2-Hydroxybiphenyl 3-monoxygenase (HbpA), the first enzyme of 2-hydroxybiphenyl degradation in *Pseudomonas azelaica* HBP1, was purified 26-fold with a yield of 8% from strain HBP1 grown on 2-hydroxybiphenyl. The enzyme was also purified from a recombinant of *Escherichia coli* JM109, which efficiently expressed the hbpA gene. Computer densitometry of scanned slab gels revealed a purity of over 99% for both enzyme preparations. Gel filtration, subunit cross-linking, and SDS-polyacrylamide gel electrophoresis showed that the enzyme was a homotetramer with a molecular mass of 256 kDa. Each subunit had a molecular mass of 60 kDa containing one molecule of noncovalently bound FAD. The monoxygenase had a pI of 6.3. It catalyzed the NADH-dependent ortho-hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl. Molecular oxygen was the source of the additional oxygen of the product. The enzyme hydroxylated various phenols with a hydrophobic side chain adjacent to the hydroxy group. All substrates effected partial uncoupling of NADH oxidation from hydroxylation with the concomitant formation of hydrogen peroxide. 2,3-Dihydroxybiphenyl, the product of the reaction with 2-hydroxybiphenyl, was a non-substrate effector that strongly facilitated NADH oxidation and hydrogen peroxide formation without being hydroxylated and also was an inhibitor. The apparent *Km* values (30 °C, pH 7.5) were 2.8 μM for 2-hydroxybiphenyl, 26.8 μM for NADH, and 29.2 μM for oxygen. The enzyme was inactivated by p-hydroxymercuribenzoate, a cysteine-blocking reagent. In the presence of 2-hydroxybiphenyl, the enzyme was partly protected against the inactivation, which was reversed by the addition of an excess of dithiothreitol. The NH₂-terminal amino acid sequence of the enzyme contained the consensus sequence GXGXXG, indicative of the ββ-fold of the flavin binding site and shared homologies with that of phenol 2-hydroxylase from *Pseudomonas* strain EST1001 as well as with that of 2,4-dichlorophenol 6-hydroxylase from *Ralstonia eutropha*.

2-Hydroxybiphenyl has been used as a fungicide for the control of postharvest diseases of various fruits since 1937 (1). The concern about its persistence in the environment was the driving force for studies aimed at elucidating its bacterial metabolism. Growing on 2-hydroxybiphenyl as the sole carbon and energy source, *Pseudomonas azelaica* HBP1 employs a meta-cleavage pathway with a broad substrate spectrum for breaking down 2-hydroxybiphenyl (2). Degradation of 2,2'-dihydroxybiphenyl, which is also a growth substrate, occurs accordingly (3). The first reaction of the pathway, the ortho-hydroxylation of 2-hydroxy- and 2,2'-dihydroxybiphenyl to 2,3-dihydroxy- and 2,2',3-trihydroxybiphenyl, respectively, is catalyzed by the NADH-dependent 2-hydroxybiphenyl 3-monoxygenase (EC 1.14.13.44). 2,3-Dihydroxy- and 2,2',3-trihydroxybiphenyl are further degraded to benzoate and salicylate via the *meta*-cleavage products 2-hydroxy-6-phenyl-6-oxo-2,4-hexadienoic acid and 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid, respectively (2, 3). These reaction intermediates are identical to those of the degradation pathways for biphenyl and dibenzofuran (4–8). In contrast to the wild type strain, *P. azelaica* HBP1 Prp is a regulatory mutant that grows on 2-propylphenol and 2-sec-butylphenol with the aid of the enzymes of the broad spectrum *meta*-cleavage pathway (9, 10).

In rats, 2-hydroxybiphenyl shows renal toxicity and causes tumors of the urinary bladder (11). The microsomal monoxygenase system hydroxylates 2-hydroxybiphenyl to 2,5-dihydroxybiphenyl (phenylhydroquinone) (12). Although the exact mechanisms of the toxic and carcinogenic effects in rats are still unclear, it is speculated that reactive oxygenation products of 2,5-dihydroxybiphenyl (phenylbenzoquinone and phenylsemiquinone) ultimately cause these effects (13).

Recently, the microbial degradation of 2-hydroxy- and 2,2'-dihydroxybiphenyl has become important to researchers involved in the desulfurization of coal and petroleum, since it was reported that 2-hydroxy- and 2,2'-dihydroxybiphenyl are the end products of the bacterial desulfurization of dibenzothiophene, a major sulfur-containing component of fossil fuels (14–16). Furthermore, the two compounds inhibit the dibenzothiophene-degrading activity in cell-free extracts of *Rhodococcus erythropolis* D-1 (17).

Previously, we partly purified the 2-hydroxybiphenyl 3-monoxygenase for producing metabolites necessary for elucidating the degradation pathway of 2,2'-dihydroxybiphenyl in *P. azelaica* HBP1 (3). We now report on the purification and characterization of the enzyme. 2-Hydroxybiphenyl 3-monoxygenase is a novel flavin-containing, NADH-dependent aromatic hydroxylase with a broad substrate spectrum. Its relationship to other members of the group of phenolic hydroxylases is discussed.
2-Hydroxybiphenyl 3-Monoxygenase from *P. azelaica* HBP1

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

*P. azelaica* HBP1 was cultured on 2-hydroxybiphenyl as described previously (2). Cultures were grown in Erlenmeyer flasks on a rotary shaker (150–170 rpm) at 30 °C. The purity of the cultures was regularly tested by streaking them out on mineral salts medium plates that were prepared by adding 2-hydroxybiphenyl as a concentrated methanolic solution to the hot mineral medium containing 15 g/liter agar. Recombinants of *Escherichia coli* JM109 were cultivated in LB medium supplemented with ampicillin (100 μg/ml) at 37 °C (18).

For the purpose of protein purification, large amounts of cells were produced in aerated 20-liter carboys equipped with a magnetic stirring bar. Cells were harvested in the late exponential growth phase (with an A600 of about 1.5) by centrifugation (15 min at 6,000 × g) at 4 °C. The culture fluid was passed through a paper filter before centrifugation to remove remaining 2-hydroxybiphenyl crystals. Cells were washed twice with an excess amount of triethanolamine-HCl buffer (10 mM, pH 7.5). Approximately 20 g of cell paste (wet weight) was obtained from one 20-liter batch. The cell paste was stored at −20 °C until further use.

**Chemicals**

2,3-Dihydroxybiphenyl was obtained from Wako Chemicals GmbH (Neuss, Germany), 2,5-Dihydroxybiphenyl, 2-ethylphenol, and 2-sec-butylphenol were purchased from Aldrich-Chemie (Steinheim, Germany). 2-sec-Butylcatechol was prepared by the enzymatic conversion of 2-sec-butylphenol with 2-hydroxybiphenyl 3-monoxygenase and by subsequent purification of the product by means of a preparative HPLC apparatus. **O2** was obtained from Euriostop, Center d’Etudes de Saclay, Gif-sur-Yvette, France. If not otherwise indicated, all other chemicals used were bought from Fluka Chemie AG (Buchs, Switzerland).

**Protein Purification**

The enzyme purification apparatus was located in a laboratory at room temperature, but the buffer reservoirs and the sample collector vials were kept on ice. The flow rate was 1 ml min⁻¹ for all chromatography steps, and the volume of the collected fractions was always 1 ml if not stated otherwise.

**Preparation of Crude Cell Extract—** Ten grams of cell paste was suspended in 50 ml of triethanolamine-HCl buffer (10 mM, pH 7.5). Crude cell extract was prepared by passing the cells through a French pressure cell (two passages at 20,000 p.s.i.) followed by ultracentrifugation (30 min at 60,000 × g) at 4 °C.

**First Anion Exchange Chromatography—** The supernatant of the above purification step was directly loaded onto an anion exchange column (1 × 15 cm; Fractogel EMD TMAE-650; Merck, Darmstadt, Germany) equilibrated with 10 mM triethanolamine-HCl buffer (pH 7.5). Elution was carried out with an increasing NaCl gradient in 10 mM triethanolamine-HCl buffer (pH 7.5). 2-Hydroxybiphenyl 3-monoxygenase eluted at an NaCl concentration of 0.05 M. The clarified enzyme solution was loaded onto a Fractogel TSK Butyl 650 S (Merck, Darmstadt, Germany) column (1 × 15 cm) equilibrated with 0.75 mM (NH4)2SO4 in 100 mM Na2HPO4 buffer (pH 7.0). The column was washed with 2–3 volumes of the equilibration buffer, and the enzyme was eluted with a linear gradient from 0.75 to 0 mM (NH4)2SO4. The fractions containing the enzyme were desalted on Sephadex G-25 M columns (Pharmacia Biotech, Uppsala, Sweden).

**Second Anion Exchange Chromatography—** The desalted enzyme solution was supplemented with FAD to a final concentration of 0.3 mM and loaded onto an anion exchange column (Fractogel EMD TMAE-650) equilibrated with 10 mM triethanolamine-HCl buffer (pH 8.2). Isocratic conditions (0.3 mM NaCl) were used to elute the 2-hydroxybiphenyl 3-monoxygenase.

**Gel Filtration—** The fractions containing active enzyme from the preceding step were incubated with 0.3 mM FAD for 30 min on ice. The solution was passed with a flow rate of 1.5 ml min⁻¹ through a Superdex 200 gel filtration column (1.6 × 80 cm; Pharmacia) equilibrated with 50 mM Na2HPO4 buffer (pH 7.5). Pooled fractions were concentrated by ultrafiltration with Centricon concentrators (Amicon Inc., Beverly, MA). The concentrated solution of the purified enzyme (3–5 mg of protein/ml) was stored at −20 °C until further use.

**2-Hydroxybiphenyl 3-Monoxygenase Assay**

Activity of the 2-hydroxybiphenyl 3-monoxygenase was measured either spectrophotometrically or polarographically as described previously (2). All measurements were done at 30 °C. The oxygen consumption rates were calculated based on saturation constants of oxygen in water (7.53 mg/liter at 30 °C and at atmospheric pressure (1.013 × 10⁵ pascals). The investigation of the pH dependence was done with the following buffers of constant ionic strength (I = 20 mM): 20 mM MES, pH 6–6.75; 20 mM NaH2PO4/KH2PO4, pH 6.75–7.75; 20 mM Tris-HCl, pH 7.75–8.5.

**Analytical Methods**

The disappearance of substrates and the formation of metabolites were monitored by high performance liquid chromatography (HPLC). Protein was removed from the samples (3 ml) by the addition of 20 μl of 8.5% H3PO4 and subsequent centrifugation. The samples were analyzed by injecting 20 μl onto a computer-controlled Gynoktek high performance liquid chromatograph consisting of a Gita 50 automated injection module, a M480 G gradient pump, an on-line degasser, and an UVD 340 S photodiode array detector (Gynoktek, Germersheim, Germany). Reverse-phase separation was achieved on a Waters Nova-Pak C-18 column (Waters-Millipore, Milford, MA) by applying a linear gradient of 60–70% B (A, 10 mM H3PO4; B, 90% ethanol, 10% 10 mM H3PO4), with a flow rate of 0.6 ml min⁻¹.

The flavin cofactor was extracted from the protein by treating a 400-μl sample of 2-hydroxybiphenyl 3-monoxygenase (2.6 mg ml⁻¹) in 20 mM phosphate buffer (pH 7.2) with 100 μl of 5% trichloroacetic acid for 5 min at room temperature followed by centrifugation at 14,000 rpm. HPLC analysis (isocratic conditions with an eluent consisting of 40% methanol and 60% 10 mM H3PO4 (v/v)) of the supernatant allowed the identification of the cofactor by comparison of the retention times and the UV-VIS spectra to authentic FAD (retention time, 10.3 min) and FMN (retention time, 14.4 min).

The protein contents of the cell extracts and the purified protein fractions were measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, München, Germany). Bovine serum albumin in the concentration range from 2 to 20 μg ml⁻¹ was used as a standard.

**Labeling with ¹⁸O₂**

Incorporation of ¹⁸O₂ into the products of the enzyme reaction was measured to confirm the monoxygenation reaction. The experiments were carried out in two 13.8-ml serum flasks, which contained 1 ml of the enzyme incubation mixture consisting of 30 milliliters of 2-hydroxybiphenyl 3-monoxygenase from *P. azelaica* HBP1, 0.3 mM NADH, and 50 mM triethanolamine-HCl buffer (pH 7.5). Both flasks were sealed with butyl rubber stoppers. A 50:50 mixture of ¹⁸O₂ to ¹⁷O₂ in one of the flasks was obtained by injecting 2.6 ml of ¹⁸O₂ into the head space (12.8 ml). The other flask contained ¹⁷O₂ from air. The enzyme reaction was started by injecting 15 μl of a methanolic solution of 2-hydroxybiphenyl (0.1 mM) through the rubber seals. After 15 min, the incubation mixtures were acidified with 5 μl of H3PO4 (8.5%) to a final pH of 2 and subsequently centrifuged to remove the precipitated protein. The metabolites were extracted from the supernatants with an equal volume of ethyl acetate and dried with anhydrous sodium sulfate. Samples were derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide and subjected to gas chromatography-mass spectrometry analysis as described before (3).

**Electrophoresis and Molecular Mass Determinations**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (19) was performed in slab gels (100 × 60 × 0.75 mm) with the separating gels containing 12.5% acrylamide. The following proteins were used as standards (Pharmacia): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie Brilliant Blue C-250 and destained with a solution of 30% (v/v) methanol and 1.5% (v/v) acetic acid in deionized water. Silver staining of SDS gels was performed according to a standard method (20). The gels were scanned and analyzed with a computing densitometer system (ImageQuant®; Molecular Dynamics, Inc.).

Isoelectric focusing gels contained 7.5% acrylamide and a broad pH range.
2-Hydroxybiphenyl 3-Monooxygenase from P azelaica HBP1

range ampholyte (Resolyte 3.5–10, BDH Laboratory Supplies, Poole, United Kingdom). Isoelectric focusing was performed in the same apparatus that was used for SDS-PAGE (20). The pH gradient was formed by filling the upper buffer chamber with catholyte solution (20 mM sodium hydroxide) and the lower buffer chamber with anolyte solution (100 mM phosphoric acid). Five micromolars of protein was loaded onto the isoelectric focusing gel, and the pI was estimated from the position of the protein band relative to the position of the bands of the marker proteins (broad pI calibration kit, 3.5–10; Pharmacia).

The molecular mass of the pure enzyme was determined under native conditions by gel filtration on a Superdex 200 gel filtration column (1.6 × 80 cm, Pharmacia) equilibrated with 20 mM phosphate buffer (pH 7.5). The column was calibrated with blue dextran 2000 and the following reference proteins (Pharmacia): thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Cross-linking

Covalent cross-linking of enzyme subunits was carried out by the method of Griffith (21) with glutaraldehyde as the reactive agent. Ten microliters of glutaraldehyde (0.5%, w/v) was added to 250 μl (0.5 mg ml⁻¹) enzyme solution and incubated for 12 h at room temperature. The treated protein sample was denatured with SDS and analyzed by SDS-PAGE.

NH₂-terminal Amino Acid Sequence

The NH₂-terminal amino acid sequence of the enzyme was determined by automated Edman degradation.

Kinetic Measurements

The specific activity of purified 2-hydroxybiphenyl 3-monooxygenase and of partly purified enzyme fractions was routinely measured in an spectrophotometric assay with NADH and as defined as the amount of NADH (μmol) that the enzyme oxidized in the presence of 2-hydroxybiphenyl/min/mg of protein. The reaction was started by the addition of the substrate. The substrate-specific oxidation rates were corrected for nonspecific NADH oxidation in the absence of substrate (endogenous rate). The kinetic parameters Vmax and Km were calculated with a computer program (IGOR Pro, WaveMetrics Inc., Lake Oswego, OR). The calculation was based on weighted nonlinear regression analysis of the Michaelis-Menten model. We confirmed by Monte Carlo simulations that the least square estimators of the parameters were close to being the Michaelis-Menten model. We confirmed by Monte Carlo simulations that the least square estimators of the parameters were close to being the Michaelis-Menten model. We confirmed by Monte Carlo simulations that the least square estimators of the parameters were close to being the Michaelis-Menten model. We confirmed by Monte Carlo simulations that the least square estimators of the parameters were close to being the Michaelis-Menten model. We confirmed by Monte Carlo simulations that the least square estimators of the parameters were close to being the Michaelis-Menten model. We confirmed by Monte Carlo simulations that the least square estimators of the parameters were close to being the Michaelis-Menten model. These results were determined by means of the method established for the wild type enzyme (Fig. 1A).

At a temperature of 4 °C, the activity of the purified enzyme remained constant for at least 12 h, and after 24 h approximately 90% of the activity was still present. The pure enzyme was stored at a concentration of 3.8 mg/ml in 50 mM phosphate buffer, pH 7.2) was incubated with various concentrations of p-hydroxymercuribenzoate at 35 °C. Aliquots of 10 μl were withdrawn from the incubation mixtures, immediately diluted in 900 μl of phosphate buffer, and assayed for enzyme activity. Reactivation of the mercurated enzyme was followed after the addition of 1 mM dithiothreitol.

Chemical Modification

2-Hydroxybiphenyl 3-monooxygenase (4–4.5 μM, in 20 mM phosphate buffer, pH 7.2) was incubated with various concentrations of p-hydroxymercuribenzoate at 35 °C. Aliquots of 10 μl were withdrawn from the incubation mixtures, immediately diluted in 900 μl of phosphate buffer, and assayed for enzyme activity. Reactivation of the mercurated enzyme was followed after the addition of 1 mM dithiothreitol.

RESULTS

Purification of 2-Hydroxybiphenyl 3-Monooxygenase—2-Hydroxybiphenyl 3-monooxygenase (HbpA) from cells of P. azelaica strain HBP1 grown on 2-hydroxybiphenyl as the only carbon and energy source was purified 26-fold with a yield of 8% (Table I). The enzyme was bright yellow and accounted for about 4% of the total cell protein in the crude cell extract. Protein samples from the final step of the purification were subjected to SDS-PAGE. The enzyme migrated as a single band. Computer densitometry of scanned slab gels revealed a purity of over 99% (Fig. 1A). To detect minor contaminating proteins in the sample, separate gels were stained with silver. No additional protein bands could be detected by silver staining.

2-Hydroxybiphenyl 3-monooxygenase was also purified from cells of the recombinant E. coli JM109 [pIV61] containing the gene (hbpA) for the 2-hydroxybiphenyl 3-monooxygenase (24) by means of the method established for the wild type enzyme (Fig. 1B).

Identification of FAD as the Prosthetic Group—The enzyme activity in the pooled fractions of the hydrophobic interaction chromatography step was increased by 30% when 10 μM FAD was present in the assay. The addition of FMN to the assay mixture had no effect on the enzyme activity. These results suggested that FAD might be the prosthetic group of the monooxygenase. To isolate the cofactor, samples of the purified enzyme were treated with 5% trichloroacetic acid. HPLC
y analysis confirmed that the cofactor of the monoxygenase was indeed FAD, since the extracted compound co-chromatographed with authentic FAD and also had an identical UV-VIS spectrum (diode array detection).

Spectral Properties of the Monoxygenase—An enzyme preparation from the final purification step (gel filtration) was taken for spectroscopic analysis. The enzyme sample was judged to be free of unbound FAD, since the specific activity did not decrease after gel filtration and the addition of FAD to the eluted enzyme did not further stimulate activity. The UV-vis spectrum of the purified 2-hydroxybiphenyl 3-monoxygenase showed maxima at 382 and 452 nm and a minimum at 410 nm. This is characteristic of a flavoprotein (Fig. 2). The ratio of the absorbance at 250 nm relative to that at 450 nm was 6.2. Above 320 nm, the spectrum closely resembled that of authentic FAD except that the ratio of the absorbance at 375 nm to the one at 450 nm was 0.92 for the enzyme as compared with 0.82 for free FAD. The absorption at 450 nm of an enzyme solution with a protein content of 2.3 mg ml$^{-1}$ was 0.37. On the basis of a molar absorption coefficient ($\varepsilon_{\text{mol}}$) of 11,300 $\text{M}^{-1} \text{cm}^{-1}$ for the FAD moiety (25), it was estimated that 1 mol of enzyme contained 3.7 mol of FAD.

Isotope Labeling Experiment—To confirm the monoxygenation reaction for the formation of 2,3-dihydroxybiphenyl from 2-hydroxybiphenyl by 2-hydroxybiphenyl 3-monoxygenase, the enzymatic reaction was carried out in the presence of $^{16}\text{O}_2$. The reaction products were isolated, derivatized, and analyzed by gas chromatography-mass spectrometry (Fig. 3). Shows the mass spectra of the TMS derivatives of 2,3-dihydroxybiphenyl from the incubation with $^{16}\text{O}_2$ (A) and with a 1:1 mixture of $^{16}\text{O}_2$ to $^{18}\text{O}_2$ (B). The fragmentation patterns of the two spectra clearly show that one atom of dioxygen was incorporated into 2-hydroxybiphenyl during the course of the reaction.

Optimal pH and Temperature Conditions for Enzymatic Activity—The effect of temperature on the activity of the 2-hydroxybiphenyl 3-monoxygenase was investigated over the range of 10–50 °C. The temperature optimum of the enzymatic reaction was at 33 °C. The monoxygenase retained more than 70% of its activity in the temperature range from 27 to 40 °C. Above 50 °C, enzymatic activity was completely absent. The monoxygenase maintained more than 80% of its activity in the pH range between pH 7.2 and 7.8 and showed a maximum activity at pH 7.5. Beyond pH 7.8, the activity of the 2-hydroxybiphenyl 3-monoxygenase declined abruptly with increasing pH.

Substrate Specificity and Uncoupling Effects of Substrates and Products—Incubation of 2-hydroxybiphenyl with the purified 2-hydroxybiphenyl 3-monoxygenase led to the formation of a reaction product that was isolated and identified as 2,3-dihydroxybiphenyl by gas chromatography-mass spectrometry analysis. The substrate specificity of the 2-hydroxybiphenyl 3-monoxygenase was investigated with a variety of ortho-substituted phenols. Table II shows initial velocities of substrate conversions determined by independent measurements of NADH oxidation, oxygen consumption, and substrate disappearance at 30 °C and pH 7.2. It is evident that for all substrates the stoichiometric coefficients for NADH oxidation (or oxygen consumption) and hydroxylation were not equal. This indicates that the substrates partially uncoupled oxygen activation from hydroxylation with the resultant reduction of both atoms of oxygen to form hydrogen peroxide. The formation of hydrogen peroxide was shown by the addition of catalase at the end of the oxygen uptake experiments. For 2-hydroxybiphenyl as the substrate, 15% of the consumed oxygen was recovered after the addition of catalase, which means that according to the stoichiometry of the catalase reaction 30% of the consumed oxygen was diverted to hydrogen peroxide. With 2-sec-butylphenol, which was more rapidly metabolized than 2-hydroxy-
biphenyl, 47% of the consumed oxygen was released as hydrogen peroxide. The uncoupling determined by the catalase-dependent oxygen production exceeded the uncoupling determined by the measurement of initial reaction velocities. Subsequent experiments showed that the product of the hydroxylation of 2-hydroxybiphenyl, 2,3-dihydroxybiphenyl was a strong effector of 2-hydroxybiphenyl 3-monoxygenase and stimulated stoichiometric NADH and oxygen consumption without undergoing hydroxylation. This explained the elevated amounts of hydrogen peroxide observed after the complete conversion of 2-hydroxybiphenyl.

Measurement of NADH oxidation with low concentrations of 2-hydroxybiphenyl and 2-sec-butylphenol revealed the contribution of the reaction products to the consumption of NADH (Table III). After the complete consumption of 10 μM 2-hydroxybiphenyl, the NADH oxidation did not cease but continued at a lower rate (1.93 μmol min⁻¹ mg of protein⁻¹). The residual activity was attributed to the uncoupling effect of 2,3-dihydroxybiphenyl, which was formed during the reaction, because it was in good agreement with the oxidation rate obtained with 10 μM 2,3-dihydroxybiphenyl as the substrate. The same observation was made with 10 μM 2-sec-butylphenol and an equimolar concentration of the hydroxylated product 2-sec-butylcatechol. Residual activities remaining after the conversion of low substrate concentrations were also observed with 2,2'-dihydroxybiphenyl, and 2-propylphenol but, interestingly enough, not with 2,5-dihydroxybiphenyl. It became obvious from these experiments that the apparent NADH oxidation rate, when determined by the standard assay method, was always the sum of two oxidation rates, one effected by the substrate and the other one by the product. For calculations concerning the kinetic constants, we therefore only used rates derived from the measurement of initial velocities. This guaranteed that product inhibition and product uncoupling did not interfere with the measurements.

Besides the aromatic compounds, oxygen and NADH were also substrates of the 2-hydroxybiphenyl 3-monoxygenase reaction. In the absence of an aromatic substrate the oxidation of NADH amounted to 0.14 ± 0.04 μmol min⁻¹ mg of protein⁻¹ in the standard assay. NADPH could replace NADH as the electron donor for the reaction, but its apparent Kₘ value was much higher (Table IV).

**TABLE II**

| Substrate range of 2-hydroxybiphenyl 3-monooxygenase and comparison of enzyme activities as determined independently by NADH oxidation, oxygen uptake, and substrate conversion. The measurements were made in air-saturated phosphate buffer (20 mM, pH 7.2) at 30 °C. Effector concentrations were 0.1 mM; the NADH concentration was 0.2 mM. |
| --- |
| | Assay substrate | Specific monooxygenase activity | Percentage of uncoupling |
| | | NADH oxidation | Oxygen uptake | Substrate conversion |
| | | μmol min⁻¹ mg protein⁻¹ | μmol min⁻¹ mg protein⁻¹ | % |
| 2-Hydroxybiphenyl | 3.49 | 3.15 | 2.72 | 13.7 |
| 2,2'-Dihydroxybiphenyl | 2.08 | 1.78 | 1.52 | 14.6 |
| 2,3-Dihydroxybiphenyl | 1.85 | 1.83 | 0.02 | 99.5 |
| 2,5-Dihydroxybiphenyl | 2.21 | 2.52 | 1.28 | 49.2 |
| 2-Methylphenol | 0.55 | 0.47 | 0.38 | 19.2 |
| 2-Propylphenol | 3.13 | 3.00 | 0.76 | 74.7 |
| 2-Ethylphenol | 1.58 | 1.16 | 0.44 | 62.1 |
| 2-sec-Butylphenol | 3.78 | 3.55 | 3.24 | 8.7 |

*The proportion of uncoupling of hydroxylation with the different substrates was calculated as the deviation of the apparent oxygen consumption from the assumed 1:1 stoichiometry with respect to substrate conversion.*

**TABLE III**

| Time of measurement | μmol min⁻¹ mg protein⁻¹ |
| --- | --- |
| NADH oxidation rate with 2-hydroxybiphenyl as the substrate | 2.91 |
| After the addition of 2-hydroxybiphenyl (10 μM) | 1.93 |
| After the consumption of 2-hydroxybiphenyl | 1.81 |
| NADH oxidation rate with 2,3-dihydroxybiphenyl as the substrate | 1.81 |
| After the addition of 2,3-dihydroxybiphenyl (10 μM) | 2.57 |
| After the consumption of 2-sec-butylphenol | 0.41 |
| NADH oxidation rate with 2-sec-butylphenol as the substrate | 0.43 |
| After the addition of 2-sec-butylphenol (10 μM) | 0.43 |

*All values were corrected for the endogenous NADH oxidation (0.14 μmol min⁻¹ mg of protein⁻¹).*  
*The end of the consumption of 10 μM 2-hydroxybiphenyl was visible as a pronounced bend in the linear decrease of the absorbance at 340 nm, corresponding to a consumption of 11.6 μM NADH up to that point in time.*  
*With 2-sec-butylphenol as the substrate, the shift in the oxidation rate was less sharply pronounced.*
and the y axis intercepts obtained from these diagrams were replotted versus [P] in two secondary diagrams (Fig. 4, B and C). The dissociation constants $K_a$ for the formation of the enzyme-product complex $[EP]$ and $K_m$ for the formation of the enzyme-substrate-product complex $[EAP]$ were represented by the respective [P] axis intercept. The values for $K_{ic}$ and $K_{ia}$ were 1.6 and 4.0 $\mu M$, respectively.

Steady state analysis carried out with 2,3-dihydroxybiphenyl as a pseudosubstrate revealed apparent $V_{max}$ and $K_m$ values of 2.6 $\mu$mol min$^{-1}$ mg$^{-1}$ and 3.7 $\mu M$, respectively. With concentrations greater than 10 $\mu M$, the activity concomitantly declined (NADH, 0.2 mM; O$_2$, 0.24 mM). The inhibition constant $K_i$ for 2,3-dihydroxybiphenyl was 0.9 $\mu M$.

**Effects of Thiol Reagent, Metal Ions, and Chloride**—A rapid inactivation of the enzyme (4.5–4.8 $\mu M$) was observed in the presence of p-hydroxymercuribenzoate (pHMB), an efficient blocker of cysteine groups (Fig. 5). The inactivation of the enzyme was a reversible reaction, since the activity was restored by the addition of 1 $\mu M$ dithiothreitol (Fig. 5A). The maximum extent of inactivation was dependent on the concentration of the inhibitor. An inactivation of 100% was achieved within 1 min upon the addition of 100 $\mu M$ pHMB, whereas an inactivation of 25% was measured in the presence of 10 $\mu M$ pHMB (Fig. 5B). Furthermore, we investigated the influence of effectors on the course of the inactivation reaction. 2-Hydroxybiphenyl (100 $\mu M$) partly protected the enzyme from inactivation, whereas the presence of 2,3-dihydroxybiphenyl (100 $\mu M$) did not protect the enzyme from inactivation by pHMB.

The extents of inhibition by heavy metal and chloride ions are listed in Table V. Upon the addition of 10 $\mu M$ of the heavy metal salts CuSO$_4$, AgNO$_3$, or HgCl$_2$, the activity of 2-hydroxybiphenyl 3-monoxygenase ceased immediately, whereas the enzyme underwent a partial inhibition in the presence of chloride ions depending on the concentration. By varying the amounts of 2-hydroxybiphenyl, an uncompetitive inhibition type was found for chloride ions with respect to the substrate 2-hydroxybiphenyl (data not shown).

**NH$_2$-Terminal Amino Acid Sequence**—The NH$_2$-terminal amino acid sequence of the purified 2-hydroxybiphenyl 3-monoxygenase from the recombinant of E. coli JM109 was determined by automated Edman degradation and compared with other previously described sequences of bacterial phenol hydroxylases (Fig. 6). The NH$_2$-terminal amino acid sequence of the enzyme purified from wild type strain HBP1 was also determined (14 amino acids) and was 100% identical to the one of the enzyme purified from the recombinant. The analyzed sequence of the monoxygenase (HbpA) contained the consensus sequence GXGXXG, indicating the fingerprint of an $\beta\alpha\beta$-fold (26).

FASTA alignments with 55,024 sequences in the Swissprot data base revealed 54.2% identity (72–amino acid overlap) with the phenol 2-hydroxylase from Pseudomonas strain EST1001, PheA (27), and 56.8% identity (74–amino acid overlap) with the 2,4-dichlorophenol 6-hydroxylase fromRalstonia eutropha, TfdB (28) (Fig. 6). The sequence of 2-hydroxybiphenyl 3-monoxygenase showed 31.2% identity (61–amino acid overlap) with that of 4-hydroxybenzoate 3-hydroxylase (29, 30).

**FIG. 4. Inhibition of 2-hydroxybiphenyl 3-monooxygenase by 2,3-dihydroxybiphenyl**. The reactions were started by the addition of 2-hydroxybiphenyl (0.1 mM in the test) to the pre-equilibrated assay mixture containing 50 mM phosphate buffer, 0.04 $\mu M$ purified 2-hydroxybiphenyl 3-monooxygenase, 0.2 mM NADH, and 2,3-dihydroxybiphenyl at the concentrations indicated. A, direct plot of 2-hydroxybiphenyl 3-monooxygenase activity versus 2-hydroxybiphenyl concentration in the presence of different 2,3-dihydroxybiphenyl concentrations: 0 (□), 1 ($\bullet$), 5 ($\blacktriangle$), and 10 $\mu M$ ($\blacksquare$). Each point represents the average of two determinations. B, secondary plot of the slopes versus the 2,3-dihydroxybiphenyl concentrations derived from double reciprocal plots of the data of panel A. Further explanations are given under “Results” (“Product and Substrate Inhibition”).

**TABLE IV**

| Substrate | $k_{cat}$ | $V_{max}$ | $K_m$ |
|-----------|----------|----------|-------|
| 2-Hydroxybiphenyl (NADH, oxygen) | 14.9 | 3.5 ± 0.1 | 2.5 ± 0.2 |
| 2-Hydroxybiphenyl (NADPH, oxygen) | 12.8 | 3.0 ± 0.2 | 3.1 ± 0.2 |
| NADH (2-hydroxybiphenyl, oxygen) | 16.2 | 3.8 ± 0.1 | 26.8 ± 1.8 |
| NADPH (2-hydroxybiphenyl, oxygen) | 18.8 | 4.4 ± 0.2 | 137.0 ± 15.0 |
| Oxygen (2-hydroxybiphenyl, NADH) | 16.2 | 3.8 ± 0.3 | 29.2 ± 7.7 |
| 2,2'-Dihydroxybiphenyl (NADH, oxygen) | 9.0 | 2.1 ± 0.1 | 4.0 ± 0.4 |
| 2,2'-Dihydroxybiphenyl (NADPH, oxygen) | 9.4 | 2.2 ± 0.1 | 3.4 ± 0.4 |
| NADH (2,2'-dihydroxybiphenyl, oxygen) | 9.8 | 2.3 ± 0.1 | 21.6 ± 2.3 |
| NADPH (2,2'-dihydroxybiphenyl, oxygen) | 11.5 | 2.7 ± 0.15 | 94.3 ± 13.5 |
| 2-sec-Butylphenol (NADH, oxygen) | 15.8 | 3.7 ± 0.1 | 5.7 ± 0.4 |
| 2-sec-Butylphenol (NADPH, oxygen) | 10.2 | 2.4 ± 0.1 | 5.7 ± 0.6 |

$^a$ The values are the best fit parameters obtained from nonlinear least square fits to the Michaelis-Menten model.

$^b$ The substrates in the parentheses were present in the assay at saturating concentrations (NADH, 0.25 mM; NADPH, 0.25 mM; O$_2$, 0.24 mM; 2-hydroxybiphenyl, 0.1 mM; 2,2'-dihydroxybiphenyl, 0.1 mM).

$^c$ This value was obtained with enzyme from a different purification batch.
mM dithiothreitol (the pathway for 2-hydroxybiphenyl degradation, is induced in the presence of 100 mM dithiothreitol). Activity was restored by the addition of 1 mM dithiothreitol (dashed line). A, an enzyme solution (4.8 mM) was incubated with 10 μM pHMB (%) 100 μM pHMB (%); 10 μM pHMB in the presence of 100 μM 2-hydroxybiphenyl (%); 10 μM pHMB in the presence of 100 μM 2,3-dihydroxybiphenyl (%); 100 μM pHMB in the presence of 100 μM 2-hydroxybiphenyl (%). B, an enzyme solution (4.8 mM) was incubated with 10 μM pHMB (%) 100 μM pHMB (%); 10 μM pHMB in the presence of 100 μM 2,3-dihydroxybiphenyl (%); 100 μM pHMB in the presence of 100 μM 2,3-dihydroxybiphenyl (%).

**FIG. 5.** Inactivation of 2-hydroxybiphenyl 3-monooxygenase by the thiol reagent pHMB. Relative enzyme activity was measured by the standard monooxygenase assay. A, an enzyme solution (4.5 μM) was incubated with 50 μM pHMB (%). Within 9 min, the enzyme activity decreased to 8% (solid line). Activity was restored by the addition of 1 mM dithiothreitol (dashed line). B, an enzyme solution (4.8 mM) was incubated with 10 μM pHMB (%); 100 μM pHMB (%); 10 μM pHMB in the presence of 100 μM 2-hydroxybiphenyl (%); 10 μM pHMB in the presence of 100 μM 2,3-dihydroxybiphenyl (%); 100 μM pHMB in the presence of 100 μM 2-hydroxybiphenyl (%); and 100 μM pHMB in the presence of 100 μM 2,3-dihydroxybiphenyl (%).

**TABLE V**

Inhibition of 2-hydroxybiphenyl 3-monooxygenase by heavy metals and chloride ions

| Addition to assay     | Inhibition % |
|-----------------------|--------------|
| CuSO4 (0.01 mM)       | 100          |
| AgNO3 (0.01 mM)       | 100          |
| HgCl2 (0.01 mM)       | 100          |
| FeSO4 (0.05 mM)       | 30           |
| NaCl (10 mM)          | 36           |
| NaCl (50 mM)          | 75           |
| NaCl (100 mM)         | 89           |

**DISCUSSION**

2-Hydroxybiphenyl 3-monooxygenase, the first enzyme of the pathway for 2-hydroxybiphenyl degradation, is induced in cells of *P. azelaica* HBP1 grown on 2-hydroxybiphenyl as the sole source of carbon and energy (2). We purified the enzyme to apparent homogeneity and, according to its physicochemical and kinetic properties presented in this paper, classified the purified 2-hydroxybiphenyl 3-monooxygenase as a member of the group of flavoprotein aromatic hydroxylases.

After 26-fold purification, the enzyme was more than 99% pure as judged by analysis of SDS gels. On the basis of determinations of the relative molecular mass carried out under denaturing and under native conditions as well as by cross-linking experiments with glutaraldehyde, we suggest that under physiological conditions, the enzyme was a 256-kDa tetramer consisting of four subunits, each with a relative molecular mass of 60 kDa. The majority of the flavoproteins so far described consist of subunits with a relative molecular mass in the range of 60–70 kDa (31) and are monomers or dimers with the exception of melilotate hydroxylase (32) and 2,4-dichlorophenol 6-hydroxylase (33), which are tetramers. The visible absorption spectrum of 2-hydroxybiphenyl 3-monooxygenase was typical of a flavoprotein. The prosthetic group was noncovalently bound FAD. Some loss of FAD during the purification of the enzyme was observed at an ionic strength higher than 0.5 M. The molecular ratio of FAD to protein was 3.7. Therefore, each subunit of the monooxygenase contained one molecule of FAD.

The purified 2-hydroxybiphenyl 3-monooxygenase catalyzed the NADH-dependent hydroxylation of 2-hydroxybiphenyl, forming 2,3-dihydroxybiphenyl as the product. Experiments with 18O2 proved the enzymatic incorporation of one oxygen atom of molecular oxygen into the substrate. As with many other flavoprotein aromatic hydroxylases, the additional hydroxy group was introduced in the ortho-position with respect to the existing hydroxy group. Although the enzyme regioselectively hydroxylated only the C-3 position of the substrate, it had a relaxed substrate specificity with respect to the hydrophobic side chain, since it was able to hydroxylate various 2-alkyl- and 2-arylphenols. As a general feature, substrates of the 2-hydroxybiphenyl 3-monooxygenase had a 2-R-phenol structure, where R is a hydrophobic carbon moiety. The substrates of the 2-hydroxybiphenyl 3-monooxygenase were different from those of other flavoprotein aromatic hydroxylases acting on substituted phenols. An exception was 2-methylphenol, which is a common substrate for several phenol 2-hydroxylases (34) as well as for 2,4-dichlorophenol 6-hydroxylase from *Acinetobacter* sp. (33). The values of the apparent Michaelis constants for the substrates (Table IV) were similar to the ones reported for the substrates of 2,4-dichlorophenol 6-hydroxylase (33, 35) and phenol 2-hydroxylase (36). Many aromatic hydroxylases have a preference for NADPH as the electron donor for the reduction of the flavin molecule, whereas some can utilize NADH (31). 2-Hydroxybiphenyl 3-monooxygenase utilized NADH as well as NADPH, and the values of the apparent Michaelis constants for the two substrates, 2-hydroxybiphenyl and 2,2′-dihydroxybiphenyl, were similar with either reduced pyridine nucleotide. However, the *Km* values for NADPH were markedly higher than those for NADH. A relaxed cofactor specificity was demonstrated for other flavoprotein aromatic hydroxylases, which oxidize NADH and NADPH with similar efficiency (37). The binding of 2-hydroxybiphenyl to 2-hydroxybiphenyl 3-monooxygenase enhanced the oxidation of NADH by the enzyme. The factor of this stimulation in rate is generally on the order of 103 to 104 with flavoprotein aromatic hydroxylases (38). For 2-hydroxybiphenyl 3-monooxygenase with 2-hydroxybiphenyl as the substrate, we only observed a 20-fold stimulation. Therefore, 2-hydroxybiphenyl 3-monooxygenase was a rather strong NADH oxidase in the absence of the aromatic substrate. Uncoupling of oxygen reduction from the hydroxylation reaction, which is observed with substrate effectors as well as with non-substrate effectors, leads to the formation of hydrogen peroxide and is a common feature of flavin monooxygenases (38). With 2-hydroxybiphenyl 3-monooxygenase, partial uncoupling occurred in the presence of each substrate, but the extent of uncoupling varied with the substrate. The same phe-
Fig. 6. FASTA alignments of the NH-terminal amino acid sequence of HbpA (2-hydroxybiphenyl 3-monooxygenase from the recombinant E. coli JM109 strain [pIV61]) with the amino acid sequences of TfdB (2, 4-dichlorophenol 6-hydroxylase from R. eutropha), PheA (phenol 2-hydroxylase from Pseudomonas strain EST-1001), and PobA (4-hydroxybenzoate 3-hydroxylase from Pseudomonas fluorescens). identical residues: : conservative mutations. Amino acid positions are noted on the right. The amino acid positions matching the ADP-binding ββββ fingerprint (26) are given in boldface letters. Positions deviating from the fingerprint residues are underlined.

nomene was reported for the substrates of phenol 2-hydroxylation from Trichosporon cutaneum (36). However, the amount of hydrogen peroxide produced in the presence of different effectors was not always consistent with the expected amount that we calculated from the disappearance of oxygen and substrate. We showed that in the case of the enzyme substrates 2-hydroxybiphenyl and 2-sec-butylphenol, the products of the 2-hydroxybiphenyl 3-monooxygenase reaction also contributed to the increase of hydrogen peroxide through uncoupling (Table III). A striking feature of the 2-hydroxybiphenyl 3-monooxygenase was the fact that the product of the reaction with 2-hydroxybiphenyl, 2,3-dihydroxybiphenyl, was a remarkably efficient non-substrate effector. 2,3-Dihydroxybiphenyl significantly stimulated the consumption of NADH and oxygen without undergoing hydroxylation. Furthermore, 2,3-dihydroxybiphenyl inhibited the conversion of 2-hydroxybiphenyl by mixed inhibition. In the case of 4-hydroxybenzoate 3-hydroxylase, 3,4-dihydroxybenzoate, the product of the hydroxylation of 4-hydroxybenzoate, acts also as a non-substrate effector (39).

Product inhibition as well as the formation of toxic amounts of hydrogen peroxide by 2,3-dihydroxybiphenyl probably did not significantly impair growth of strain HBP1. The 2,3-dihydroxybiphenyl dioxygenase, the next enzyme of the 2-hydroxyphenylacetate 6-hydroxylase (37). Heavy metal ions, which are known to build complexes with the flavoquinone form of free flavins (44), immediately abolished activity of 2-hydroxybiphenyl 3-monooxygenase.

The NH-terminal amino acid sequence of 2-hydroxybiphenyl 3-monooxygenase contained the GXGXXG sequence, which is the core of a fingerprint of 11 amino acids at crucial positions within a stretch of 29–32 amino acid residues (26). Proteins that contain a match to the fingerprint fold into an ADP-binding ββββ unit involved in FAD and NAD binding. The putative fingerprint regions of both 2-hydroxybiphenyl 3-monooxygenase and 4-hydroxybenzoate 3-hydroxylase did not exactly match the requested sequence. They deviated in the last two amino acid positions from the predicted fingerprint and did not have the obligatory acidic residue at position 32. However, the presence of this acidic residue seems not to be absolutely necessary, since 4-hydroxybenzoate 3-hydroxylase contains a ββββ-fold in the FAD-binding domain at the NH2 terminus of the enzyme despite the lack of this residue (41).

The inhibition of the monooxygenase by chloride ions was not competitive. Monovalent ions are noncompetitive inhibitors of milliota hydroxylase (32), but on the other hand they are competitive inhibitors of phenol 2-hydroxylase (43) and 3-hydroxyphenylacetate 6-hydroxylase (37). Heavy metal ions, which are known to build complexes with the flavoquinone form of free flavins (44), immediately abolished activity of 2-hydroxybiphenyl 3-monooxygenase.

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The NH-terminal amino acid sequence of 2-hydroxybiphenyl 3-monooxygenase had a high degree of sequence identity to that of 2,4-dichlorophenol 6-hydroxylase from R. eutropha (28) and phenol 2-hydroxylase from Pseudomonas sp. strain EST1001 (27). Furthermore, 2-hydroxybiphenyl 3-monooxygenase shared many molecular and catalytic properties with the 2,4-dichlorophenol 6-hydroxylase from Alcaligenes sp. (33), which is also a tetramer composed of identical subunits with a relative molecular mass of 63 kDa.

We purified and described a novel flavin monooxygenase, which is able to hydroxylate a large number of 2-alkyl- and 2-arylnaphthols. It shares many similarities with other enzymes of the group of external flavoprotein aromatic hydroxylases but displays an exceptionally wide substrate spectrum for region-selective hydroxylation.

Acknowledgments—We thank Gerhard Frank for performing the NT-terminal amino acid sequencing and Willem van Berkel for several stimulating discussions and suggestions.

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