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Purification and properties of ferredoxin$_{BPH}$, a component of biphenyl 2,3-dioxygenase of *Pseudomonas* sp strain LB400

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The ferredoxin component (ferredoxin$_{BPH}$) of biphenyl 2,3-dioxygenase was purified to homogeneity from crude cell extract of *Pseudomonas* sp strain LB400 using ion exchange, hydrophobic interaction and gel filtration column chromatography. The protein was a monomer with a molecular weight of 15 000 and contained 2 gram-atoms each of iron and acid-labile sulfur. Ultraviolet-visible absorbance spectroscopy showed peaks at 325 nm and 460 nm with a broad shoulder around 575 nm. The spectrum was partially bleached in the visible region upon reduction by reductase$_{BPH}$ with NADPH as the source of electrons. Electron paramagnetic resonance spectrometry showed no signals for the oxidized protein. Upon reduction with sodium dithionite, signals with $g_x = 1.82$, $g_y = 1.92$ and $g_z = 2.02$ were detected. These results indicate that the protein contains a Rieske-type (2Fe-2S) iron-sulfur center. Ferredoxin$_{BPH}$ was required for the oxidation of biphenyl by the terminal oxygenase component of the enzyme and is probably involved in the transfer of reducing equivalents from reductase$_{BPH}$ to the terminal oxygenase during catalysis.

Keywords: ferredoxin; dioxygenase; biphenyl; polychlorinated biphenyl; electron transport; Rieske iron-sulfur protein; *Pseudomonas*

Introduction

Biphenyl is utilized as a growth substrate by aerobic bacteria that possess enzymes that utilize molecular oxygen to activate and cleave the aromatic ring (Figure 1). These enzymes will also accept some polychlorinated biphenyls (PCBs) as substrates; however, complete degradation is usually blocked at one or more steps, thus preventing most PCBs from supporting growth. PCBs are stable, manmade compounds containing from 1–10 chlorine substituents. Commercial PCB mixtures are widely dispersed in the environment as a result of 50 years of heavy industrial use and their resistance to biodegradation [20]. Biphenyl has been used as an enrichment and growth substrate to select for aerobic bacteria that cooxidize lightly chlorinated PCB congeners [1,3,12]. The aerobic degradation pathway for biphenyl and PCBs is initiated by biphenyl 2,3-dioxygenase, a multicomponent enzyme that incorporates both atoms of molecular oxygen at the 2,3-ring position [14,17]. The product of the reaction is (+)*cis*-(2*R*,3*S*)-dihydroxy-2,3-dihydro-1-phenylcyclohexa-4,6-diene (*cis*-biphenyl 2,3-dihydrodiol) [15,33]. *Pseudomonas* sp strain LB400 was previously isolated from a PCB-contaminated site [5]. This bacterium has an exceptional ability to attack a broad range of PCB congeners, including those with chlorine substituents located at the usual site of attack by biphenyl 2,3-dioxygenase [3]. Strain LB400 contains a three-component biphenyl 2,3-dioxygenase that hydroxylates 2,5,2′,5′-tetrachlorobiphenyl and 2,5,2′-trichlorobiphenyl at the 3,4-ring position. In addition, the enzyme attacks the 2,3-position of 2,2′- and 2,4′-dichlorobiphenyl and the 2′,3′-position of 2,5,2′-trichlorobiphenyl causing spontaneous elimination of a chlorine substituent [16,26]. The unusual reactions catalyzed by this multicomponent enzyme have prompted our investigations of the structural and functional properties of the protein components [13,15–17]. Here, we report the purification and characterization of ferredoxin$_{BPH}$, an electron transport component of the biphenyl 2,3-dioxygenase of strain LB400.

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Materials and methods

Organism and growth conditions

Pseudomonas sp strain LB400 was provided by Herman L Finkbeiner, Research and Development Center, General Electric Company, Schenectady, NY, USA. The organism was cultured in a basal salts medium with biphenyl as the carbon source, as previously described [17].

Preparation of cell extract

Crude cell extract was prepared from 25 g (wet weight) of cells suspended in 75 ml of 50 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-Tris) buffer, pH 7.0, containing 5% (v/v) ethanol and 5% (v/v) glycerol (BEG). The cells were lysed by passage through a chilled French pressure cell at 20 000 p.s.i. followed by ultracentrifugation to remove membranes and cell debris as previously described [17]

Protein purification

All steps were performed at 4°C. Crude cell extract (1.62 g of protein) was applied to a 5 cm × 16 cm Q-Sepharose Fast Flow column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA), equilibrated with BEG buffer. Protein was eluted with a linear salt gradient to 400 mM KCl (total gradient volume, 1.7 L). Fractions containing ferredoxin$_{BPH}$ were

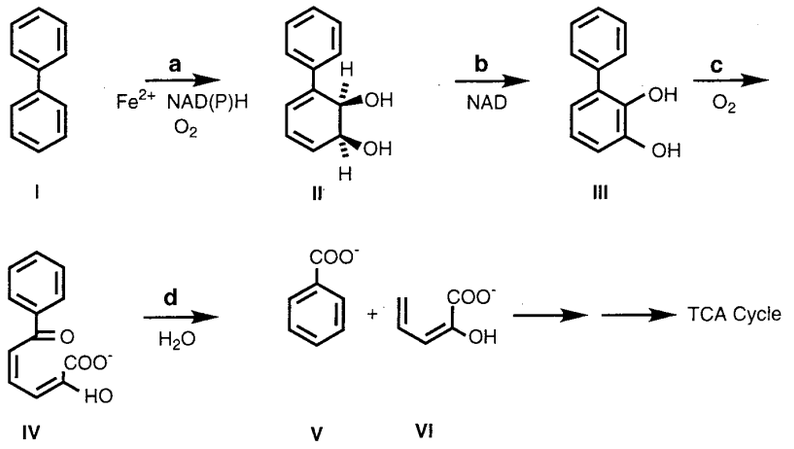

Figure 1 Pathway for the degradation of biphenyl by aerobic bacteria. I, biphenyl; II, *cis*-biphenyl 2,3-dihydrodiol; III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; V, benzoate; VI, 2-hydroxypenta-2,4-dienoate. (a) biphenyl 2,3-dioxygenase; (b) *cis*-biphenyl 2,3-dihydrodiol dehydrogenase; (c) 2,3-dihydroxybiphenyl dioxygenase; (d) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase.

pooled and concentrated by ultrafiltration using a 10-kDa cut-off membrane filter (Amicon, Danvers, MA, USA). Ammonium sulfate was added to the concentrated protein solution to a concentration of 2.4 M and the solution was centrifuged at 23 000 × *g* for 30 min. The supernatant was applied to a 2.6 cm × 10 cm phenyl-Sepharose column (Pharmacia) equilibrated with 2.2 M ammonium sulfate in BEG. Bound protein was eluted with a 1-L linear gradient from 2.2 M to 0 M ammonium sulfate. Fractions containing ferredoxin$_{BPH}$ were concentrated by ultrafiltration as described above and applied to a 2.6 cm × 67 cm S-300 gel filtration column (Pharmacia) equilibrated with BEG buffer containing 150 mM KCl. Fractions containing ferredoxin$_{BPH}$ were concentrated by ultrafiltration, equilibrated with BEG buffer and stored at −70°C.

Activity

Fractions containing ferredoxin$_{BPH}$ were routinely assayed by measuring the reduction of cytochrome *c* in the presence of partially purified preparations of NAD(P)H : ferredoxin$_{BPH}$ oxidoreductase (reductase$_{BPH}$) as described previously [17]. Reductase$_{BPH}$ requires the presence of ferredoxin$_{BPH}$ for reduction of cytochrome *c*. Cytochrome *c* functions as an artificial electron acceptor in the assay and reduction is determined by the increase in absorbance at 550 nm. Each assay mixture contained 87 μM horse heart cytochrome *c*, 300 μM NADPH, 130 μg of partially pure reductase$_{BPH}$ [17] and appropriate amounts of ferredoxin$_{BPH}$. The reaction was carried out in 1.0 ml of 50 mM Bis-Tris, pH 7.0. An extinction coefficient of 21 000 M^{-1} cm^{-1} for reduced minus oxidized cytochrome *c* was used to calculate activity [30]. Biphenyl 2,3-dioxygenase activity was determined by measuring the amount of ^{14}C-labeled *cis*-biphenyl 2,3-dihydrodiol formed after incubation with 160 μM ^{14}C-labeled biphenyl (added in dimethylformamide), 400 μM NADPH, 400 μM ferrous ammonium sulfate, 130 μg partially pure reductase$_{BPH}$, 91 μg of the pure terminal oxygenase component (ISP$_{BPH}$) [15] and appropriate amounts of ferredoxin$_{BPH}$. The reaction was carried out in 0.25 ml of 50 mM 2-(*N*-morphilino)ethanesulfonic acid buffer, pH 6.0, as previously described [17].

Protein concentration

Protein concentration was determined by the method of Bradford [6] with bovine serum albumin as the standard.

Molecular weight

The molecular weight of ferredoxin$_{BPH}$ was determined with a 1.0 cm × 50 cm Superdex 75 column (Pharmacia) equilibrated with BEG buffer containing 150 mM KCl. The column was calibrated with the following protein standards (molecular weight) : bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000) and ribonuclease A (13 700). The molecular weight was also determined by electrophoresis on a sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) (12% total monomer, 0.75 mm × 5 cm) by the method of Laemmli [21]. The standard molecular weight proteins were phosphorylase *b* (97 000), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400).

N-terminal amino acid sequence

The *N*-terminal amino acid sequence was determined by Edman degradation and analysis on an automated sequencer (Applied Biosystems, Foster City, CA, USA) at the University of Iowa protein sequencing facility, Iowa City, IA.

Ultraviolet-visible absorption spectra

Spectra of ferredoxin$_{BPH}$ were recorded under an N_2 atmosphere in 1 ml of 50 mM Bis-Tris, pH 7.0 at a protein concentration of 0.29 mg ml^{-1}. The oxidized spectrum was recorded for the protein as isolated and the reduced spectrum was recorded after reduction with 400 nmol of NADPH in the presence of catalytic quantities (49 μg) of partially purified reductase$_{BPH}$ [17].

Electron paramagnetic resonance (EPR) spectra

Spectra of liquid nitrogen frozen ferredoxin$_{BPH}$ (0.2 mg) were recorded as isolated (oxidized) and after reduction with sodium dithionite (25 scans each), at 5.02 mW microwave power, 3650 G centerfield, 1000 G scan range, 9.29 GHZ modulation frequency, 335.5 s sweep time, and

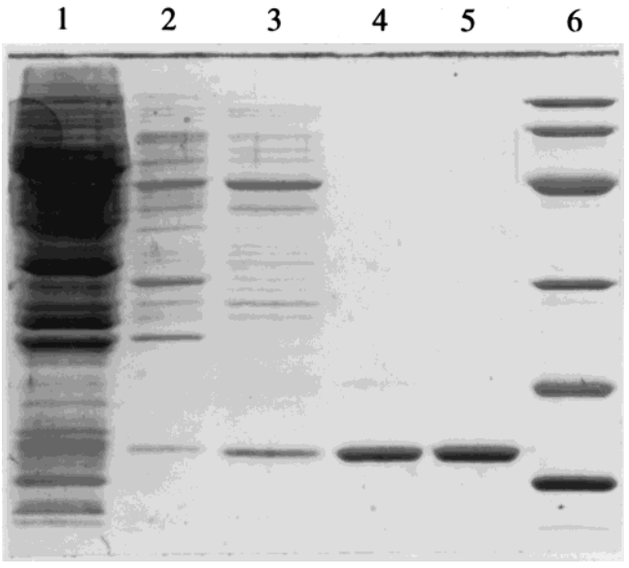

Figure 2 SDS-PAGE analysis of samples taken after each step of the purification ferredoxin$_{BPH}$. Lane 1, 30 μg of cell extract protein; lane 2, 15 μg of protein following the Q Sepharose column step; lane 3, 5 μg of protein following equilibration with 2.4 M ammonium sulfate; lane 4, 2 μg of protein following the phenyl-Sepharose column step; lane 5, 2 μg of protein following the S-300 column step; lane 6, molecular weight standards. Protein was stained with Coomassie brilliant blue R250.

1.0×10^5 (reduced spectrum) and 6.3×10^5 (oxidized spectrum) receiver gain.

Iron and acid-labile sulfur content

Iron and acid-labile sulfur were determined by the methods of Zabinski *et al* [32] and Beinert [4].

Results and discussion

Ferredoxin$_{BPH}$ was purified to homogeneity from the soluble fraction of crude cell extract of *Pseudomonas* sp strain LB400 cells grown with biphenyl as the carbon and energy source (Figure 2). The final purification step yielded 2.4 mg of pure protein (Table 1). The calculated recovery of activity was 30% of the activity present following the first column step. High nonspecific cytochrome *c* reductase activity in crude cell extract prevented accurate measurement of ferredoxin$_{BPH}$-dependent activity prior to the first column step. Based on the molecular weight of 12 000 mg mmol^{-1} derived from the deduced amino acid sequence [10], 0.2 mmol of ferredoxin$_{BPH}$ were recovered from 1.62 g of cell extract protein. This contrasts with the 1.6 mmol of pure ISP$_{BPH}$ obtained from 1.97 g of cell extract protein [15]. Normalized for the starting protein concentration, the molar yield of ferredoxin$_{BPH}$ was 6.7-fold lower than that of ISP$_{BPH}$ based on a molecular weight of 73 600 mg mmol^{-1} per $\alpha\beta$ heterodimer [10]. The relatively low recovery of ferredoxin$_{BPH}$ may have resulted from loss or instability of the protein during purification. Pure ferredoxin$_{BPH}$ was stable when stored at −70°C in BEG buffer, however. Alternatively, *bphF*, the gene encoding ferredoxin$_{BPH}$ [10] may have a lower level of expression than *bphA,E* which encode the two subunits of ISP$_{BPH}$. Biphenyl 2,3-dioxygenase activity was increased by addition of ferredoxin$_{BPH}$ and reductase$_{BPH}$ to crude cell extract [15] suggesting that their concentration may be limiting. However, Tan *et al* [29] found that the concentration of the ferredoxin component of benzene dioxygenase limited dioxygenase activity *in vitro* but not *in vivo* because of high intracellular concentrations of the ferredoxin in whole cells.

Biphenyl 2,3-dioxygenase activity was detected only in the presence of ferredoxin$_{BPH}$, reductase$_{BPH}$ and ISP$_{BPH}$, while only background counts were detected when ferredoxin$_{BPH}$ was eliminated from the assay. Activity showed a hyperbolic response when the ferredoxin concentration was varied. The apparent K_m for ferredoxin$_{BPH}$ was 1.25 μM and the V_{max} was 230 nmol *cis*-biphenyl 2,3-dihydrodiol produced min^{-1} per mg of ISP$_{BPH}$.

Ferredoxin$_{BPH}$ consists of a single polypeptide with an estimated molecular weight of 15 000 and 15 100 as determined by gel filtration column chromatography and SDS-PAGE. These values are 25% higher than the value estimated from the nucleotide sequence [10]. Similar results were reported for ferredoxin$_{TOL}$ [27] and ferredoxin$_{NAP}$ [18], components of toluene dioxygenase and naphthalene dioxygenase. Solutions of the pure protein were reddish-brown in color and the absorbance spectrum of the protein showed maxima (ϵ, mM^{-1} cm^{-1}) at 325 nm (11.6) and 460 nm (4.5) with a broad shoulder around 575 nm (2.3) (Figure 3). The spectrum was partially bleached and a new

Table 1 Steps of the procedure used to purify ferredoxin$_{BPH}$

Step	Protein	Activity[a]		Yield	Purification
	(mg)	(U)	(U mg^{-1})	(%)	(-fold)
Cell extract	1620	—[b]			
Q Sepharose	95	124	1.3	100	1.0
Ammonium sulfate	37	158	4.3	127	3.3
Phenyl Sepharose	4.1	78	19	60	15
S-300	2.4	37	15	30	12

[a]1 U = 1 μmol cytochrome *c* reduced per min.
[b]High endogenous cytochrome *c* reductase activity in cell extract prevented measurement of ferredoxin$_{BPH}$ dependent activity.

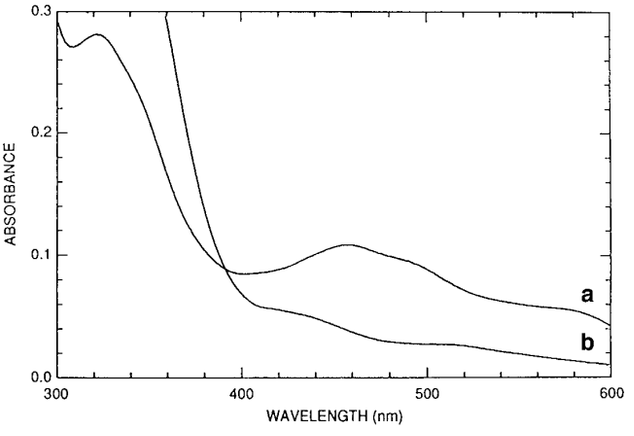

Figure 3 Absorption spectra of ferredoxin$_{BPH}$. (a) oxidized; (b) reduced.

peak at 435 nm was present upon reduction with NADPH in the presence of a catalytic amount of reductase$_{BPH}$.

The EPR spectrum of the oxidized protein showed no signals from 3150–4150 G, but signals were detected with g-values of 2.02, 1.92 and 1.82 after reduction with sodium dithionite (Figure 4).

The absorbance and EPR spectral data are consistent with the presence of a Rieske-type (2Fe-2S) iron-sulfur center [24] that accepts and donates one electron during the transfer of reducing equivalents from reductase$_{BPH}$ to ISP$_{BPH}$. One of the antiferromagnetically-coupled high-spin ferric iron atoms of the iron-sulfur center is reduced to the ferrous state by the transferred electron [22]. The presence of iron and sulfur were confirmed by chemical analyses which gave 2.06 ± 0.02 ($n = 6$) and 1.98 ± 0.06 ($n = 3$) g-atoms of iron and acid-labile sulfur, respectively. The iron-sulfur center of Rieske-type proteins is thought to be coordinated by two cysteine and two histidine ligands [7,11], and the midpoint potential of Rieske proteins is generally more positive than that of plant-type (2Fe-2S) iron-sulfur-containing ferredoxins [22]. Thus, the transfer of electrons from a flavin-containing reductase to a Rieske-type ferredoxin would be more favorable than to a plant-type ferredoxin.

The first 10 amino acids of the amino-terminus were MKFTRV(C)DRR. This sequence is the same as that deduced from the nucleotide sequence of *bphF* of strain LB400 [10] and *bphA3* from *P. pseudoalcaligenes* KF707 [28]. The nucleotide sequences of *bphF* and *bphA3* are 100% identical [9].

Biphenyl 2,3-dioxygenase of *Pseudomonas* sp strain LB400 is a three-component enzyme that incorporates one molecule of oxygen and two reducing equivalents into the aromatic ring to produce *cis*-biphenyl 2,3-dihydrodiol (Figure 5) [14,17] The enzyme consists of an iron-sulfur protein (ISP$_{BPH}$) that has spectral properties similar to those of proteins containing a Rieske-type (2Fe-2S) center [24]. This component is thought to contain the substrate-binding

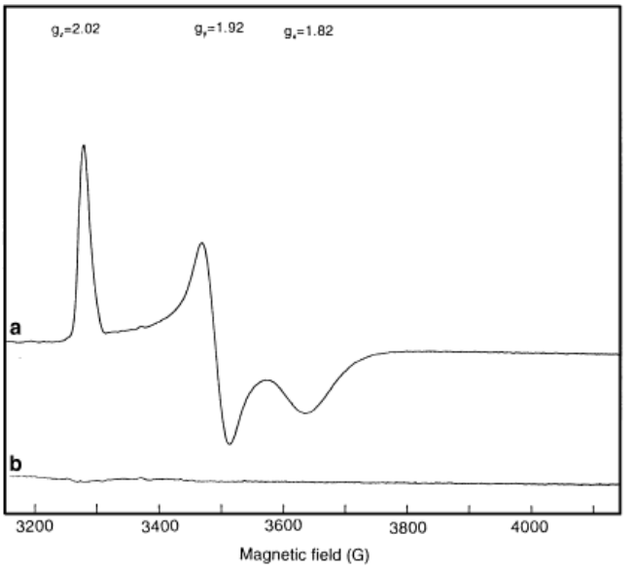

Figure 4 EPR spectra of ferredoxin$_{BPH}$. (a) reduced; (b) oxidized.

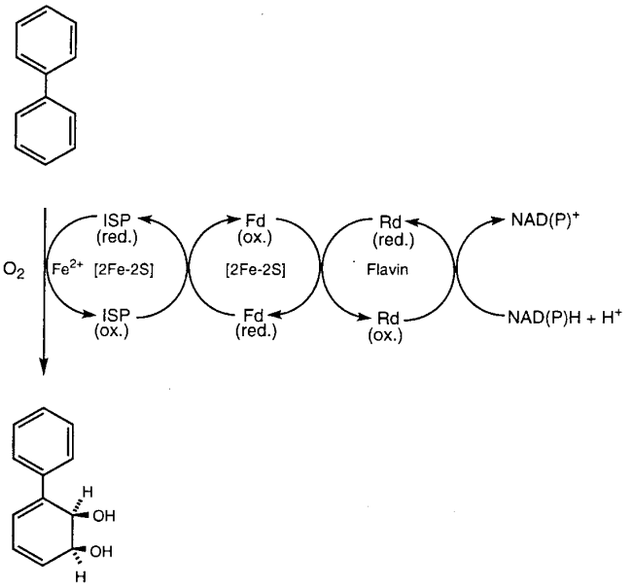

Figure 5 Proposed arrangement of the components of biphenyl 2,3-dioxygenase in *Pseudomonas* sp strain LB400. ISP, iron-sulfur protein; Fd, ferredoxin; Rd, reductase, ox, oxidized; red, reduced.

and catalytic sites of the enzyme, while ferredoxin$_{BPH}$ functions to transfer reducing equivalents from reductase$_{BPH}$ to ISP$_{BPH}$. A three-component biphenyl dioxygenase with similar properties has been partially purified from *Comamonas testosteroni* B-356 [19]. The ferredoxin component was unstable and contained less than one gram-atom of iron and acid-labile sulfide per mole of protein.

Rieske-type ferredoxins of multicomponent dioxygeneases involved in the oxidation of toluene [27], benzene [8], naphthalene [18] and *ortho*-halobenzoate [25] have also been purified. Toluene-4-monooxygenase isolated from *Pseudomonas mendocina* KR1 contains a Rieske-type ferredoxin that may be evolutionarily related to those present in the toluene, benzene and naphthalene dioxygenases [23,31]. It is likely that ferredoxin$_{BPH}$ is a component of a class IIB electron transfer chain, as is the case for the toluene dioxygenase and benzene dioxygenase ferredoxins [2]. These proteins are members of two-component electron transfer chains that transfer reducing equivalents from reductases containing FAD as a coenzyme to their respective terminal oxygenase component. Purification and characterization of reductase$_{BPH}$ from strain LB400 is currently underway to test this hypothesis.

Acknowledgements

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