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1 **Characterization of a naphthalene dioxygenase endowed with an exceptionally broad**
2 **substrate specificity towards polycyclic aromatic hydrocarbons†**

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13 Running title : A broad specificity ring-hydroxylating dioxygenase

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15

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25

1 **Abbreviations**

2 GC-MS, gas chromatography coupled to mass spectrometry;

3 HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid!;

4 HPLC, high performance liquid chromatography;

5 Ht, His-tagged;

6 IMAC; immobilized metal affinity chromatography;

7 IPTG, isopropyl- β -D-thiogalactopyranoside;

8 PAH; polycyclic aromatic hydrocarbon;

9 Red_{B356}, reductase component of the biphenyl dioxygenase from *C. testosteroni* B356;

10 RHD, ring hydroxylating dioxygenase

11

1 **Abstract**

2 In *Sphingomonas* CHY-1, a single ring-hydroxylating dioxygenase is responsible for the
3 initial attack of a range of polycyclic aromatic hydrocarbons (PAHs) composed of up to five
4 rings. The components of this enzyme were separately purified and characterized. The
5 oxygenase component (ht-PhnI) was shown to contain one Rieske-type [2Fe-2S] cluster and
6 one mononuclear Fe center per alpha subunit, based on EPR measurements and iron assay.
7 Steady-state kinetic measurements revealed that the enzyme had a relatively low apparent
8 Michaelis constant for naphthalene ($K_m = 0.92 \pm 0.15 \mu\text{M}$), and an apparent specificity
9 constant of $2.0 \pm 0.3 \mu\text{M}^{-1} \text{s}^{-1}$. Naphthalene was converted to the corresponding 1,2-
10 dihydrodiol with stoichiometric oxidation of NADH. On the other hand, the oxidation of eight
11 other PAHs occurred at slower rates, and with coupling efficiencies that decreased with the
12 enzyme reaction rate. Uncoupling was associated with hydrogen peroxide formation, which is
13 potentially deleterious to cells and might inhibit PAH degradation. In single turnover
14 reactions, ht-PhnI alone catalyzed PAH hydroxylation at a faster rate in the presence of
15 organic solvent, suggesting that the transfer of substrate to the active site is a limiting factor.
16 The four-ring PAHs chrysene and benz[a]anthracene were subjected to a double ring-
17 dihydroxylation, giving rise to the formation of a significant proportion of *bis-cis*-
18 dihydrodiols. In addition, the dihydroxylation of benz[a]anthracene yielded three
19 dihydrodiols, the enzyme showing a preference for carbons in positions 1,2 and 10,11. This is
20 the first characterization of a dioxygenase able to dihydroxylate PAHs made up of four and
21 five rings.

22

1 Ring-hydroxylating dioxygenases (RHDs) are widely spread bacterial enzymes that play a
2 critical role in the biological degradation of a large array of aromatic compounds, including
3 polycyclic aromatic hydrocarbons (PAHs)(1, 2). RHDs catalyze the initial oxidation step of
4 such compounds, which consists in the hydroxylation of two adjacent carbon atoms of the
5 aromatic ring, thus generating a *cis*-dihydrodiol. This reaction converts hydrophobic, often
6 toxic, molecules, into more hydrophilic products, allowing for their subsequent metabolism
7 by other bacterial enzymes. Some RHDs were found to attack highly recalcitrant
8 environmental pollutants, including dibenzo *p*-dioxin (3, 4), polychlorobiphenyls (5), and
9 PAHs (6-8), thus promoting studies on this type of enzymes with the ultimate goal of
10 improving bioremediation processes (2, 9). RHDs are multi-component enzymes, generally
11 composed of a NADH-oxidoreductase, a ferredoxin and an oxygenase component that
12 contains the active site. Sometimes, the reductase and the ferredoxin are fused in a single
13 polypeptide. The oxygenase component is a multimeric protein, with either an $\alpha_n\beta_n$ ($n=2$ or 3)
14 or α_3 structure, that contains one [2Fe-2S] Rieske cluster and one non-heme iron atom per α
15 subunit (1). During a catalytic cycle, two electrons from the reduced pyridine nucleotide are
16 transferred, via the reductase, the ferredoxin and the Rieske center, to the Fe(II) ion at the
17 active site. The reducing equivalents allow the activation of molecular oxygen, which is a
18 prerequisite to dihydroxylation of the substrate (10).

19 So far, only a few RHDs have been purified and extensively characterized, including
20 phthalate dioxygenase (11, 12), naphthalene dioxygenase (13, 14) and biphenyl dioxygenase
21 (15). None of these enzymes is able to oxidize substrates with more than three fused rings,
22 and data on the mechanism, kinetics and efficiency of the oxidation of high molecular weight
23 PAHs by bacterial dioxygenases are relatively scarce (16). However, the four-ring PAHs
24 chrysene and benz[a]anthracene, and the five-ring benzo[a]pyrene are of particular concern
25 because they are well-documented carcinogens (17). Recently, a *Sphingomonad* endowed

1 with the remarkable ability to grow on chrysene as sole carbon and energy source was
2 isolated in our laboratory (18). In this strain, called *Sphingomonas* sp. CHY-1, a single
3 dioxygenase was shown to be responsible for the oxidation of polycyclic hydrocarbons made
4 of 2 to 4 rings (6). In the present study, the three components of the dioxygenase were
5 purified and characterized, and the catalytic properties of the enzyme with respect to the
6 oxidation of nine PAHs were examined. Due to the broad specificity of this enzyme, the
7 kinetics and coupling efficiency of the dioxygenase-catalyzed reaction with 2 to 5-ring PAHs
8 could be compared for the first time. Steady-state kinetic parameters were determined for
9 representative 2-ring PAHs. In addition, the reactivity and regioselectivity of the enzyme
10 towards benz[a]anthracene was further investigated by means of single turnover chemistry
11 and EPR spectroscopy.

12

1 MATERIALS AND METHODS

2 *Bacterial strains and growth conditions*

3 Strains of *Escherichia coli* and *Pseudomonas putida* carrying the relevant expression
4 plasmids, as well as general culture conditions, have been previously described (6). Large-
5 scale cultures required for the purification of the enzyme components were grown on rich
6 medium, either Luria-Bertani or Terrific broth (19), in a 12-L fermentor (Discovery 100, SGI-
7 Inceltech/New Brunswick Scientific, Paris, France). Cultures destined to the overproduction
8 of the oxygenase or the ferredoxin component were supplemented with 50 μ M ferrous
9 ammonium sulfate. The medium was inoculated with 400 ml of an overnight culture, then
10 incubated at 37°C under constant aeration and agitation (500 rpm), until the bacterial density
11 (OD_{600}) reached about 1.0. The temperature was then lowered to 25°C, IPTG was added to
12 0.2 mM final concentration, and the culture was further incubated for 20 h before being
13 harvested by centrifugation. The bacterial pellet was washed with 50 mM Tris-HCl buffer (pH
14 7.5), and kept frozen until use.

15

16 *Protein purification*

17 All purification procedures were carried out under argon, using buffers equilibrated for at
18 least 24 h in a glove box maintained under anoxic conditions ($O_2 < 2$ ppm, Jacomex, France).
19 The temperature was kept at 0-4 °C except when otherwise indicated. Crude extracts were
20 prepared by thawing the bacterial pellets in twice as much lysis buffer by volume, followed
21 by lysozyme treatment (0.5 mg/ml) for 15 min at 30°C. The lysis buffer was either 50 mM
22 Tris-HCl, pH 7.5 (oxygenase preparation), 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10%
23 glycerol (reductase preparation) or 50 mM potassium phosphate, pH 7.5, 0.5 M NaCl, 10%
24 glycerol, 2 mM β -mercaptoethanol (ferredoxin preparation). The suspension was then
25 subjected to ultrasonication for a total time of 5 min at 80% of maximal intensity, using a

1 Vibra Cell apparatus run in pulse mode at 5 s/pulse (Fisher Bioblock Scientific, Illkirch,
2 France). The lysate was centrifuged at 12,000 g for 30 min, and the resulting cell extract was
3 used as the starting material for protein purification.

4 ***Purification of the oxygenase component PhnI***

5 A cell extract was prepared as described above from *P. putida* KT2442 carrying plasmid
6 pSD9 (6). The extract obtained from approx. 50 g of cells was diluted two-fold with TGE
7 buffer (25 mM Tris-HCl, pH 7.5, containing 5% glycerol, 5% ethanol, and 2 mM β -
8 mercaptoethanol), and applied to a 40-ml column of DEAE-cellulose (DE52, Whatman)
9 equilibrated with TGE buffer. After washing the column with four bed volumes of the same
10 buffer, the oxygenase was eluted as a brown band with buffered 0.3 M NaCl. The eluate was
11 immediately applied to a small column (7 ml) of immobilized metal affinity chromatography
12 (IMAC) resin loaded with Co^{2+} (TALON, BD Biosciences Ozyme, France). The column was
13 washed successively with 8 bed volumes of TGE buffer containing 0.5 M NaCl, and 5 bed
14 volumes of the same buffer supplemented with 20 mM imidazole. A brown protein fraction
15 was then eluted with TGE buffer containing 0.15 M imidazole. This fraction was diluted 6-
16 fold with TGE buffer and applied to a small column of DEAE-cellulose (4 ml). The purified
17 protein was eluted in a small volume of TGE buffer containing 0.3 M NaCl, and frozen as
18 pellets in liquid nitrogen. This preparation, designated ht-PhnI, was judged to be at least 95%
19 pure by SDS-PAGE.

20 ***Purification of the ferredoxin component PhnA3***

21 PhnA3 was overproduced in *E. coli* BL21AI (Invitrogen) carrying plasmid pEBA3 (15). The
22 cell extract prepared from 154 g packed cells was loaded onto two columns of IMAC-
23 TALON, 13 ml each, equilibrated in PG buffer (50 mM potassium phosphate, pH 7.5, 10%
24 glycerol, 2 mM β -mercaptoethanol), containing 0.5 M NaCl. Each column was washed with
25 100 ml of equilibration buffer, followed by 50 ml of buffered 20 mM imidazole. A brown

1 protein fraction was eluted with PG buffer containing 0.15 M imidazole. This fraction was
2 immediately diluted 5-fold with PG buffer, and loaded onto a 10-ml DEAE cellulose column.
3 After washing with two bed volumes of PG buffer, the brown ferredoxin fraction was eluted
4 with buffered 0.3 M NaCl in a volume of 8.6 ml. This preparation was designated ht-PhnA3.
5 Part of the purified His-tagged protein (4.7 μ moles) was cleaved by incubation with thrombin
6 (10 U/ μ mole) for 16 h at 20°C, in buffer containing 0.15 M NaCl and 2 mM CaCl₂, pH 8.2.
7 The digested protein was passed through a 2-ml IMAC-TALON column, diluted 5-fold with
8 PG buffer, then loaded onto a 2-ml column of DEAE-cellulose. The ferredoxin was eluted in
9 a small volume PG buffer containing 0.3 M NaCl, and frozen as pellets in liquid nitrogen.
10 This preparation was referred to as rc-PhnA3.

11

12 ***Purification of the reductase component PhnA4***

13 PhnA4 was overproduced in *E. coli* BL21(DE3) carrying plasmid pEBA4 (15). The crude
14 extract from 16 g of cells was applied to a 2-ml column of IMAC-TALON equilibrated in TG
15 buffer (Tris-HCl, pH 8.0, 10% glycerol) containing 0.5 M NaCl. The column was washed
16 with 10 bed volumes of equilibration buffer, and 3 bed volumes of TG buffer containing 0.5
17 M NaCl and 10 mM imidazole. A yellow protein fraction was then eluted with TG buffer
18 containing 0.15 M imidazole. This fraction was dialyzed for 16 h against TG buffer, and
19 further purified on a second column of IMAC-TALON (1 ml). The column was successively
20 washed with TG buffer containing 0.5 M NaCl (10 ml), and the same buffer containing 10
21 mM (9 ml), and 20 mM (4 ml) imidazole. The reductase was eluted in a small volume of TG
22 buffer containing 0.15 M imidazole, dialyzed as above, and concentrated to 0.8 ml by
23 ultrafiltration using an Ultrafree centrifugal device with 30-kDa cut-off (Millipore, Amilabo,
24 France). The purified protein was stored as pellets in liquid nitrogen.

25

1 ***Purification of ht-Red_{B356}***

2 The reductase component of the biphenyl dioxygenase from *C. testosteroni* B-356, designated
3 as ht-Red_{B356}, was purified from *E. coli* SG12009(pREP4)(pEQ34::*bphG*) (20), as previously
4 described (21).

5

6 ***Enzyme assays***

7 Dioxygenase activity was assayed either by following NADH oxidation at 340 nm or by
8 measuring the rate of O₂ consumption using a Clark-type O₂ electrode (Digital model 10;
9 Bioblock Scientific, Illkirch, France). Polarographic measurements (standard assay) were
10 performed at 30°C in reaction mixtures (1 ml) containing 0.13 μM ht-PhnI, 1.57 μM PhnA3,
11 0.40 μM ht-Red_{B356}, and 0.5 mM NADH in 50 mM potassium phosphate buffer, pH 7.0. The
12 PAH substrate was supplied at 0.1 mM from a concentrated solution in acetonitrile. The
13 concentration of all three enzyme components was doubled for assays with 4- and 5-ring
14 PAHs. The reaction was initiated by injecting, with a gas-tight syringe, a 100-fold
15 concentrated mixture of the proteins kept under argon on ice in phosphate buffer containing
16 10% glycerol, 10 mM dithiothreitol and 0.05 mM ferrous ammonium sulfate. The enzyme
17 activity was determined from the initial rate of O₂ consumption and expressed as μmol O₂ per
18 min per mg ht-PhnI. Reaction rates were calculated from duplicate assays and corrected for
19 the O₂ consumption measured in control assays carried out in the absence of PAH substrate.

20 The O₂ electrode was also used to determine the coupling between PAH oxidation and O₂
21 consumption as follows. After approx. 50 nmol O₂ had been consumed, 300 U of bovine liver
22 catalase (Sigma) was added as a means to estimate the amount of H₂O₂ generated during the
23 reaction. Then, 0.6 ml of reaction mixture was withdrawn and immediately mixed with an
24 equal volume of ice-cold acetonitrile. The dihydrodiols present in these samples were directly

1 quantified by HPLC as described below. When appropriate, the dihydrodiols were extracted
2 with ethyl acetate, derivatized and analyzed by GC-MS (see below).

3 To determine the coupling efficiency between NADH and PAH oxidation, some reactions
4 were carried out in Eppendorf tubes containing 0.1 mM of substrate and 0.2 mM of NADH in
5 0.6 ml of reaction mixture. In those assays, the concentrations of the enzyme components
6 were 0.38 μM (ht-PhnI), 6 μM (PhnA3) and 2 μM (ht-Red_{B356}). After an incubation time of 2
7 to 10 min at 30°C, depending on the substrate, the reaction was stopped by addition of an
8 equal volume of acetonitrile. Residual NADH, and the dihydrodiols formed during the
9 enzymatic reaction, were separated and quantified by HPLC.

10 Steady-state kinetic parameters of the dioxygenase-catalyzed reaction were determined from
11 sets of enzyme assays where the substrate concentration was varied over a 0.5-100 μM range.
12 The component ratio was the same as in the standard assay, but the protein concentration in
13 the assays was 1.67-fold higher. The initial NADH concentration was 0.2 mM. Reactions
14 were carried out at 30°C in quartz cuvettes, and the absorption at 340 nm was recorded at 0.1-
15 s intervals over 1 min with a HP8452 spectrophotometer (Agilent Technologies, Les Ulis,
16 France). The enzyme activity was calculated from the initial linear portion of the time course,
17 using an absorption coefficient of 6,220 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for NADH. When biphenyl was used as
18 substrate, NADH oxidation was recorded at 360 nm ($\epsilon_{360} = 4,320 \text{ M}^{-1}\cdot\text{cm}^{-1}$), because biphenyl
19 2,3-dihydrodiol absorbed at 340 nm. All assays were performed in duplicate, and at least 12
20 concentrations were tested per substrate. Plots of the initial reaction rate versus substrate
21 concentration were fitted to the Michaelis-Menten equation using the curve fit option of
22 Kaleidagraph (Synergy Software). Only curve fits showing correlation coefficients better than
23 0.98 were considered.

24

1 *Single turnover reactions*

2 Ht-PhnI was diluted to 57 μ M in 20 mM HEPES, pH 7.0 containing 10% glycerol and 5 μ M
3 methyl viologen under argon, then reduced with a stoichiometric amount of dithionite. The
4 reduction was checked by monitoring the protein absorbance in the 300-600 nm range. A
5 portion of the reduced protein (50 μ L) was diluted in 0.55 ml of air-saturated HEPES buffer
6 containing 0.1 mM of PAH substrate. In some experiments, the buffer also contained a
7 proportion of acetonitrile, as indicated. After incubation at 30°C for up to 10 min, the reaction
8 was stopped by mixing with an equal volume acetonitrile containing 0.8% acetic acid. The
9 mixture was heated for 2 min at 90°C, centrifuged and subjected to HPLC analysis as
10 described below. Part of the solution was also extracted with ethyl acetate, and analyzed by
11 GC-MS.

13 *Identification and quantification of reaction products*

14 Determination of dihydrodiols and the residual NADH concentration at the end of the
15 dioxygenase-catalyzed reactions was performed by HPLC using a Kontron system equipped
16 with F430 UV detector. Samples (0.2 ml) were injected onto a 4 \times 150-mm C8 reverse-phase
17 column (Zorbax, Agilent Technologies, France) run at 0.8 ml/min. The column was eluted
18 with water for 2 min, then with a linear gradient to 80% acetonitrile for 8 min, and finally
19 with 80% acetonitrile for 5 min. Detection was carried out at 340 nm (for residual NADH),
20 and one of the following wavelengths, which was varied as a function of the absorbance
21 maxima of the PAH dihydrodiols: 220 nm (naphthalene), 303 nm (biphenyl), 260 nm
22 (phenanthrene), 244 nm (anthracene), 263 nm (benz[a]anthracene), 278 nm (chrysene), 280
23 nm (benzo[a]pyrene). Quantification was performed on the basis of peak area using
24 calibration curves obtained by injecting known amounts of each dihydrodiol. Residual NADH
25 was determined from the peak eluting at 2.6 min. The three benz[a]anthracene dihydrodiols

1 formed by ht-PhnI were not resolved under the HPLC conditions used, and were estimated as
2 a sum of their individual contribution, given that their absorbance coefficients at 263 nm were
3 close to $31,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Purified 1,2-dihydroxy-1,2-dihydrobenz[a]anthracene was used for
4 HPLC calibration. The extent of oxidation of fluorene and fluoranthene by the dioxygenase
5 was determined from HPLC measurements of the amount of residual substrate. Wavelengths
6 used for their detection were 262 and 236 nm, respectively.

7 PAH oxidation products generated by PhnI were also analyzed by GC-MS. Ethyl acetate
8 extracts of samples were dried on sodium sulfate, evaporated under N_2 , and derivatized with
9 bis(trimethylsilyl)trifluoroacetamide :trimethylchlorosilane (99:1) from Supelco (Sigma-
10 Aldrich), prior to GC-MS analysis using a HP6890/HP5973 apparatus (Agilent
11 Technologies). Operating conditions were as previously described (22), and mass spectrum
12 acquisitions were carried out either in the total ion current or the single ion monitoring mode.

13

14 *Determination of the iron content of proteins*

15 To extract iron from proteins, samples (150 μl) were treated with 2.5 N HCl for 30 min at
16 95°C , then diluted with 0.7 volume of water. Iron was reacted with bathophenanthroline
17 disulfonate (Sigma-Aldrich), and the complex formed was assayed by absorbance
18 measurements at 536 nm (23). Assays were performed in triplicates. A calibration curve was
19 generated by assaying serial dilutions of a standard solution of ferric nitrate, containing 1g/L
20 of iron (Merck).

21

22 *Protein analyses*

23 Routine protein determinations were performed using the Bradford assay (24), or the
24 bicinchoninic acid reagent kit (Pierce) using bovine serum albumin as a standard. The protein
25 concentration of purified preparations of ht-PhnI was determined by a modification of the

1 biuret assay (25). The absorbance coefficient of ht-PhnI at 458 nm was calculated to be
2 12,500 M⁻¹.cm⁻¹, on the basis of the latter assay. The concentrations of ht-PhnA3 and rc-
3 PhnA3 were estimated from absorbance measurement at 460 nm, using an absorbance
4 coefficient of 5,000 M⁻¹.cm⁻¹. SDS-PAGE on mini-slab gels was performed as previously
5 described (26). The molecular masses of purified ht-PhnI and rc-PhnA3 were determined by
6 size-exclusion chromatography on HR 10/30 columns of Superdex SD200 and SD75,
7 respectively (both from Amersham Biosciences). The columns were run at a flow rate of 0.2
8 ml/min and calibrated with the following protein markers: Ferritin (443 kDa), catalase (240
9 kDa), aldolase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and
10 myoglobin (17 kDa), aprotinin (6.5 kDa), all from Sigma-Aldrich, and ferredoxin VI from
11 *Rhodobacter capsulatus* (11.58 kDa; (27)).

12

13 ***EPR spectroscopy***

14 Protein samples were adjusted to a concentration of 20-40 μM (ht-PhnI) or 100-500 μM
15 (PhnA3) in argon-saturated phosphate buffer, pH 7.0, containing 10% glycerol. The redox
16 status of the protein sample was checked by recording the absorbance spectrum, and, when
17 appropriate, the protein was fully oxidized by injecting stoichiometric amounts of air with a
18 gas-tight syringe. Ht-PhnI-nitrosyl complexes were prepared in a glove-box under argon
19 (Jacomex) by incubating 190 μl of protein sample with 10 μl of 20 mM diethylamine NO-
20 NOate (Cayman Chemical, Interchim, France) for 15 min. Samples were then introduced into
21 EPR tubes and frozen in liquid nitrogen. In some experiments, protein samples were
22 preincubated for 10 min with a PAH (0.1 mM) or a dihydrodiol, prior to NO-NOate addition.
23 For analysis of the Rieske clusters, protein samples were reduced with an excess of sodium
24 dithionite (1 mM). Full reduction was checked by absorbance recording prior to transferring
25 the samples in EPR tubes and freezing them in liquid nitrogen. Spectra were recorded at a

1 temperature set between 4 and 20 K with an X-band EMX Bruker spectrometer equipped with
2 an ESR900 liquid helium cryostat (Oxford Instruments). Spin quantification was performed
3 by integrating the appropriate signal, and comparing the signal intensity to that of the [2Fe-
4 2S] ferredoxin (FdVI) from *Rhodobacter capsulatus*, taken as a reference (0.1 mM; (27)). The
5 iron content of this reference sample was checked by chemical assay. All EPR tubes were
6 calibrated in diameter.

7

8 **Chemicals**

9 NAD⁺, NADH, PAHs, and most other chemicals were purchased from Sigma-Aldrich (Saint-
10 Quentin-Fallavier, France). The *cis*-dihydrodiols used in this study were prepared from
11 cultures of *E. coli* recombinant strains overproducing the PhnI dioxygenase, and incubated
12 with a PAH. The purification and characterization of the diol compounds will be described
13 elsewhere. The dihydrodiol concentrations were calculated using the following absorption
14 coefficients: $\epsilon_{262} = 8,114 \text{ M}^{-1}.\text{cm}^{-1}$ for *cis*-1,2-dihydroxy 1,2-dihydronaphthalene (28); $\epsilon_{252} =$
15 $38,300 \text{ M}^{-1}.\text{cm}^{-1}$ and $\epsilon_{260} = 43,000 \text{ M}^{-1}.\text{cm}^{-1}$ for *cis*-3,4-dihydroxy 3,4-dihydrophenanthrene
16 (29); $\epsilon_{244} = 55,600 \text{ M}^{-1}.\text{cm}^{-1}$ and $\epsilon_{287} = 17,000 \text{ M}^{-1}.\text{cm}^{-1}$ for *cis*-1,2-dihydroxy 1,2-
17 dihydroanthracene (29); $\epsilon_{278} = 57,650 \text{ M}^{-1}.\text{cm}^{-1}$ for *cis*-3,4-dihydroxy 3,4-dihydrochrysene
18 (30); $\epsilon_{280} = 66,500 \text{ M}^{-1}.\text{cm}^{-1}$ for *cis*-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene (31); $\epsilon_{263} =$
19 $31,000 \text{ M}^{-1}.\text{cm}^{-1}$ for *cis*-1,2- dihydroxy-1,2-dihydrobenz[a]anthracene and $\epsilon_{275} = 37,000 \text{ M}^{-1}.$
20 cm^{-1} for *cis*-10,11-dihydroxy-10,11-dihydrobenz[a]anthracene (32).

21

22

1 RESULTS

2 *Purification and properties of the oxygenase component PhnI*

3 The His-tagged oxygenase component of strain CHY-1 dioxygenase, hereafter referred to as
4 ht-PhnI, was anaerobically purified from *P. putida* KT2442(pSD9) in three steps as described
5 under Materials and Methods. The procedure yielded approx. 12 mg of purified protein per
6 liter of culture. The oxygenase was also produced in *E. coli* BL21(DE3)(pSD9), but the
7 purification resulted in a lower yield. In addition, strain BL21(DE3) always produced a
8 variable amount of insoluble recombinant protein (inclusion bodies), which was not the case
9 when using strain KT2442 as host (data not shown). The latter strain was therefore preferred
10 for overproduction and subsequent purification of ht-PhnI. SDS-PAGE analysis revealed that
11 the ht-PhnI preparation was at least 95% pure, and was composed of two subunits with
12 apparent M_r of 52.000 and 20.000 (Fig. 1), consistent with the molecular masses of the
13 polypeptides deduced from relevant gene sequences (6). Purified ht-PhnI exhibited a
14 molecular mass of approx. 200 kDa by gel filtration chromatography, indicating that it is an
15 $\alpha_3\beta_3$ hexamer. The brown protein showed a UV-visible absorbance spectrum with maxima at
16 280, 458 nm and a shoulder near 570 nm (data not shown), which is typical of proteins
17 containing Rieske-type [2Fe-2S] clusters. The absorbance coefficient at 458 nm was found to
18 be $12,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ on average, as calculated from the protein content of three independent
19 preparations of ht-PhnI with a similar content of [2Fe-2S] cluster (see Table 1). In contrast to
20 related oxygenases previously characterized, ht-PhnI did not show a well-defined absorption
21 band near 325 nm, but instead a shoulder likely resulting from two poorly resolved absorption
22 bands. The A_{280}/A_{458} ratio was relatively high (26.9) compared to that of naphthalene
23 dioxygenase (17.6, (33)), a feature which might be partly explained by the higher content of
24 aromatic residues of PhnI (Trp and Tyr account for 2.70 and 4.46 % of the total number of
25 residues in PhnI versus 2.18 and 3.73% in naphthalene dioxygenase). EPR analysis of the

1 reduced protein gave a rhombic signal with apparent g values at 2.02, 1.92 and 1.71, which is
2 characteristic of Rieske-type [2Fe-2S] clusters (data not shown).

3 The iron content of the oxygenase was found to vary between 1.73 and 2.55 Fe atoms per pair
4 of $\alpha\beta$ subunits depending on preparations (Table I). In order to estimate the proportion of iron
5 in each metal center, the ht-PhnI preparations were subjected to two independent EPR
6 measurements. Upon reaction with NO, ht-PhnI gave rise to the formation of an Fe(II)-
7 nitrosyl complex which was detected as an heterogeneous $S = 3/2$ EPR signal near $g=4$ (see
8 Fig. 3). Based on the integration of that signal, the occupation rate of Fe(II) at the active site
9 of the enzyme was found to vary between 0.20 and 0.92 (Table I). On the other hand, the
10 estimation of the ratio cluster/ $\alpha\beta$, calculated from the integration of the $S = 1/2$ signal in fully
11 reduced protein samples, yielded values ranging between 0.75 and 0.85. Remarkably, the iron
12 content of the preparations calculated from the sum of the two EPR determinations was in
13 fairly good agreement with the total iron found by chemical assay.

14 The specific activity of the dioxygenase increased as a function of its iron content, but no
15 clear correlation was observed between activity and the occupation rate of the active site
16 (Table 1). In addition, preincubation of ht-PhnI with ferrous ions under reducing conditions
17 prior to enzyme assay resulted in a marginal increase of activity (data not shown).

18

19 *Purification of the ferredoxin and reductase components*

20 The ferredoxin component was anaerobically purified as a His-tagged recombinant protein by
21 IMAC chromatography, and designated ht-PhnA3. The purification procedure described
22 herein yielded about 20 mg of ferredoxin per liter of culture, when strain BL21AI was used as
23 a host for expression. Lower yields were observed with strain BL21(DE3)(pEBA3). The
24 preparation was >90% pure as judged from SDS-PAGE. Cleavage of the protein with
25 thrombin, followed by two short purification steps, gave an essentially pure preparation

1 containing a 12-kDa polypeptide (Fig.1). The molecular mass of this protein, referred to as rc-
2 PhnA3, was 13.5 kDa by gel filtration, which was slightly higher than the theoretical mass of
3 the polypeptide calculated from the *phnA3* gene sequence (11,225 Da, (6)), but indicated that
4 the ferredoxin was monomeric. Both ht-PhnA3 and rc-PhnA3 exhibited absorbance spectra
5 indicative of partial reduction upon isolation under anoxic conditions, but rapidly oxidized in
6 air. In the oxidized state, the two preparations of ferredoxin had identical spectra, featuring
7 absorbance maxima at 278, 325 and 460 nm. The iron content of the ht-PhnA3 and rc-PhnA3
8 preparations was estimated to be 1.5 and 1.7 mol/mol of ferredoxin, respectively. EPR
9 analysis of the reduced ferredoxin gave a signal with g values at 2.02, 1.90 and 1.82, which
10 integrated to 0.86 spin/molecule. Taken together, these data provide strong evidence that the
11 ferredoxin component contains one Rieske-type [2Fe-2S] cluster.

12 The reductase component of the dioxygenase encoded by *phnA4* was overproduced as a 45
13 kDa polypeptide in *E. coli* BL21(DE3)(pEBA4). However, a large proportion of the
14 recombinant protein accumulated in the cells as inclusion bodies, and this problem was not
15 solved by changing the host strain, or by lowering the temperature during induction. Although
16 a low level of the reductase was recovered from the soluble cell extract, the recombinant
17 protein was purified as a His-tagged fusion (ht-PhnA4) by affinity chromatography (0.2 mg/L
18 of culture). The isolated ht-PhnA4 protein was yellow in color, and showed an absorbance
19 spectrum typical for a flavoprotein, with absorbance maxima at 375 and 450 nm. Attempts to
20 overexpress the reductase in *P. putida*, either intact or as a His-tagged fusion, under
21 conditions similar to those described for PhnI, were unsuccessful (data not shown). These
22 observations suggested that PhnA4 was an unstable protein. For our studies on the catalytic
23 activity of the dioxygenase, we replaced PhnA4 by the more stable component, Red_{B356}, of
24 the biphenyl dioxygenase from *C. testosteroni* (20). Enzyme assays performed under standard
25 conditions showed that Red_{B356} efficiently substituted for PhnA4, and titration experiments

1 with increasing concentrations of the reductase indicated that the two isoforms had almost
2 identical affinities for rc-PhnA3 (data not shown).

3

4 *Catalytic properties of the dioxygenase complex : dependence of activity on electron carrier* 5 *concentrations*

6 Purified ht-PhnI catalyzed the oxidation of naphthalene to *cis*-1,2-dihydroxy-1,2-
7 dihydronaphthalene, in a reaction that required the presence of the reductase (Red_{B356}) and
8 ferredoxin (ht-PhnA3) components. When the reductase concentration was varied, at constant
9 concentrations of the ferredoxin and the oxygenase, activity reached half-saturation for a
10 reductase concentration of 0.05 μ M (data not shown). When the ht-PhnA3 concentration was
11 varied, half-saturation was obtained when ferredoxin was added to an approx. 14-fold molar
12 excess over the oxygenase concentration (4.5 μ M). rc-PhnA3 was found to be equally active,
13 indicating that the His-tag did not alter the enzyme function. At a ferredoxin concentration
14 close to saturation (20 μ M; 60-fold molar excess), the specific activity of the enzyme complex
15 was calculated to be 1.25 ± 0.04 U/mg ht-PhnI. For most of the assays performed in this
16 study, the reductase and ferredoxin concentrations were set at concentrations 2.4-fold and 12-
17 fold higher than that of PhnI, respectively. A suboptimal level of ferredoxin was chosen to
18 limit non-specific NADH oxidation by the protein mixture in the absence of PAH substrate.

19

20 *Specific activity and coupling efficiency*

21 To examine the ability of the dioxygenase to oxidize PAHs, the enzyme activity was first
22 determined by measuring the initial rate of oxygen consumption in the presence of an excess
23 of substrate. The dihydrodiol products formed in the reaction mixture were quantified by
24 HPLC as described under Materials and Methods. When fluorene and fluoranthene were
25 tested, substrate oxidation was rather estimated by measuring the amount of residual PAH at

1 the end of the enzymatic reaction, because the oxidation products of these PAHs have not yet
2 been fully characterized (see below and Table 3). In a second and independent set of
3 experiments, the coupling efficiency of the PAH oxidation reactions catalyzed by the
4 dioxygenase was determined by measuring the rates of NADH oxidation and dihydrodiol
5 formation during catalysis. Table 2 compares the results obtained for nine PAHs in terms of
6 specific activity, and reaction coupling between oxygen consumption, NADH oxidation and
7 dihydrodiol formation.

8 Naphthalene appeared to be the only substrate yielding a stoichiometry close to 1, indicating a
9 tight coupling between substrate and cofactor oxidation. It was also the best substrate as it
10 gave the highest rates of O₂ consumption or NADH oxidation. Other substrates were utilized
11 at rates that decreased with the number of fused rings, in reactions that gave rise to significant
12 uncoupling between NADH oxidation and dihydrodiol formation. Chrysene appeared to be
13 the worst substrate in terms of both oxidation rate and coupling efficiency. Although
14 discrepancies were observed with some substrates when comparing enzyme activities assayed
15 by O₂ consumption and NADH oxidation, the coupling efficiencies calculated as either
16 dihydrodiol/O₂ or dihydrodiol/NADH ratios were similar within experimental error, except
17 for phenanthrene and anthracene. It is unclear why different ratios were obtained in the two
18 latter cases.

19 During steady-state catalysis, hydrogen peroxide was produced, with a H₂O₂/O₂ ratio that
20 increased with the uncoupling of the reaction (Table 2). Depending on the substrate, the
21 fraction of oxygen utilized for dihydrodiol formation varied between 8% (chrysene) and
22 100% (naphthalene), the balance of O₂ consumed being mainly allocated to H₂O₂ formation.
23 However, some peroxide was produced even in the tightly coupled naphthalene hydroxylation
24 reaction, the amount of which corresponded to the background O₂ consumption observed in
25 the absence of PAH. Since the enzyme was saturated with naphthalene, the involvement of

1 ht-PhnI in H₂O₂ formation was unlikely, suggesting that the electron carriers were responsible
2 for this side reaction. In a control experiment, we observed that electron carriers alone gave
3 rise to an O₂ consumption of 3.9 nmol.min⁻¹ compared to 5.1 nmol.min⁻¹ for the complete
4 enzyme system, and generated 0.42 H₂O₂ per O₂ consumed (versus 0.57 for the complete
5 system). Hence, a large proportion of the peroxide produced during *in vitro* catalysis of PAH
6 hydroxylation was contributed by the electron carriers alone, most likely through air-
7 oxidation of the reduced PhnA3 ferredoxin component.

8

9 *Steady-state kinetics*

10 Using naphthalene and biphenyl as substrates, the steady state rate of the PhnI-catalyzed
11 reaction was determined in the 0.5-100 μM concentration range. The reaction was monitored
12 spectrophotometrically, by measuring the kinetics of NADH oxidation. This assay method
13 was preferred to the polarographic method, since at low substrate concentrations, the response
14 time of the oxygen electrode was too long to account for the rapid consumption of the
15 substrate. The dioxygenase exhibited a Michaelis-type behavior with respect to substrate
16 concentration, and results indicated that the enzyme had an apparent K_m as low as 0.92 ± 0.15
17 μM for naphthalene. The apparent turnover number for this substrate was $1.82 \pm 0.03 \text{ s}^{-1}$. The
18 enzyme showed a similarly low K_m for biphenyl ($0.42 \pm 0.20 \text{ μM}$), the latter value being only
19 an estimate as enzyme kinetics were extremely short (<4 s) and difficult to calculate
20 accurately at substrate concentrations below 1.0 μM. The turnover number, expressed in
21 terms of rate of dihydrodiol formed, was smaller ($1.01 \pm 0.04 \text{ s}^{-1}$), taking into account a
22 dihydrodiol/NADH ratio of 0.67 in the calculation (Table 2). The apparent specificity
23 constant was calculated to be $2.0 \pm 0.3 \text{ μM}^{-1} \text{ s}^{-1}$ for naphthalene, and $2.4 \pm 1.0 \text{ μM}^{-1} \text{ s}^{-1}$ for
24 biphenyl.

25

1 *Dihydroxylations and monohydroxylations catalyzed by PhnI*

2 GC-MS analysis of the PAH oxidation products revealed that a single dihydrodiol was
3 generated by the dioxygenase in most cases, except when fluorene, fluoranthene, chrysene
4 and benz[a]anthracene were used as substrates (Table 3 and Fig. 2). Biphenyl, naphthalene,
5 phenanthrene were hydroxylated at positions 2,3-, 1,2- and 3,4-, respectively. as previously
6 determined (6), whereas anthracene was most likely converted to the 1,2-dihydrodiol, as
7 found for the dioxygenase present in *S. yanoikuyae* B1 (29). Fluorene oxidation gave rise to
8 the formation of five detectable products, four of which had mass spectra corresponding to
9 monohydroxylated derivatives (Table 3). While 9-fluorenol resulted from a monohydroxylation,
10 the other products might have arisen from either a single hydroxylation or spontaneous
11 dehydration of unstable dihydrodiols primarily produced by the enzyme, as proposed in a
12 previous study on fluorene oxidation by naphthalene dioxygenase (34). Fluoranthene
13 oxidation yielded only one detectable product with a mass spectrum characteristic of a
14 monohydroxylated molecule (the prominent fragment at $m/z=290$ in Table 3 corresponds to
15 the mass of the trimethylsilyl derivative of hydroxyfluoranthene), and a UV absorbance
16 spectrum identical to that of 8-hydroxyfluoranthene (35). This result suggested that the
17 dioxygenase catalyzed a monohydroxylation of fluoranthene on the C8 position. With
18 chrysene, the major product detected was the *cis*-3,4-dihydrodiol, as determined by
19 comparison of GC-MS and UV absorption data with those of the previously characterized diol
20 (30). A more polar compound was also detected by HPLC, which accounted for less than 10%
21 of the total products based on peak area. This compound, which gave a trimethylsilyl derivative
22 with a mass of 584 (Table 3), had the same chromatographic properties as the 3,4,9,10-*bis*-
23 *cis*-chrysene dihydrodiol. We have independently identified this product based on proton and
24 ^{13}C NMR (Jouanneau, Meyer, and Duraffourg, unpublished results). Finally, the dioxygenase-
25 catalyzed oxidation of benzo[a]pyrene yielded a single product with a mass spectrum

1 characteristic of a dihydrodiol derivate (Table 3). The UV spectrum of this product was
2 identical to that of the *cis* -9,10-benzo[a]pyrene dihydrodiol (31).

3 ***Dihydroxylation of benz[a]anthracene***

4 Benz[a]anthracene was converted by CHY-1 dioxygenase to three *cis*-dihydrodiol isomers
5 and one *bis-cis*-dihydrodiol (Table 3 and Fig. 2). The three dihydrodiols have been
6 independently purified and identified as the 1,2-, 8,9- and 10,11-isomers, based on a good
7 match of GC-MS and UV absorbance data with previously published data (32). Quantitative
8 analysis of the diols by GC-MS in several experiments showed that the 1,2-isomer was most
9 abundant ($68 \pm 7\%$), with the 8,9- and the 10,11-isomers representing $9 \pm 3\%$ and $23 \pm 4\%$ of
10 the diols formed, respectively (average of 6 determinations). The proportion of *bis-cis*-
11 dihydrodiol increased during the course of the enzymatic reaction, suggesting that at least one
12 of the dihydrodiol reacted a second time with the enzyme to form the *bis-cis*-dihydrodiol. To
13 test this hypothesis, the 1,2- and 10,11-isomers were independently provided as substrates to
14 the dioxygenase. Interestingly, the two dihydrodiols triggered a fast and uncoupled oxidation
15 of NADH, with small amounts of *bis-cis*-dihydrodiol produced (Table 2). Nevertheless, since
16 the two isomers yielded the same product as judged from HPLC and GC-MS analysis, it is
17 inferred that the *bis-cis*-dihydrodiol bore hydroxyls on carbons in positions 1, 2, 10 and 11 of
18 the benz[a]anthracene molecule. The enzymatic reaction generated hydrogen peroxide at a
19 rate much higher than that attributed to the electron carriers, indicating that, in this case, the
20 formation of H₂O₂ was mainly due to futile cycling of the oxygenase.

21

22 ***Reactivity of ht-PhnI toward benz[a]anthracene as investigated by single turnover*** 23 ***experiments***

24 To further investigate the reactivity of the dioxygenase toward benz[a]anthracene, single
25 turnover reactions were carried out under conditions similar to those previously described for

1 naphthalene dioxygenase (10). In these experiments, the oxygenase component alone was
2 allowed to react with the substrate in air-saturated buffer, and a rapid formation of
3 dihydrodiol was expected at the enzyme active site. In a control experiment with naphthalene
4 as substrate, the formation of dihydrodiol was observed on a time scale lower than 1 min.
5 Surprisingly, the conversion of benz[a]anthracene was much slower and reached completion
6 only after approx. 20 min. In addition, *bis-cis*-dihydrodiol was detected and its concentration
7 increased linearly during the course of the reaction. These results suggested that the rate of the
8 reaction was limited by the solubility of the substrate, which in turn reduced the accessibility
9 of the substrate to the enzyme active site. On the other hand, the dihydrodiols produced which
10 are soluble in water, might compete with the PAH for enzyme active sites, thus explaining the
11 formation of *bis-cis*-dihydrodiol. This interpretation was tested in experiments where the
12 solubility of the PAH was increased by adding an organic solvent to the reaction (Table 4).
13 By carrying out the reaction in 20% acetonitrile, the solubility of benz[a]anthracene was
14 increased 100-fold, and the reaction was completed in less than 1 min. In 30% acetonitrile, the
15 reaction was also fast, but the product yield was lower, probably because of enzyme
16 inactivation. GC-MS analysis of the diols formed showed that the 10,11-isomer was most
17 abundant, and no *bis-cis*-dihydrodiol was detectable in reactions carried out in the presence of
18 solvent. These results contrasted with those obtained in steady-state experiments, since in the
19 latter case, the 1,2-isomer was the predominant product. However, the two sets of data could
20 be reconciled by assuming that, under steady state conditions, the 10,11-isomer is converted
21 to *bis-cis*-dihydrodiol faster than the 1,2-isomer (see below). The results of single turnover
22 experiments demonstrate that the oxygenase preferentially hydroxylates benz[a]anthracene on
23 carbons in positions 10,11. It is also shown that the reactivity of the enzyme towards water-
24 insoluble substrates, which is limited by substrate transfer to the active site, could be

1 enhanced by carrying out the reaction in aqueous medium containing up to 20% organic
2 solvent.

3
4 ***Interaction of ht-PhnI with benz[a]anthracene and dihydrodiols as probed by EPR***
5 ***spectroscopy***

6 The high level of uncoupling observed when benz[a]anthracene dihydrodiols were incubated
7 with the dioxygenase indicated that these compounds did not interact correctly with the
8 enzyme active site. As a means to probe this interaction, we carried out EPR analysis of
9 complexes between the active site Fe(II) and NO, in the presence or absence of substrate (Fig.
10 3). The spectrum of the substrate-free enzyme showed a complex signal centered at $g=4.0$,
11 which might reflect the existence of more than one NO binding site. Alternatively, the signals
12 might arise from different conformations due to different orientations of the Fe-NO bond.
13 Interestingly, two pairs of resonance lines at 3.68/4.40 and 3.98/4.07, which were prominent
14 in the spectrum of the free enzyme, underwent dramatic changes upon substrate binding (Fig.
15 3). These lines almost disappeared in the spectrum of the benz[a]anthracene-bound enzyme,
16 and were undetectable in the case of the naphthalene-bound enzyme complex. The shape of
17 the signals obtained for the dihydrodiol-bound enzyme complexes showed patterns
18 intermediate between the substrate-free and the benz[a]anthracene-bound enzyme, the
19 spectrum of the 10,11-isomer-bound enzyme being closer to the latter. These observations
20 could be taken as indirect evidence that the dihydrodiols were not correctly oriented in the
21 substrate-binding pocket to allow for a productive catalytic conversion into *bis-cis*-
22 dihydrodiol. Based on these EPR data, the 10,11-isomer would bind the active site in a more
23 favorable position than the 1,2-isomer. Alternatively, our data might indicate that the enzyme
24 was not saturated by the dihydrodiols, although concentrations in molar excess over the
25 enzyme catalytic sites were used. Accordingly, in experiments where the concentration of the
26 dihydrodiols was doubled (0.2 mM), we observed that the relative intensity of the resonance

1 lines at 3.68/4.40 and 3.98/4.07, attributed to the free enzyme (see above), was significantly
2 reduced compared to that found in spectra b and c in Fig. 3. The spectra of the enzyme-
3 dihydrodiol complexes were then only slightly different from those obtained with naphthalene
4 or benz[a]anthracene as ligands (data not shown). We therefore conclude that the differences
5 seen in spectra b and c, compared to spectrum d, are likely due, in great part, to the
6 contribution of substrate-free enzyme, thus reflecting a relatively low affinity of the enzyme
7 for the two benz[a]anthracene dihydrodiols.

8

9 **DISCUSSION**

10

11 The ring-hydroxylating dioxygenase described in this study exhibits one of the broadest
12 substrate specificities toward PAHs ever reported. It is a rare example of an enzyme able to
13 attack aromatic substrates composed of 2 to 5 rings, which gave us an opportunity to compare
14 the kinetics of dioxygenation for a wide range of PAHs. Consistent with previous *in vivo*
15 observations (6), the specific activity of strain CHY-1 dioxygenase was highest with
16 naphthalene, and declined as a function of substrate size. Ironically, while strain CHY-1 was
17 isolated for its ability to grow on chrysene as sole carbon source (18), chrysene appeared to be
18 the worst substrate in terms of both oxidation rate and coupling efficiency (Table 2). This
19 finding suggested that a dioxygenase other than PhnI might be responsible for the initial
20 attack of chrysene in strain CHY-1. However, such a hypothesis can be ruled out on the basis
21 of our previous work showing that a mutant strain lacking PhnI failed to grow on and oxidize
22 chrysene, as well as any other PAH used as substrate by the parental strain CHY-1 (6). Hence,
23 PhnI is essential for growth on chrysene, and its low activity towards this substrate is
24 probably one of the main reason why strain CHY-1 shows slow growth and poor cell yields
25 when provided with chrysene as sole carbon source (18). In comparison, benz[a]anthracene,
26 another 4-ring PAH which was oxidized at a much higher rate, failed to support growth of

1 strain CHY-1 (18). Possible reasons which might explain this paradox are discussed below.
2 The present study also unveiled interesting new features of strain CHY-1 dioxygenase,
3 including its ability to utilize fluoranthene and benzo[a]pyrene. Hence, this enzyme has the
4 remarkable potential to initiate the degradation of at least half of the 16 EPA priority PAHs,
5 including the carcinogenic benz[a]anthracene and benzo[a]pyrene. A dioxygenase activity
6 with a similarly broad substrate specificity has only been found in *S. yanoikuyae* B1, but the
7 corresponding enzyme has not yet been described (36).

8 The dioxygenase from strain CHY-1 is a three-component enzyme that shares many of
9 the biochemical properties of counterparts found in other bacteria degrading aromatic
10 hydrocarbons. Based on the properties of the associated electron carriers, it would belong to
11 class IIB of the dioxygenase classification proposed by Batie et al. (37), together with
12 benzene and biphenyl dioxygenases (1). However, amino acid sequence analysis of the PhnI
13 α and β subunits rather indicated that the enzyme was more closely related to naphthalene and
14 phenanthrene dioxygenases (6), consistent with the substrate specificity of the dioxygenase
15 determined herein. Purified ht-PhnI contained Rieske [2Fe-2S] and mononuclear Fe (II)
16 centers as expected, but quantitative analysis based on EPR spectroscopy revealed that the
17 two types of metal binding sites were not fully occupied. We cannot rule out metal loss during
18 purification, despite employing conditions likely to minimize such losses. However, it is also
19 possible that the biosynthesis of the oxygenase in *Pseudomonas* recombinant cells yielded a
20 protein that did not have its full content of metal centers. Examples of purified oxygenases
21 having a full complement of iron have occasionally been reported (10, 38, 39), but enzyme
22 preparations partially lacking iron are more frequently obtained (4, 40-42). Hence, our EPR-
23 based method to determine the occupancy of both iron binding sites might be of general
24 interest for the characterization of such types of enzymes.

1 The *in vitro* activity of the dioxygenase was highly dependent on the component ratio
2 ferredoxin over oxygenase, half saturation occurring for a 14-fold molar excess of the
3 ferredoxin. Likewise, a high molar excess of ferredoxin was required to reach maximal
4 activity in the case of naphthalene (10), and biphenyl dioxygenase (39). As a consequence,
5 comparison of the apparent specific activities or k_{cat} of enzymes from different sources should
6 be regarded with caution. At a ferredoxin/oxygenase ratio of 14, the CHY-1 dioxygenase
7 showed an apparent k_{cat} of $1.82 \pm 0.03 \text{ s}^{-1}$ and a specificity constant of $2.0 \pm 0.3 \mu\text{M}^{-1} \text{ s}^{-1}$ with
8 naphthalene as substrate. A velocity constant 2.5-fold as high (4.48 s^{-1}) was found at a molar
9 ratio of 60. In comparison, the biphenyl dioxygenase from *C. testosteroni* exhibited a k_{cat} of
10 $7.0 \pm 0.2 \text{ s}^{-1}$ and a specificity constant of $1.2 \pm 0.1 \mu\text{M}^{-1} \text{ s}^{-1}$, at a ratio of 23 (39). An apparent
11 k_{cat} of 2.4 s^{-1} and a specificity constant of $7.0 \mu\text{M}^{-1} \text{ min}^{-1}$ (equivalent to $0.11 \mu\text{M}^{-1} \text{ s}^{-1}$), was
12 reported for 2-nitrotoluene dioxygenase from *Comamonas* JS765, at a ratio of 3.7 (42).

13 The present study revealed that the coupling between substrate oxidation and O_2 (or
14 NADH) utilization varied widely depending on PAHs. While the conversion of naphthalene
15 to dihydrodiol by the dioxygenase involved a stoichiometric amount of O_2 , the oxidation of
16 all other PAHs, except fluorene, gave rise to significant uncoupling. This uncoupling was
17 associated with the release of H_2O_2 , although a large proportion of the peroxide could be
18 attributed to auto-oxidation of the electron carriers alone. Assuming that most of the peroxide
19 detected in our *in vitro* assays is an artifact due to the great molar excess of electron carriers
20 used in these assays, it is unclear whether peroxide would be produced in significant amounts
21 *in vivo* as a consequence of the dioxygenase-catalyzed oxidation of PAHs, given that electron
22 carriers are certainly in limiting amounts in natural host cells. Nevertheless, because of this
23 uncoupling, part of the energy recovered from the catabolism of PAHs as NAD(P)H, is
24 probably lost as unproductive transfer of reducing equivalents to O_2 . This energy burden
25 might affect growth yield, and could explain, at least in part, the higher resistance of 4- and 5-

1 ring PAHs to bacterial biodegradation, inasmuch as the coupling efficiency of the
2 dioxygenase reaction was lowest with those PAHs. Other dioxygenase systems were found to
3 give rise to partially uncoupled reactions when challenged with poor substrates, and this was
4 associated to a release of H₂O₂. Uncoupling occurred when naphthalene dioxygenase was
5 incubated with benzene, and the peroxide formed was found to irreversibly inactivate the
6 enzyme, probably because of the damage done by the product of the Fenton reaction between
7 peroxide and the active site Fe(II) (33). Biphenyl dioxygenase also catalyzed uncoupled
8 reactions and H₂O₂ production when provided with certain dichlorobiphenyls, which might
9 result in the inhibition of the dioxygenation of other chlorobiphenyls. This effect, combined
10 with the deleterious action of peroxide on cells, was predicted to inhibit the microbial
11 catabolism of polychlorobiphenyls (39). Interestingly, some PAH-degrading bacteria were
12 shown to specifically induce a catalase-peroxidase when grown on PAHs, thereby providing a
13 means to cope with the dioxygenase-mediated formation of peroxide (43).

14 The oxidation of benz[a]anthracene by the dioxygenase from strain CHY-1 is of
15 particular interest because this PAH was converted into three dihydrodiols, two of which
16 were subjected to a second dihydroxylation in a highly uncoupled reaction. The amount of
17 *bis-cis* dihydrodiol formed in this secondary reaction within the time of an assay (around 5
18 min) was estimated to be between 20 and 40 % of the total amount of diols primarily
19 produced by the enzyme. These observations have several implications as for the reactivity
20 and the coupling efficiency of the enzyme with respect to benz[a]anthracene. The rapid
21 accumulation of *bis*-dihydrodiol indicated that the 1,2- and 10,11-dihydrodiols competed with
22 benz[a]anthracene for the enzyme active site, which they reached faster than the PAH because
23 of their much higher water solubility. This interpretation is supported by single turnover
24 experiments showing that organic solvent accelerated benz[a]anthracene oxidation and
25 suppressed *bis*-dihydrodiol formation. Because the dihydrodiols are poor substrates for the

1 dioxygenase, as confirmed by EPR probing of nitrosyl-enzyme complexes, the competition
2 they exert on benz[a]anthracene oxidation was expected to alter the coupling efficiency. A
3 ratio dihydrodiol/O₂ of 0.31 was calculated without taking into account the formation of *bis*-
4 dihydrodiol (Table 2). A calculation of the O₂ consumed for *bis*-dihydrodiol formation,
5 assuming that 2 O₂ molecules were required per each molecule formed, allowed to bracket the
6 ratio (*bis*-dihydrodiol + dihydrodiols)/O₂ between 0.42 and 0.55. This is definitely higher than
7 the dihydrodiol/O₂ ratios found for chrysene and benzo[a]pyrene. Hence, based on coupling
8 efficiency and oxidation rates, benz[a]anthracene appeared to be a better substrate than
9 chrysene, and yet, it could not support growth of strain CHY-1. In *S. yanoikuyae* B1, a strain
10 which cannot grow on benz[a]anthracene either, previous studies showed that the oxidation
11 of this PAH led to the accumulation of three metabolites identified as 1-hydroxyanthranic
12 acid, 2-hydroxy 3-phenanthroic acid and 3-hydroxy 2-phenanthroic acid (44). These
13 metabolites were predicted to arise from five similar degradation steps of benz[a]anthracene,
14 involving an initial dihydroxylation on positions 1,2-, 8,9- and 10,11-, respectively. The data
15 suggested that a subsequent step in the catabolic pathway of this PAH might be too slow to
16 allow efficient processing of the metabolites, thereby preventing bacterial growth.
17 Alternatively, the possibility was considered that the peroxide formed in the dioxygenase-
18 catalyzed oxidation of benz[a]anthracene, or in the secondary oxidation of dihydrodiols, could
19 inhibit growth. The secondary reaction generating *bis*-dihydrodiol is highly uncoupled, but it
20 is unknown whether it would occur *in vivo* in strain CHY-1. *S. yanoikuyae* B1 did not
21 produce any detectable *bis*-dihydrodiol when degrading benz[a]anthracene (44), indicating
22 that dihydrodiols were rapidly metabolized. Accordingly, we have recently characterized a
23 dihydrodiol dehydrogenase which can efficiently convert the three dihydrodiol isomers of
24 benz[a]anthracene to corresponding catechols (22). Hence, in PAH-degrading
25 Sphingomonads, the coupling between the first and the second enzymatic step of the catabolic

1 pathway likely prevents the dioxygenase from catalyzing unproductive and potentially
2 deleterious reactions.

3 In this work, we have purified and characterized a ring-hydroxylating dioxygenase with
4 an exceptionally broad substrate specificity, which provides a good model for further
5 structure-function studies on this class of enzymes. The oxygenase component has been
6 recently crystallized and subjected to X-ray diffraction analysis. The structure of the protein
7 has been solved to 1.85 Å resolution and will be described elsewhere (J. Jakoncic, Y.
8 Jouanneau, C. Meyer, V. Stojanoff, unpublished data).

9

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Table 1 : Specific activity and iron content of different preparations of ht-PhnI .

Iron was determined by chemical analysis and EPR spectroscopy. The standard error for each determination was less than 10%.

Ht-PhnI preparation	1	2	3
Concentration ^a (μM)	21.0	25.7	19.7
Total Fe (atoms/αβ)	1.73	2.54	2.55
Fe(II)-NO (spin/αβ)	0.20	0.78	0.92
[2Fe-2S] (spin/αβ)	0.75	0.85	0.75
Specific activity ^b (U/mg)	0.41 ± 0.035	0.86 ± 0.01	0.58 ± 0.01

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^a The protein concentration were estimated from the microbiuret assays, using a molecular mass of 215 kDa for ht-PhnI. Based on these determinations, the absorbance coefficient of ht-PhnI at 458 nm was calculated to be $\epsilon_{458} = 12,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$

^b as determined by the NADH oxidation assay with naphthalene as substrate. The molar ratio PnA3/PhnI was approx. 9.5 in these assays.

Table 2 : Specific activity and coupling efficiencies of the dioxygenase as a function of PAH substrates^a

Substrate	O ₂ consumption	Dihydrodiol formed	Dihydrodiol/O ₂ ^b	H ₂ O ₂ / O ₂	NADH oxidation	Diol/NADH
	nmol.min ⁻¹ .mg ⁻¹	nmol.min ⁻¹ .mg ⁻¹			nmol.min ⁻¹ .mg ⁻¹	
Naphthalene	460 (20)	418 (20)	1.03 (0.07)	0.122 (0.010)	510 (50)	0.92 (0.06)
Biphenyl	410 (40)	260 (12)	0.70 (0.01)	0.28 (0.015)	290 (35)	0.67 (0.03)
Phenanthrene	370 (30)	300 (30)	0.83 (0.03)	0.128 (0.028)	465 (10)	0.46 (0.01)
Anthracene	360 (30)	130 (10)	0.36 (0.02)	0.53 (0.02)	141 (6)	0.62 (0.01)
Fluorene	91.5 (7)	91 (5) ^c	0.99 (0.05)	-	-	-
Fluoranthene	147 (24)	76 (5) ^c	0.51 (0.04)	-	-	-
Benz[a]anthracene	167 (20)	52 (5) ^d	0.31 (0.04)	0.48 (0.04)	46 (2)	0.40 (0.07)
Chrysene	49 (2)	2.5 (0.3)	0.082 (0.011)	0.59 (0.08)	9.6 (0.6)	0.098 (0.007)
Benzo[a]pyrene	43 (8)	7.8 (0.7)	0.33 (0.02)	0.50 (0.005)	10 (1)	0.19 (0.01)
1,2-Benz[a]anthracene diol	2500 (400)	11 (2)	0.0044 (0.001)	0.83	-	-
10,11-Benz[a]anthracene diol	1500 (150)	16.5 (0.5)	0.011 (0.0005)	0.66	-	-

^a The indicated values represent means obtained from two to four determinations, with standard deviations given in parentheses. - means not determined. O₂ consumption and NADH oxidation represent initial rates corrected for the background activity observed in the absence of PAH

substrate. The rates of dihydrodiol formation are average rates calculated over the duration of the assay which lasted between 4 min(naphthalene) and 11 min (benzo[a]pyrene).

^b Values were calculated as ratios between the rates of dihydrodiol formation (column 3), and the average rates of O₂ consumption over the duration of the assay, which are lower than the initial rates of O₂ consumption given in column 2.

^c These values represent rates of substrate oxidation, because the oxidation products from florene and fluoranthene could not be measured accurately (see text).

^d Three diol isomers were produced, which were not separated by HPLC and quantified as a mixture (see Methods). The contribution of *bis-cis*-dihydrodiol, also produced in the reaction, was not taken into account in the calculations.

Table 3 : GC-MS identification of PAH oxidation products formed by CHY-1 dioxygenase.

Substrate	Properties of trimethylsilyl derivatives of products			Identification
	GC retention time (min)	Relative peak area (%)	m/z and relative abundance (%) of major fragments	
Fluorene	15.18	6	254(M ⁺ , 41), 239(8), 166(15), 165(100)	9-FluorenoI ^a
	16.18	6	254(M ⁺ , 100), 239(39), 224(19), 223(89), 178(8), 165(13)	Hydroxyfluorene
	16.49	14	254(M ⁺ , 100), 239(70), 223(3), 195(2.5), 178(11), 165(63)	Hydroxyfluorene
	16.67	16	254(M ⁺ , 100), 239(67), 223(2), 195(2), 178(10), 165(25)	Hydroxyfluorene
	17.70	58	342(M ⁺ , 100), 327(4), 253(62), 238(5), 178(5), 164(5), 163(5)	Dihydroxyfluorene
Fluoranthene	20.74	100	290(M ⁺ , 100), 275(70), 219(11), 215(11), 201(14), 200(14), 189(15)	8-Hydroxyfluoranthene
Chrysene	23.08	>90 ^b	406(M ⁺ , 28), 317(14), 316(18), 303(32),	3,4-dihydrodiol ^c

			244(14), 228(40), 226(33), 215(66), 191(100)	
	24.55	<10 ^b	584(M ⁺ , 8), 393(8), 355(8), 282(13), 281(40), 207(100), 191(73)	3,4,9,10- <i>bis</i> -dihydrodiol ^c
Benz[a]anthracene	22.65	68	406(M ⁺ , 28), 316(26), 303(30), 281(14), 228(28), 226(25), 215(36), 191(50), 73(100)	1,2-Dihydrodiol ^c
	22.95	23	406(M ⁺ , 32), 316(29), 303(59), 228(21), 226(14), 191(46), 73(100)	10,11-Dihydrodiol ^c
	23.43	9	406(M ⁺ , 31), 316(27), 303(80), 281(16), 228(18), 226(14), 215(29), 191(68), 73(100)	8,9-Dihydrodiol ^c
	23.53	20-40 ^d	584(M ⁺ , 46), 481(21) 392(9), 355(16), 281(22), 207(48), 191(100)	1,2,10,11- <i>bis</i> -Dihydrodiol ^c
Benzo[a]pyrene	25.88	100	430(M ⁺ , 5.5), 415 (1.5), 341(5), 327(8), 281(20), 252(8), 207(100)	9,10-Dihydrodiol ^c

^a Identification based on match of mass spectrum and GC retention time with those of an authentic sample.

^b Percentages estimated from HPLC peak area.

^c Identification based on comparisons of the GC-MS and UV absorbance data of the products with previously published data (see text).

^d The indicated range represents an estimation of the *bis*-dihydrodiol/dihydrodiols ratio, as indicated in the text.

Table 4 : Solvent-facilitated formation of benz[a]anthracene *cis*-dihydrodiols catalyzed by PhnI in single turnover reactions.

Acetonitrile % in the reaction	Benz[a]anthracene solubilized (μM)	Incubation time (min)	Total diols/PhnI	Percentage of dihydrodiols produced as ^a		
				1,2-diol	10,11-diol	8,9-diol
0	0.025	1	0.44			
		10	2.12 ^b	28	64	8
20	2.5	1	2.01	30	53	17
		10	1.94	36	50	14
30	27.0	1	1.12	29	43	28
		10	0.96	28.5	54	17.5

^a As determined from the peak area of trimethylsilyl derivatives analyzed by GC-MS

^b Some *bis-cis*-dihydrodiol was also produced which accounted for about 1.5% of the total diols formed, based on HPLC determination.

Figure legends

FIGURE 1: SDS-PAGE of the purified components of the ring-hydroxylating dioxygenase of *Sphingomonas* strain CHY-1. Polypeptides were electrophoresed on a 15% polyacrylamide slab gel. Lane 1: molecular mass markers. Lane 2: ht-PhnI, 3.2 μ g. Lane 3: ht-PhnA3, 1.0 μ g. Lane 4: ht-PhnA4, 1.8 μ g.

FIGURE 2 : Dioxygenation reactions of four-ring PAHs catalyzed by ht-PhnI.

Benz[a]anthracene was converted into three dihydrodiols isomers (a), in proportions which varied depending on experimental conditions (see text). The 1,2- and 10,11-isomers were subjected to a second dihydroxylation, yielding the same *bis-cis*-dihydrodiol (b). Chrysene was oxidized to a single dihydrodiol, which could subsequently react with the dioxygenase to yield the 3,4,9,10-*bis-cis*-dihydrodiol (c). Reaction products were identified as indicated in the text. Stereochemical configurations were assumed to be identical to those reported in previous studies on the *S. yanoikuyae* enzyme (32, 45).

FIGURE 3 : EPR spectra of nitrosyl complexes of ht-PhnI in the presence or absence substrates.

Protein samples contained 22.0 μ M ht-PhnI in 0.18 ml of 50 mM potassium phosphate, pH 7.5, and, either no substrate (spectrum a) or one of the following substrates (0.1 mM) added in 10 μ l acetonitrile : 1,2-benz[a]anthracene dihydrodiol (spectrum b); 10,11-benz[a]anthracene dihydrodiols (spectrum c); benz[a]anthracene (spectrum d); naphthalene (spectrum d). After 15 min at room temperature under argon, nitrosyl complexes were prepared (see Materials and Methods), samples were transferred into EPR tubes and frozen. Acquisition conditions : Temperature; 4K; Microwave power: 250 μ W; modulation frequency: 100 kHz; modulation amplitude: 1mT. Relevant g values are indicated.

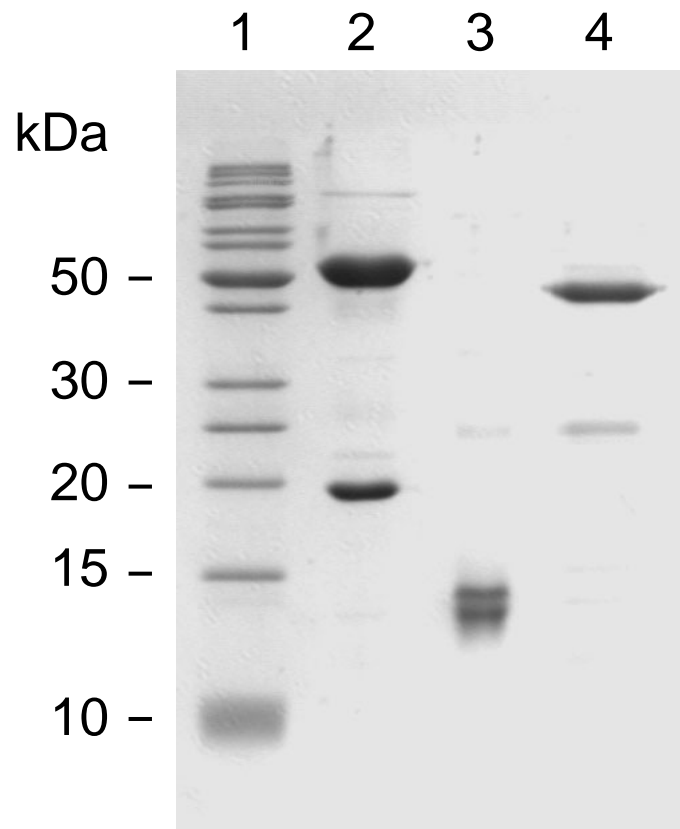


Fig. 1

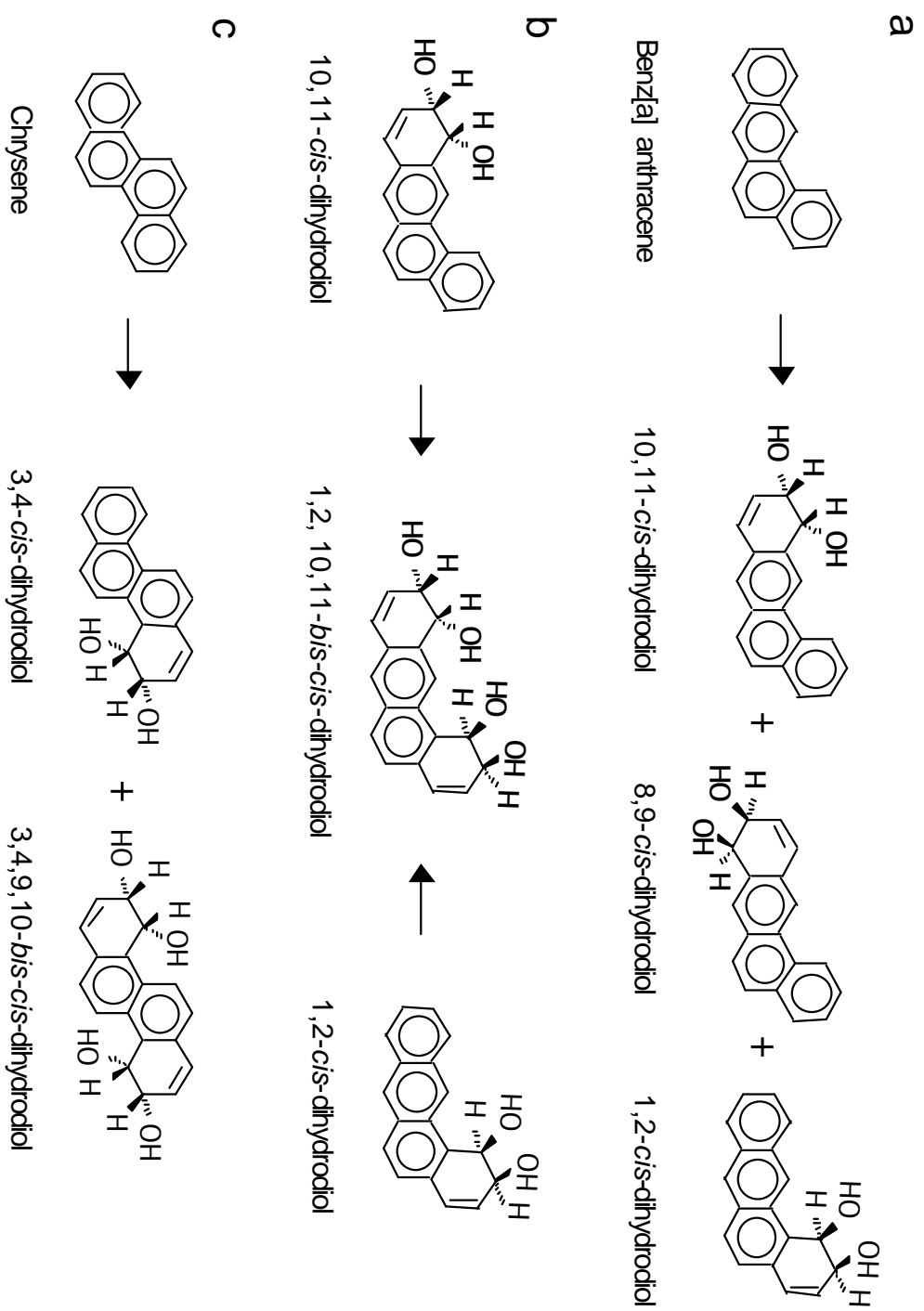


Fig. 2

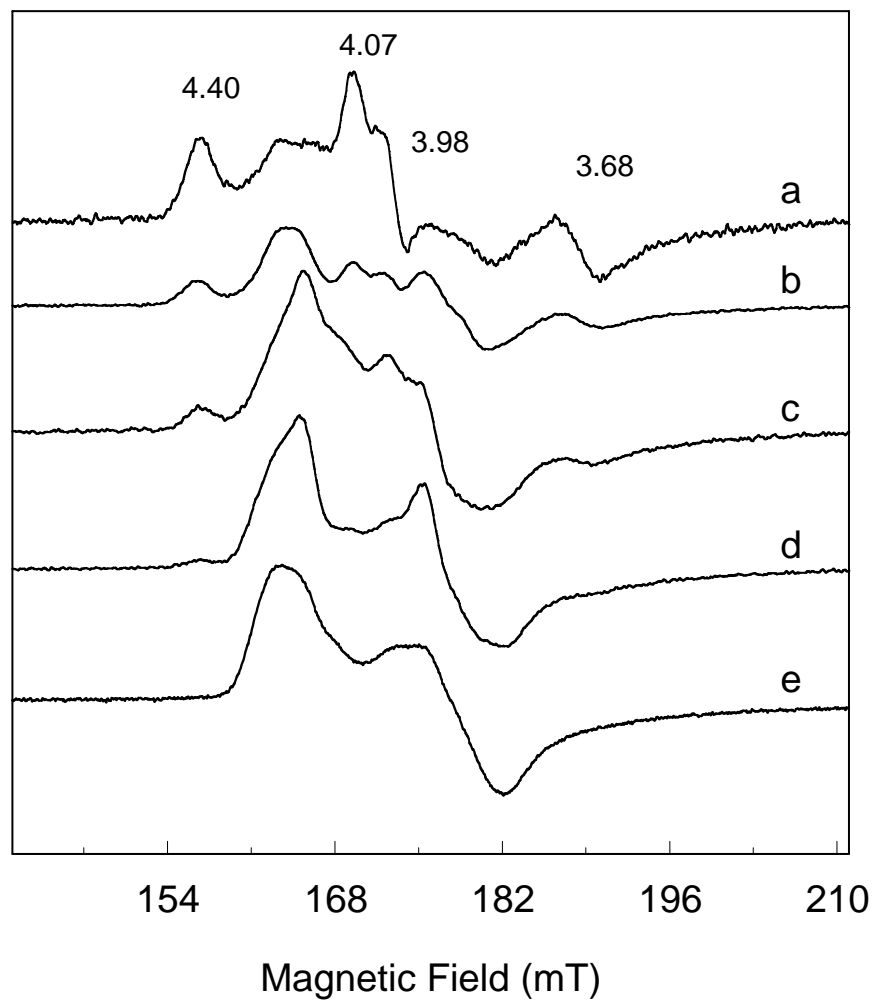


Fig. 3

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Characterization of a naphthalene dioxygenase endowed with an exceptionally broad specificity towards polycyclic aromatic hydrocarbons†

By Yves Jouanneau ^{*}, [‡], Christine Meyer[‡], Jean Jakoncic[§], Vivian Stojanoff[§], Jacques Gaillard^{||}

