are arranged in two tetrameric planes, staggered with respect to one another. The preference for the enzyme for 3-methylcatechol over 4-methylcatechol is consistent with the enzyme's inability to cleave 3,4-dihydroxybiphenyl and indicates that 2,3-dihydroxybiphenyl 1,2-dioxygenase has a preference for catechols with groups immediately adjacent to the hydroxyl substituents.

It has been established that low concentrations of various organic solvents, including ethanol, isopropyl alcohol, glycerol, and acetone, stabilize the activity of extradiol dioxygenases (23). In the case of 2,3-dihydroxybiphenyl 1,2-dioxygenase, acetone was the most effective of these solvents in stabilizing the activity (data not shown). EPR measurements indicate that the ferrous center has *g*-values typical of an extradiol dioxygenase, but it is clear from atomic absorption spectroscopy measurements that the purified enzyme contains only 50% of its full complement of iron (i.e. half the subunits contain no iron) (data not shown). Dilute solutions of the dioxygenase lose activity within days, even in the presence of acetone. It can be reasonably assumed that the loss of activity during the purification procedure (Table 1) is due not only to incomplete recovery of the dioxygenase at each step, but also to inactivation of the enzyme due to oxidation and/or loss of the iron. Nevertheless, the purified 2,3-dihydroxybiphenyl 1,2-dioxygenase reported here has twice the specific activity of a highly homologous enzyme isolated from another pseudomonad (14).

Extradiol dihydroxybiphenyl dioxygenases have been purified from two other sources: *Pseudomonas* sp. strain KF707 (14) and *Pseudomonas putida* (24). The structural gene of the former has been sequenced (25) and shows a single nucleotide

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2. R. Kappel, personal communication.
**FIG. 6.** Electron microscope presentation of negatively stained purified enzyme. Identical survey views of the enzyme particles are shown: the elastic bright field at 0 eV (a) and at 115 eV at the uranium edge (b). Molecules showing four distinct masses represent the top view projection. c, gallery of selected views of enzyme particles in the top view projection at higher magnification. These views reveal four planar tetragonally arranged equivalent protein masses. d, corresponding selected side view projections. Single protein masses of the two layers are indicated by short lines. e, a computer-generated model of the enzyme summarizes the data. The top view projection (f), an intermediate 45° tilted view (g), and the side view projection (h) are simulated. Bars represent 40 nm (a and b) and 20 nm (c–e).

Difference from the bphC gene of *Pseudomonas* sp. strain LB400. This corresponds to a single amino acid substitution in the polypeptide sequence (Ala-185 → Val). The KF707 enzyme was reported to be octameric and to have a substrate specificity very similar to that of the 2,3-dihydroxybiphenyl 1,2-dioxygenase described here (i.e. weaker catalytic activity against catechol, 3-methylcatechol, and 4-methylcatechol, and no activity against 3,4-dihydroxybiphenyl). However, no substrate inhibition and an 11-fold higher $K_M$ value for 2,3-dihydroxybiphenyl were reported. Whether these differences reflect differences in the measurement conditions or the single amino acid difference remains to be established. Substrate inhibition in a dihydroxybiphenyl dioxygenase has been reported for an enzyme originating from *Acinetobacter* sp. strain P6 (9).

The second (*P. putida*) dihydroxybiphenyl dioxygenase displayed substrate inhibition, but has a substantially different subunit structure, being a heterotetramer ($\alpha_2\beta_2$) with a molecular mass of 135 kDa. At least on the quaternary structure level, the *P. putida* dihydroxybiphenyl dioxygenase seems to be more closely related to protocatechuate 4,5-dioxygenase (26).
possessed varying extents of same disorder.

*Pseudomonas* sp. LB400 is capable of metabolizing a number of PCB congeners (7). Whereas most congeners are initially attacked by a dioxygenase at positions 2 and 3 of one of the rings, evidence exists that in strain LB400, some of these congeners, such as 2,5,2',5'-tetrachlorobiphenyl, are hydroxylated at positions 3 and 4 of one of biphenyl rings (27). Whereas these compounds are apparently not substrates for the *bphB* gene product, the inability of 2,3-dihydroxybiphenyl 1,2-dioxygenase to cleave 3,4-dihydroxybiphenyl indicates that this enzyme probably would not catalyze the further degradation of chlorinated 3,4-dihydroxybiphenyl analogues. Complete mineralization of these compounds by the LB400 *bph* pathway may be eventually achieved by appropriate mutagenesis of 2,3-dihydroxybiphenyl 1,2-dioxygenase or by recruitment of an extradiol dioxygenase capable of cleaving 3,4-dihydroxylated biphenyls. Such enzymes have been isolated from two sources (22, 28). In any event, the availability of such enzymes would greatly increase our knowledge of the function of this enzyme and facilitate the manipulation of its properties.

**Acknowledgments**—We thank Silke Fischer for skilled technical assistance, Rita Getzlaff for performing the NH₂-terminal amino acid sequencing, and Dr. Shige Harayama for useful discussions.

**REFERENCES**