The Effect of Ferredoxin$_{BED}$ Overexpression on Benzene Dioxygenase Activity in *Pseudomonas putida* ML2

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The benzene dioxygenase from *Pseudomonas putida* ML2 is a multicomponent complex comprising a flavoprotein reductase, a ferredoxin, and a terminal iron-sulfur protein (ISP). The catalytic activity of the isolated complex shows a nonlinear relationship with protein concentration in cell extracts, with the limiting factor for activity in vitro being ferredoxin$_{BED}$. The relative levels of the three components were analyzed by using ^{125}I-labelled antibodies, and the functional molar ratio of ISP$_{BED}$, ferredoxin$_{BED}$, and reductase$_{BED}$ was shown to be 1:0.9:0.8, respectively. The concentration of ferredoxin$_{BED}$ was confirmed by quantitative electron paramagnetic resonance spectroscopy of the 2Fe-2S centers in ferredoxin$_{BED}$ and ISP$_{BED}$ of whole cells. These results demonstrate that the ferredoxin$_{BED}$ component is a limiting factor in dioxygenase activity in vitro. To determine if it is a limiting factor in vivo, a plasmid (pJRM606) overproducing ferredoxin$_{BED}$ was introduced into *P. putida* ML2. The benzene dioxygenase activity of this strain, measured in cell extracts, was fivefold greater than in the wild type, and the activity was linear with protein concentration in cell extracts above 2 mg/ml. Western blotting (immunoblotting) and electron paramagnetic resonance spectroscopic analysis confirmed an elevated level of ferredoxin$_{BED}$ protein and active redox centers in the recombinant strain. However, in these cells, the increased level of ferredoxin$_{BED}$ had no effect on the overall rate of benzene oxidation by whole cells. Thus, we conclude that ferredoxin$_{BED}$ is not limiting at the high intracellular concentration (0.48 mM) found in cells.

Many microorganisms initiate the oxidation of aromatic compounds through the action of dioxygenases (12). Dioxygenases that oxidize such compounds as biphenyl, anthracene, phenanthrene, naphthalene, toluene, and benzene have been reported in the literature. As a class, these enzymes are important because of their ability to introduce molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols. One of the best-studied dioxygenases is benzene dioxygenase (EC 1.14.12.3) from *Pseudomonas putida* ML2 (NCIB 12190), which incorporates two electrons and both atoms of dioxygen into benzene to form *cis*-benzene dihydrodiol (1). The enzyme consists of a flavoprotein reductase (reductase$_{BED}$) and a ferredoxin (ferredoxin$_{BED}$) which transfer electrons from NADH to an iron-sulfur protein that functions as the terminal dioxygenase (ISP$_{BED}$) (5, 10). The latter consists of two dissimilar subunits arranged in an $\alpha_2\beta_2$ configuration (26). Both the ferredoxin$_{BED}$ and ISP$_{BED}$ components contain 2Fe-2S clusters, which have been examined by Mössbauer and electron paramagnetic resonance (EPR) spectroscopy (9, 11) and which from sequence information (15, 18, 24) are probably of the Rieske type, with histidine as well as cysteine ligands (17). Although we have some understanding of the function of each individual component, little is known about the interactions involved among the three components in the overall reaction. As with many other multicomponent oxygenases, attempts to determine a meaningful specific activity for the enzyme have proved difficult because of a marked nonlinearity

of activity in vitro with respect to protein concentration (16). Purification of the enzyme results in separation of any noncovalent complex into its individual components. Kinetic evidence for such systems suggests that the ferredoxin$_{BED}$ component functions as a mobile electron shuttle in which ferredoxin$_{BED}$ first binds to the reductase$_{BED}$ and accepts an electron from NADH-reduced reductase$_{BED}$. Reduced ferredoxin$_{BED}$, which dissociates from the complex, then binds to oxidized ISP$_{BED}$ to transfer its electron. Finally, oxidized ferredoxin$_{BED}$ dissociates from reduced ISP$_{BED}$, and the cycle continues. Such mechanisms have been demonstrated for other multicomponent systems such as putidaredoxin and P-450$_{cam}$ from *P. putida* (25) and mitochondrial cytochrome P-450$_{scc}$ (13).

In this study, we examined the role of ferredoxin$_{BED}$ as a limiting factor in the activity of benzene dioxygenase. The effect of increasing the concentration of ferredoxin$_{BED}$ was determined both in vitro by supplementation with purified protein and in vivo by expression of additional copies of the gene encoding the ferredoxin (*bedB*). Evidence is presented that although dioxygenase activity can be stimulated in vitro by enhanced levels of ferredoxin$_{BED}$, it does not limit activity in vivo. We intend to show that absolute concentration in addition to relative concentration is important when one is dealing with complex enzymes such as dioxygenases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* strains were grown with benzene as the sole carbon source at 30°C (26); *Escherichia coli* strains were grown on LB medium in the presence of the appropriate antibiotics at 37°C. Strains containing plasmids pJRM606, pJRM754, and pRK2013 were

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TABLE 1. Characteristics of the bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
P. putida ML2 (NCIB 12190)	Bed^{+a} prototroph bearing plasmid pHMT112	23
E. coli DH5α	F$^-$ φ80d*lac*ZΔM15 Δ(*lacZYA argF*)U169 *deoR recA1 endA1 hsdR17* (r$_K^-$ m$_K^+$) *SupE44*, λ$^-$ *thi-1 gyrA96 relA1*	Bethesda Research Laboratories
Plasmids		
pKK223-3	Ap^r, expression vector	Pharmacia
pJRD215	Km^r, broad-host-range cosmid vector	6
pRK2013	Km^r	8
pJRM754	pJRD215 with *tac* promoter	This work[b]
pHMT181	pUC18 with 4.5-kb *Pst*I insert encoding *bedC1C2B* and truncated *bedA*	23
pJRM506	*bedB* gene cloned into pKK223-3	This work[b]
pJRM606	*bedB* gene cloned into pJRM754	This work[b]

[a] Bed$^+$ indicates the ability to grow on benzene as the sole carbon and energy source.
[b] For details of construction, see Materials and Methods.

grown in the presence of kanamycin (50 μg/ml). Strains containing plasmids pJRM506 and pHMT181 were grown in the presence of ampicillin (100 μg/ml).

Subcloning and transformation of the bedB gene. The ferredoxin$_{BED}$ *bedB* gene was isolated from plasmid pHMT181, containing genes *bedC1C2BA* (23, 24). The *bedB* gene was excised from pHMT181 by restriction with the enzymes *Dde*I and *Cfo*I and gel purified by using glass milk (Geneclean; BIO 101 Inc., La Jolla, Calif.), and the resultant 0.5-kb fragment was blunt ended with T4 DNA polymerase. *Eco*RI linkers (Pharmacia) were added, restricted with *Eco*RI, and ligated into the multiple cloning site of the vector pKK223-3 (Pharmacia) previously restricted with *Eco*RI and treated with HK phosphatase (Epicentre Technologies, Madison, Wis.). To express the *bedB* gene in *P. putida*, it was subcloned into the broad-host-range vector pJRD 215 (6). The *tac*:*bedB* gene construct was isolated from plasmid pJRM506 by *Bam*HI digestion, and the gel-purified 668-bp fragment was ligated into the multiple cloning site of plasmid pJRD215, previously restricted with *Bam*HI and treated with phosphatase. The recombinant plasmid in *E. coli* containing the *bedB* gene under the control of the *tac* promoter (pJRM606) was introduced into *P. putida* ML2 by triparental mating (7). Selection on minimal medium plates with benzene as the carbon source and kanamycin as the selecting antibiotic gave rise to the recombinant strain *P. putida* ML2(pJRM606). The control plasmid pJRM754 was constructed by subcloning the *tac* promoter alone into pJRD215 in a similar manner.

Preparation of cell extracts. Cells used for the preparation of cell extracts were disrupted by sonication as described previously (26) except that cells were resuspended in 1 ml of buffer per g (wet weight) of cells.

Protein determination. Protein concentration was determined by the modified Lowry method of Hess et al. (14), with bovine serum albumin (BSA) as the standard.

Assay of benzene dioxygenase activity. Benzene dioxygenase activity in cell extracts was measured polarographically, using a Clark-type oxygen electrode at 30°C in a total volume of 1 ml by the method of Geary et al. (10). Whole-cell benzene dioxygenase activity was also measured by using an oxygen electrode; cells with an optical density at 660 nm of 0.1 to 0.6 were washed in 50 mM phosphate buffer (pH 7.2). One milliliter was allowed to equilibrate to 30°C for 5 min, and the assay was initiated by the addition of 50 μl of benzene-saturated water (1 μl of benzene in 1 ml of water). Activity was expressed as micromoles of O_2 consumed per minute per milligram (dry weight) of protein.

Purification of ferredoxin$_{BED}$, ISP$_{BED}$, and reductase$_{BED}$. The components of benzene dioxygenase, reductase$_{BED}$, ferredoxin$_{BED}$, and ISP$_{BED}$, were purified as described by Geary et al. (10).

Western blotting (immunoblotting) and quantification of Western blots by using ^{125}I-labelled antibodies. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with antibodies raised to the purified benzene dioxygenase subunits as described by Zamanian and Mason (26) and Geary et al. (10). Quantitation of the Western blots and iodination of antibodies were performed as described by Geary et al. (10).

EPR spectroscopy. EPR measurements were recorded on a Bruker ESP300 spectrometer fitted with an Oxford Instruments ESR900 liquid helium flow cryostat. All samples were reduced by the addition of 4 mM sodium dithionite after a freeze-thaw cycle, and interfering manganese signals in the whole-cell sample were broadened by the addition of 5 mM EDTA. Spectra were baseline corrected by subtraction of a cavity spectrum run under identical conditions.
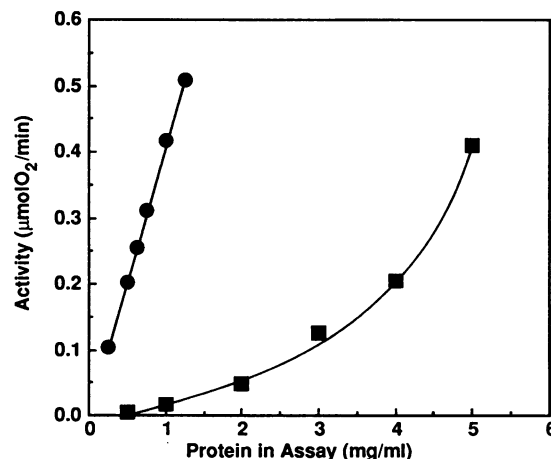

FIG. 1. Benzene dioxygenase activity in cell extracts and whole cells of *P. putida* ML2. The activity of benzene dioxygenase in whole cells (●) and cell extracts (■) was estimated with increasing amounts of biomass and extract protein as described in Materials and Methods. Values are means of at least four replicates with a standard error of <5%.
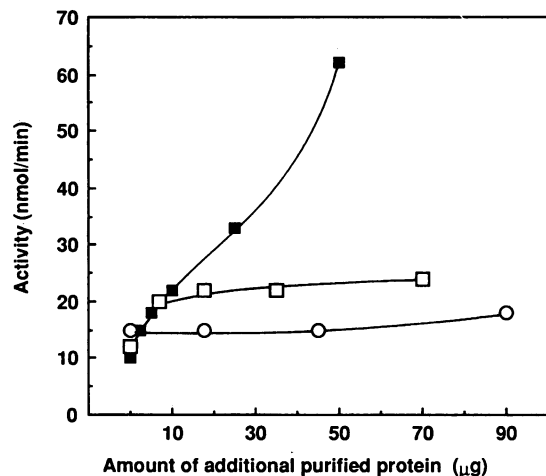
FIG. 2. Effect on benzene dioxygenase activity of adding pure subunits of ferredoxin$_{BED}$, ISP$_{BED}$, and reductase$_{BED}$. The purified proteins ferredoxin$_{BED}$ (■), reductase$_{BED}$ (□), and ISP$_{BED}$ (○) at given concentrations were added to 1 mg of cell extract, incubated for 5 min at 30°C, and assayed for benzene dioxygenase activity.

Identification of the signals compared with pure ISP$_{BED}$ and ferredoxin$_{BED}$ in whole cells was performed as described by Cooper et al. (3). Cells were washed and concentrated by centrifugation and resuspended in EPR tubes at a final concentration of 2×10^{11} cells per ml. The absolute number of spins in the samples was determined by estimating how much of the pure protein spectrum had to be subtracted from the whole-cell spectrum to reduce the respective signal to zero. The number of spins in the pure ISP$_{BED}$ and ferredoxin$_{BED}$ spectra were estimated by measurement under nonsaturating conditions and comparing the double-integrated intensities against a CuII-EDTA standard.

RESULTS

Specific activity of benzene dioxygenase. The activity of the enzyme benzene dioxygenase in whole cells and cell extracts was measured polarographically from the rate of benzene-dependent oxygen uptake. In whole cells, the activity was found to increase linearly with respect to biomass. However, with cell extracts, the activity showed a marked nonlinearity, such that a 5-fold increase in protein concentration (1 to 5 mg/ml) resulted in a 27-fold increase in activity (Fig. 1). Such kinetics are characteristic of multicomponent enzyme systems in which the dilution effect upon cell disruption causes a pronounced decrease in activity.

We performed assays in which the concentration of cell extract was fixed at 1 mg/ml and increasing amounts of the three purified components (reductase$_{BED}$, ferredoxin$_{BED}$, and ISP$_{BED}$) were added separately to the assays (Fig. 2). Addition of ISP$_{BED}$ had no significant effect on enzyme activity, but stimulation was observed with both reductase$_{BED}$ and ferredoxin$_{BED}$. Addition of reductase$_{BED}$ caused a twofold increase in activity which saturated above 20 μg of additional purified component per ml. This stimulation was even more pronounced with the addition of ferredoxin$_{BED}$, with activity continuing to increase up to the maximum amount of ferredoxin$_{BED}$ added. No stimulation in activity was observed upon addition of BSA, heat-inactivated cell extract, or cell extract prepared from a strain of *P. putida* lacking benzene dioxygenase (data not shown).

Relative concentrations of subunits. To determine the relative amounts of the three components in cells of *P. putida* ML2, cell extracts of benzene-grown cells were quantitatively analyzed by Western blotting, using ^{125}I-labelled antibodies raised to the purified components of benzene dioxygenase. Preliminary experiments were performed to estimate the recovery of the three components in cell extracts. In the case of ferredoxin$_{BED}$ and ISP$_{BED}$, greater than 95% of material was recovered in the soluble fraction, but for reductase$_{BED}$, 20% was found to be membrane associated (data not shown). The relative amounts of the three components present in the soluble cell extract are shown in Table 2. It is clear from these data that the molar ratio of reductase$_{BED}$ to ferredoxin$_{BED}$ to terminal oxygenase (ISP$_{BED}$) is approximately 0.64:0.92:1.00. As stated above, the low figure for reductase$_{BED}$ is probably due to only 80% of this component being recovered in the soluble fraction, which would give a corrected ratio of 0.8:0.9:1.

Relative amounts of iron-sulfur clusters. Western blotting gives an estimate of the amounts of the three components but gives no information about their relative activities in whole cells. As an approach to answering this question, EPR spectroscopy was performed on whole cells to estimate quantitatively the iron-sulfur clusters in ferredoxin$_{BED}$ and ISP$_{BED}$. The ratio of iron-sulfur clusters in the cells was estimated by subtracting the pure protein spectrum until there was a flat baseline. This is a relatively stringent test, since any changes in the linewidth of the spectrum will cause the appearance of second-derivative features in the difference spectrum. The fact that an almost flat baseline was obtained after subtraction indicates that the EPR spectra of the Rieske-type 2Fe-2S clusters in ferredoxin$_{BED}$ and ISP$_{BED}$ are similar to those in the purified proteins. The relative concentrations of active clusters present in the two iron-sulfur proteins are shown in Table 2. From these data, it is possible to determine the intracellular concentrations of the two components to be 1.3

TABLE 2. Relative concentrations of ferredoxin$_{BED}$, reductase$_{BED}$, and ISP$_{BED}$ in cell extract and whole cells of *P. putida* ML2[a]

Subunit	Immunological assay in cell extract			Fe-S centers in whole cells		
	Concn (μg/mg of protein)	Extract concn (M)	Molar ratio	μM spin	Intracellular concn (M)	Molar ratio
ISP$_{BED}$	131.6 ± 19	7.8×10^{-5}	1.00	157 ± 8	1.3×10^{-3}	1.00
Ferredoxin$_{BED}$	14.2 ± 3	3.6×10^{-5}	0.92	57 ± 3	4.8×10^{-4}	0.75
Reductase$_{BED}$	72.5 ± 12	5.0×10^{-5}	0.64			

[a] The amount of each subunit in the cell extract (30 mg of protein per ml) was quantified by Western blotting using ^{125}I-labelled antibodies to each of the subunits (8). Values are means ± standard errors of the means with three replicate determinations. Estimation of active iron-sulfur clusters in whole cells for ferredoxin$_{BED}$ and ISP$_{BED}$ was carried out by using EPR spectroscopy and quantified by comparing the double-integrated intensities against a CuII-EDTA standard. The intracellular concentration of enzyme is calculated on the basis of an intracellular volume of 6×10^{-16} liters per cell (20). The molar ratio estimation is based on a ferredoxin$_{BED}$ monomer (M_r of 11,939) and dimers of the ISP$_{BED}$ α subunit (dimer M_r of 102,210) and reductase$_{BED}$ (dimer M_r of 87,168).
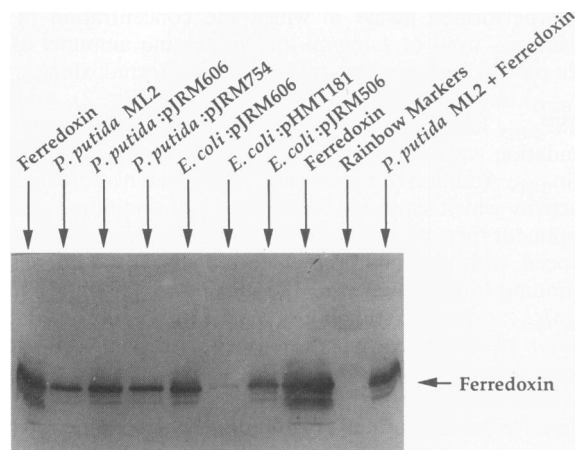
FIG. 3. Western blotting to determine expression of the cloned *bedB* gene. Cell extract (10 μg) prepared from cells used in the cloning procedure was resolved by SDS-PAGE, electroblotted onto nitrocellulose, and probed with antibodies raised against ferredoxin$_{BED}$. Amounts of pure ferredoxin$_{BED}$ protein loaded in tracks labeled "Ferredoxin" were 0.4 and 1 μg, respectively. The method was that described by Tan and Mason (23).

and 0.48 mM for ISP$_{BED}$ and ferredoxin$_{BED}$, respectively. These data indicate that there is significantly less active to ferredoxin$_{BED}$ than ISP$_{BED}$ in the cells; however, since the ISP$_{BED}$ α subunit exists as a dimer, the ratio of ISP$_{BED}$α$_2$ to ferredoxin$_{BED}$ is estimated to be 1:0.75.

Effect of increasing the concentration of ferredoxin$_{BED}$ in vivo. The gene encoding benzene dioxygenase ferredoxin$_{BED}$ (*bedB*) was subcloned first into the vector pKK223-3 to place the gene under the control of the *tac* promoter (pJRM506). After confirming the correct orientation of the *bedB* gene by restriction with endonucleases, we subcloned the gene and the attached *tac* promoter into the broad-host-range vector pJRD215 to form a plasmid designated pJRM606. This plasmid was then transferred into *P. putida* ML2 by triparental mating. Cell extracts were prepared from both *E. coli* and *P. putida* strains containing these constructs, and the expression of ferredoxin$_{BED}$ was analyzed by Western blotting. It is evident from Fig. 3 that expression in *E. coli* was significantly enhanced when the *bedB* gene was placed under the control of the *tac* promoter, rather than its natural *Pseudomonas* promoter, in both pKK223-3 (pJRM506) and pJRD215 (pJRM606). When the latter plasmid was transferred into *P. putida* ML2, there was a significant enhancement in the amount of immunodetectable ferredoxin$_{BED}$ (a two- to three-fold enhancement, as judged by comparison of known amounts of purified ferredoxin$_{BED}$ with cell extracts). EPR spectroscopy of the cells showed that the addition of the cloned ferredoxin$_{BED}$ gene (pJRM606) resulted in an increase in the peak associated with the 2Fe-2S clusters of ferredoxin$_{BED}$ relative to the ISP$_{BED}$ (Fig. 4, spectrum c). Cloning of the promoter sequence alone (pJRM754) did not increase the ratio relative to the wild-type cells (Fig. 4, spectra a and b). The additional feature at *g* = 1.92 in the difference spectrum (Fig. 4, spectrum c minus spectrum b) is from an endogenous iron-sulfur protein in the host cells, probably succinate dehydrogenase (4), which is differentially expressed in the recombinant strain. Quantification of these data indicates expression of ferredoxin$_{BED}$ in the strain containing plasmid pJRM606 is enhanced twofold (Table 3), resulting in a shift of the molar ratio of ISP$_{BED}$α$_2$ to ferredoxin$_{BED}$ from 1:0.75 to 1:1.75.
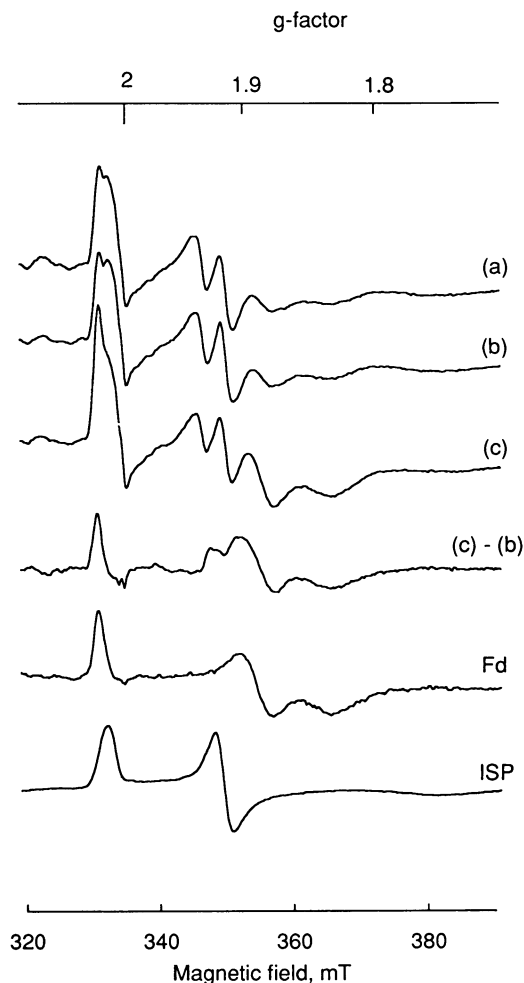

FIG. 4. EPR spectra of *P. putida* ML2 cells in the presence and absence of cloned ferredoxin$_{BED}$. a, *P. putida* ML2 cells (10.8 mg of protein per ml); b, *P. putida* ML2(pJRM754) (8.8 mg of protein per ml); c, *P. putida* ML2(pJRM606) (9.4 mg of protein per ml). Cells were grown in minimal medium liquid cultures with benzene as the sole carbon source, harvested at 10,000 × *g* (10 min), resuspended in 200 ml of 50 mM phosphate buffer (pH 7.2), and prepared for EPR as described in Materials and Methods. EPR conditions were as follows: temperature, 30 K; microwave power, 2 mW; scan rate 1.2 mT/s; receiver gain, 1.6 × 10^4; microwave frequency, 9.35 GHz; modulation amplitude, 0.5 mT. The calculated difference spectrum c − b is corrected for differences in protein concentration and amplified 1.5 times relative to the original spectra. Spectra of dithionite-reduced ferredoxin and ISP are included for comparison.

To determine the effect of this increased expression of ferredoxin$_{BED}$ on dioxygenase activity, cell extracts were prepared and the enzyme was assayed polarographically in the presence of increasing amounts of extract. Figure 5 clearly shows that activity is significantly enhanced in *P. putida* ML2(pJRM606) compared with the wild-type strain and that above 2 mg/ml of extract in the assay, the enzyme achieves a maximum specific activity of 108 nmol/min/mg. No such effect, however, was observed in whole cells. The substrate stimulated rate of oxygen uptake with washed cells of *P. putida* ML2 was determined to be 312 ± 12 nmol/min/mg (three replicate determinations), and this showed no significant stimulation in the strain expressing the higher level of ferredoxin$_{BED}$ [*P.*

TABLE 3. Amounts of iron-sulfur centers in *P. putida* ML2 cells in the presence and absence of cloned ferredoxin

P. putida strain	Mean amt of dioxygenase component (μM spins) ± SEM (n = 3)		Ratio, ISP$_{BED}$α$_2$/ ferredoxin$_{BED}$
	ISP$_{BED}$	Ferredoxin$_{BED}$	
ML2	157 ± 8	57 ± 3	1:0.75
ML2(pJRM754)	157 ± 7	57 ± 2	1:0.75
ML2(pJRM606)	157 ± 8	143 ± 7	1:1.75

putida ML2(pJRM606); 328 ± 12 nmol/min/mg]. The value for *P. putida* ML2(pJRM754) was 302 ± 12 nmol/min/mg.

DISCUSSION

In this work, we have demonstrated the overproduction of a component of benzene dioxygenase by the use of a broad-host-range expression vector in *P. putida* ML2. The relative amounts of the protein components have been determined by immunological assay. It has also been demonstrated that the iron-sulfur clusters of the dioxygenase system may be detected quantitatively in whole cells by EPR spectroscopy.

The activity of benzene dioxygenase in cell extracts of *P. putida* ML2 was found to be nonlinear with respect to protein concentration and was stimulated markedly by addition of purified ferredoxin. We attempted, therefore, to determine the amount of the benzene dioxygenase components in whole cells of *P. putida*. The protein concentrations of the three components were estimated using ^{125}I-labelled antibodies, and EPR was used to detect the reduced iron-sulfur clusters in ISP$_{BED}$ and ferredoxin$_{BED}$. Protein estimation indicated a molar excess of ISP$_{BED}$ such that the ISP$_{BED}$ α subunit/ferredoxin$_{BED}$/ reductase$_{BED}$ ratio was 1:0.45:0.8. Thus, the amount of ferredoxin$_{BED}$ was approximately half that of the other components despite all of the subunits being encoded on a single operon and presumably giving rise to a single mRNA transcript. This may be explained by differences in the efficiency of translation initiation. To this end, it is significant that sequence analysis shows that the putative Shine-Dalgarno sequence (AGTGA) preceding the *bedC1* and *bedA* genes encoding the ISP$_{BED}$α and reductase$_{BED}$ subunits, respectively, differs from that upstream of the *bedB* ferredoxin gene (AGGAG) (24). Although the molar ratio of ferredoxin$_{BED}$ to ISP$_{BED}$α and reductase$_{BED}$ monomer differs substantially, this may not reflect the functional ratio. Since the latter two components are both dimers in the enzyme complex, the functional ratio would be 1:0.9:0.8. This result is interesting in the light of studies on similar systems such as the 4-sulfobenzoate 3,4-dioxygenase (16) and dibenzofuran 4,4a-dioxygenase (2) systems, which show low molar concentrations of reductases. However, in all of these systems, no evidence is presented for the ratio in whole cells, with the consequence that the differences may be due to poor or differential extraction. In addition, in 4-sulfobenzoate 3,4-dioxygenase, although the reductase B component is present at 30% the molar concentration of the oxygenase component (16), this corresponds to only one of two reductases (B and C) in this system, and it is possible that reductase C is the authentic electron donor. Significantly, maximum activity of this system could be achieved only with a molar excess of the reductase.

The immunological results concerning the amount of the three benzene dioxygenase components were supported by estimation of the amount of 2Fe-2S cluster in the ISP$_{BED}$ and
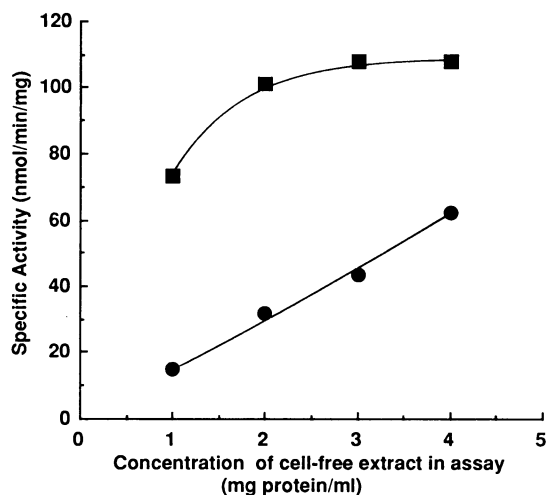

FIG. 5. Benzene dioxygenase activity in cell extracts of strain *P. putida* ML2 (●) and *P. putida* ML2 containing plasmid pJRM606 (■). Values are means of three replicates with a standard error of <5%.

ferredoxin$_{BED}$ of whole cells. In this case, the amount of ferredoxin cluster was slightly lower than that estimated for the protein. Both the immunological assays and EPR spectroscopic measurements were standardized by reference to purified protein components. A possible reason for this apparent discrepancy is that a fraction of the ferredoxin$_{BED}$ is partly denatured or that the assembly of the iron-sulfur clusters in the protein is incomplete. This latter possibility would imply that the insertion of iron-sulfur clusters into ferredoxin$_{BED}$ and ISP$_{BED}$ may be a limiting factor of the dioxygenase in vivo. The mechanism of insertion of iron-sulfur clusters into proteins is not known in general, though there is some evidence for the involvement of a specific mechanism in chloroplasts (21, 22).

From the results of the EPR spectroscopic measurements, the intracellular concentrations of ferredoxin$_{BED}$ and ISP$_{BED}$ were estimated to be 0.48 and 1.3 mM, respectively. These values are approximately 2 orders of magnitude higher than the maximum concentration present in enzyme assays in vitro. To examine the effect of increasing the amount of ferredoxin in vivo on enzyme activity, plasmid pJRM606, encoding the *bedB* gene under the control of the *tac* promoter, was transferred into *P. putida* ML2. Western blotting demonstrated clearly that the ferredoxin was expressed efficiently in both *E. coli* and *P. putida*. In *P. putida*, this resulted in an approximate doubling of the amount of ferredoxin protein in the cell and a 2.3-fold increase in the amount of iron-sulfur EPR signal. This elevation in the intracellular concentration of ferredoxin resulted in a substantial increase in the in vitro activity of benzene dioxygenase in cell extracts, enabling an estimation of the specific activity of the enzyme. Thus, it can be seen from Fig. 5 that an increase in ferredoxin$_{BED}$ concentration in the assay from 14 to 33 μg/ml (present in 1 mg of *P. putida* ML2 and *P. putida* ML2(pJRM606) cell extracts, respectively) results in an increase in specific activity from 13 to 72 nmol/min/mg. This relates to an increase from 10 to 50 nmol/min/mg when pure ferredoxin$_{BED}$ protein was added to an assay (Fig. 2). However, there was no increase in the whole-cell oxidation of benzene by *P. putida* ML2(pJRM606), confirming that in intact cells, ferredoxin does not limit enzyme activity. Similar results were obtained by Murdock et al. with the naphthalene dioxygenase (19). An increase in the intracellular concentration of ferredoxin had no initial effect on whole-cell dioxygenase

activity although it did enable sustained indigo biosynthesis, probably by compensating for the fraction of ferredoxin inactivated by indigo. It is interesting that in the present work, the maximum activity that was obtained in vitro in the presence of additional ferredoxin was still only 30% of that achieved in vivo. This is due to dioxygenase activity in vitro no longer being limited by ferredoxin but being limited by another component of the system, probably the reductase, as indicated by the twofold enhancement in activity in the presence of additional purified reductase$_{BED}$ (Table 2). In conclusion, when one is dealing with complex enzymes such as dioxygenase, both absolute and relative concentrations of components must be considered when one is extrapolating from in vitro to in vivo studies.

ACKNOWLEDGMENTS

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