Biphenyl (BPH) dioxygenase oxidizes BPH to 2,3-dihydro-2,3-dihydroxybiphenyl in Comamonas testosteroni B-356. The enzyme comprises a two-subunit iron-sulfur protein (ISP$_{BPH}$), a ferredoxin FER$_{BPH}$, and a ferredoxin reductase RED$_{BPH}$. RED$_{BPH}$ and FER$_{BPH}$ transfer electrons from NADH to an Fe-S active center of ISP$_{BPH}$ which activates molecular oxygen for insertion into the substrate. In this work B-356 ISP$_{BPH}$ complex and its $\alpha$ and $\beta$ subunits were purified from recombinant Escherichia coli strains using the His-bind QIAGEN system. His-tagged B-356 ISP$_{BPH}$ construction carrying a single His tail on the N-terminal portion of the $\alpha$ subunit was active. Its major features were compared to the untagged enzyme. In both cases, the native form is an $\alpha$-$\beta$ heteromer, with each $\alpha$ subunit containing a [2Fe-2S] Rieske center (\$\epsilon_{\text{abs}}$ = 8,300 m$^{-1}$ cm$^{-1}$) and a mononuclear Fe$^{2+}$. Although purified His-tagged $\alpha$ subunit showed the characteristic absorption spectra of Rieske-type protein, reassociation of this enzyme component and His-tagged $\beta$ subunit to reconstitute active ISP$_{BPH}$ was weak. However, when His-tagged $\alpha$ and $\beta$ subunits were reassembled in vitro in crude cell extracts from E. coli recombinants, active ISP$_{BPH}$ could be purified on Ni-nitrilotriacetic acid resin.

Biphenyl dioxygenase (BPH dox)$^\dagger$ catalyzes the first step of the bacterial BPH degradation pathway. The enzyme introduces molecular oxygen into the ortho-meta positions on one of the aryl rings to generate 2,3-dihydro-2,3-dihydroxybiphenyl. In a previous study (1), we have reported the purification and characterization of Comamonas testosteroni strain B-356-BPH dox system. The enzyme comprises three components which are: the terminal oxygenase, an iron-sulfur protein (ISP$_{BPH}$) made up of an $\alpha$-subunit (M$_{r}$ = 51,000) and a $\beta$-subunit (M$_{r}$ = 22,000), encoded by bpHa and bpHe, respectively; a ferredoxin (FER$_{BPH}$, M$_{r}$ = 12,000) encoded by bpF; and a ferredoxin reductase (RED$_{BPH}$, M$_{r}$ = 43,000) encoded by bpG. FER$_{BPH}$ and RED$_{BPH}$ were found to be involved in electron transfer from NADH to ISP$_{BPH}$ (1). The Rieske center of the oxygenase component is then believed to receive the electron and pass it to a mononuclear Fe$^{2+}$ which activates molecular oxygen for insertion into the substrate (2, 3).

The ISP$_{BPH}$ component has been purified from BPH-induced bacteria of strain B-356 (1) and from Pseudomonas sp. LB400 (4). Since active purified FER$_{BPH}$ and RED$_{BPH}$ were difficult to obtain from cell extracts of parental strains (1, 4), these enzyme components were purified from Escherichia coli recombinant clones using the His-bind QIAGEN system (1). Both His-tagged FER$_{BPH}$ and His-tagged RED$_{BPH}$ from strain B-356 were able to transfer electrons from NADH to B-356-ISP$_{BPH}$. However, purification of the individual active ISP$_{BPH}$ $\alpha$ and $\beta$ subunits has not yet been reported.

Understanding the various factors that contribute to the strain selectivity pattern toward substrate should help the modeling of new mutants with increased ability to degrade BPH analogs such as polychlorinated biphenyls. The BPH dox reactivity pattern is a major determinant affecting the performance of bacterial polychlorinated biphenyl degraders. The BPH dox-congener selectivity pattern is partly affected by the position of attack on the aromatic ring. For example, the capacity of Pseudomonas sp. LB400 to preferentially degrade the ortho-substituted polychlorinated biphenyl congeners was attributed to its ability to oxygenate BPH at ring positions 3 and 4 in addition to 2 and 3. Haddock et al. (5) have recently shown that partially purified LB400-BPH dox was able to attack 2,2',5,5'-tetrachlorobiphenyl (for which there is no available ortho-meta sites for oxygenation) in a 3,4-position suggesting that the same enzyme catalyzes both type of attacks. Using site-directed mutagenesis, Erickson and Mendelo (6) have provided evidence that minor structural differences of the ISP$_{BPH}$ $\alpha$ subunit component are responsible for major changes in the reactivity pattern of strain LB400-BPH dox.

Although the substrate selectivity of strain B-356 distinguishes it from strain LB400, we have recently shown that strain B-356 dox is also able to oxygenate BPH at both 2,3- and 3,4-positions (1).

Other studies have suggested that additional determinants are affecting the reactivity pattern of various strains toward BPH analogs. For example, Hirose et al. (7) have found that the BPH dox components of Pseudomonas pseudoalcaligenes strain FD707 are to some extent, interchangeable with those of the Pseudomonas putida F1-toluene dioxygenase system encoded by the tod operon. Several of the recombinant enzymes tested were found to manifest new degradative abilities that neither of the original enzymes possessed. Their results suggest that the oxygenase $\beta$ subunit is specific to the system to which it belongs. However, to date, the role of the ISP$_{BPH}$ $\beta$ subunit in enzyme activity remains unknown.

At this time, it is clear that further investigation is required at the molecular level to identify the role of the BPH dox components regarding catalytic activity and substrate selectivity. However, because most of these enzyme components are unstable and cannot sustain the large number of manipulations needed for their purification, new approaches are needed.

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$^\dagger$The abbreviations used are: BPH dox, biphenyl dioxygenase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Ni-NTA, nickel-nitrilotriacetic acid.
to obtain purified active enzyme. In this work we have identified some of the major features of purified strain B-356 His-tagged ISPBPH and of individual His-tagged ISPBPH subunits. We have also found that the His-tagged ISPBPH subunits can be reassembled in vitro to produce an active oxygenase component.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Culture Media, and General Protocols**—The bacterial strains used in this study were E. coli M15[repP4] and SG13009[pREP4] (both from QiAGEN Inc., Chatsworth, CA) and C. testosteroni B-356 (8). The media used were Luria-Bertani (LB) broth (9), H-plates, and minimal medium No. 30 (MM30) (10) containing 0.05% (w/v) BPHE.

Plasmid DNA from E. coli, restriction endonuclease digestions, ligations, agarose gel electrophoresis, and transformation of E. coli cells were done according to protocols described by Sambrook et al. (9). Polymerase chain reactions (PCR) were performed using Pwo DNA polymerase following the method given by Boehringer Mannheim.

**Purification Protocols**—The procedures to obtain purified preparations of strain B-356-BPH dox ISP components from BPH-induced B-356 cells and to obtain purified His-tagged FERBPH and REDBPH from recombinant E. coli cells have already been described (1). The His-tagged B-356-ISP complex or individual His-tagged ISP subunits were expressed in appropriate recombinant strains of E. coli M15[repP4] and SG13009[pREP4] using a protocol similar to the one described for His-tagged REDBPH (1). The oligonucleotides used for PCR were chosen on the basis of the known DNA sequences of the genes to be amplified (II) and they were as follows: oligonucleotide I (BamHI), 5'-CGGGATCCGATGAGTTCGACTATGA-3'; oligonucleotide II (KpnI), 5'-GGGGTACCCCTCAAAAGAACACGCT-3'; oligonucleotide III (BamHI), 5'-GGGGATCCGGATGAGTTCGACTATGA-3'; oligonucleotide IV (KpnI), 5'-GGGGTACCCCTCAAAAGAACACGCT-3'. To obtain purified His-tagged B-356-ISP sub, the coding region of B-356-bphe was PCR-amplified from a cloned DNA fragment using the oligonucleotides I and IV. To obtain the individual His-tagged α and β subunits of B-356-ISP sub, the coding region of bphA or bphE were amplified using the oligonucleotides I and II, and III and IV, respectively.

The PCR products were digested with BamHI and KpnI. A 1.9-kilobase DNA fragment (for bphe), a 1.3-kilobase DNA fragment (for bphA), and a 0.6-kilobase DNA fragment (for bphE) containing the entire coding sequences were isolated and cloned into the compatible sites of pQE31. Constructions were such that the His tail added 13 amino acids (MRGSHHHHHHTDP) to the protein at its N-terminal portion. When both the α and β subunits were produced together in the same clone, the His tail was attached to the α subunit only.

Protein Characterization—SDS-PAGE gels were developed according to Laemmli (12). Proteins were stained with Coomassie Brilliant Blue (9). M, values of native ISP sub, His-tagged ISP sub, and individual α and β subunits were determined by HPLC using a Perkin-Elmer Series 1 chromatograph and a Waters Protein Pak 300 SW column (7.8 x 300 mm). The column was eluted at 1 ml/min with 100 mM potassium phosphate buffer, pH 7.0. The UV detector was a Perkin-Elmer LC6ST and it was set at 280 nm. The column was calibrated with catalase (M), 232,000, bovine serum albumin (M), 67,000, albumin (M), 46,000, and RNase A (M), 13,700.

The iron content of protein was evaluated by the ferrazine colorimetric method modified by Batie et al. (13). Acid-labile sulfide was determined according to the method of Fogo and Popowsky (14) as modified by King and Morris (15). Protein concentrations were estimated by the methods of Lowry (16) and Bradford (17) using bovine serum albumin as standard.

**BPH Dox Assays—**Enzyme assays were performed in a 200-μl volume as described previously (1), except that the reaction was stopped by adding 400 μl of methanol. After centrifugation for 30 s in a microcentrifuge, 50 μl of the supernatant were injected into a Hewlett-Packard ODS Hypersil II (5 μm) reverse phase column (4 mm x 25 cm) that had been equilibrated with water-methanol-acetonitrile (50:25:25). The column was eluted for 5 min at 1 ml/min with the same solvent, followed by a 3-min linear gradient to methanol-acetonitrile (60:40) and then eluted for 5 min with the final solvent mixture. The activity was evaluated from measurement of substrate disappearance and metabolite production. They were detected using a Perkin-Elmer LC95-UV/visible detector set at 356 nm for 2,3-dihydro-2,3-dihydroxybenzyl alcohol (254 nm for biphenyl). Confirmation of the identity of the metabolites was obtained by gas chromatographic-mass spectrometric analysis using protocols described previously (1).

**RESULTS**

**Comparative Features of His-tagged B-356-ISP sub, Construction**—The His-tagged B-356-ISP construction was such that a single (His)x tail was added to the N-terminal portion of the ISP sub, construction. Both subunits were expressed in isopropyl-1-thio-β-D-galactopyranoside-induced E. coli recombinant cells as demonstrated by SDS-PAGE. Moreover, the presence of a single His tail was sufficient to obtain a purified preparation comprising both the His-tagged α and β subunits through a one-step purification performed on the Ni-nitrotri-acetic acid resin. This suggests that strong bonds are involved in the association between the subunits in the native protein and that the His tail of the β subunit did not significantly affect this association. Moreover, as shown below, purified His-tagged ISP sub was active in the BPH dox assay with added His-tagged FERBPH and His-tagged REDBPH.

The yield of purified His-tagged ISP sub was in the order of 1.5 mg/g cell paste (wet weight), which is slightly lower than the 3 mg/g cell paste obtained for ISP sub from B356. The best preparations showed two major bands on SDS-PAGE (Fig. 1). Their M, values were estimated to be 53,600 and 25,200, which correspond to the M, values of the ISP sub, the plus the His tail addition) and β subunits, respectively. The M, of the native ISP sub and His-tagged ISP sub were estimated by HPLC gel filtration and they were found to be of 234,000 and 186,000, respectively. These values indicate that the native conformation of B-356 ISP sub is αβ2 and corroborate previously published data obtained for strain LB400-ISP sub (4). The lower native M, of His-tagged ISP sub remains unexplained. However, the difference in migration in the gel could be attributed to an electrostatic interaction between the His tail and the diol residues of the stationary phase.

The purity of different preparations of His-tagged ISP sub were evaluated by scanning the SDS-PAGE gels, and these values were used as a correction factor to evaluate the actual protein concentration of His-tagged ISP sub. Using these data,
the average \( \varepsilon_{455} \) value was calculated to be 8,300 \( \text{M}^{-1} \text{cm}^{-1} \)
(based on the determination on seven preparations). Purified His-tagged ISP\(_{BPH}\) contained 2.6 ± 0.5 iron and 1.9 ± 0.1 sulfur per mol of \( \alpha/\beta \) heterodimer. Therefore, the number of iron atoms per mol of protein for His-tagged ISP\(_{BPH}\) preparations was close to the theoretical value of 3 iron that should be expected for a [2Fe-2S] Rieske-type center carrying a mononuclear Fe\(^{2+}\). As further evidence that the enzyme had retained most of its mononuclear Fe\(^{2+}\), we found that BPH dox specific activity of His-tagged ISP\(_{BPH}\) preparations increased by only 30–40% when an excess of iron was added to the reaction mixture.

On the other hand, the ISP\(_{BPH}\) preparations obtained from B-356 cells were somewhat more altered than the His tail preparations. For example, based on the \( \varepsilon_{455} \) value of 8,300 \( \text{M}^{-1} \text{cm}^{-1} \), we calculated that on average, 85% of the protein in the preparations had retained an intact Rieske center. This estimation was confirmed by the observation that these ISP\(_{BPH}\) preparations contained 1.7 ± 0.2 iron and 1.7 ± 0.3 sulfur atom/mol of \( \alpha/\beta \) heterodimer. It thus appears that the ISP\(_{BPH}\) preparations obtained from strain B-356 had lost all their mononuclear iron and that a portion of the Rieske center was destroyed.

ISP\(_{BPH}\) remained active for months at −70 °C. However, the His-tagged ISP\(_{BPH}\) that were reduced in activity showed a different subunit association pattern where the preparation contained \( \alpha \) monomer and \( \beta \) homodimer with minor amounts of \( \alpha/\beta \) heterodimer. On the other hand, \( \alpha/\beta \) heterodimer was the only form observed in the case of B-356 ISP\(_{BPH}\). This suggested that \( \alpha/\beta \) is the only active form of ISP\(_{BPH}\).

We found that a 20-min preincubation of His-tagged ISP\(_{BPH}\) with 5 mmol dithiothreitol on ice can restore activity of older preparations. The same phenomenon was observed with FER\(_{BPH}\). However, it is not clear whether this reactivation occurred by restoring the Rieske center or by changing some other feature of the molecule. On the other hand, fresh preparations were at their optimal level of activity when the required supplemental mononuclear Fe\(^{2+}\) was added to the assay. Aditional of dithiothreitol did not significantly affect their activity.

Kinetic parameters of the recombinant versus parental protein were compared when ISP\(_{BPH}\) in the reaction mixture containing His-tagged RED\(_{BPH}\) and His-tagged FER\(_{BPH}\) was replaced by His-tagged ISP\(_{BPH}\). For both preparations, 40 °C was the optimal temperature for BPH dox activity and the reaction was optimal at pH 5.5–6.0. When the proportion of the enzyme components were varied in the assay, the reaction was optimal for equimolar amounts of each one. Under the conditions described under “Experimental Procedures,” the \( K_m \) and \( V_{max} \) for the BPH dox activity were, respectively, 94 \( \mu \text{M} \) and 1.5 nmol min\(^{-1} \) \( \mu \text{g}^{-1} \) for ISP\(_{BPH}\) and 100 \( \mu \text{M} \) and 1.9 nmol min\(^{-1} \) \( \mu \text{g}^{-1} \) for His-tagged ISP\(_{BPH}\).

In previous work, gas chromatography-mass spectroscopic analysis of metabolites produced from B-356-BPH dox reaction suggested that both 2,3- and 3,4-dihydro-dihydroxybiphenyl were produced from BPH (1). Both metabolites were also produced when B-356-ISP\(_{BPH}\) was replaced by the recombinant ISP\(_{BPH}\) in the BPH dox assay (results not shown).

Purification and Characterization of Individual His-tagged ISP\(_{BPH}\) Subunits—Individual His-tagged \( \alpha \) and His-tagged \( \beta \) subunits were expressed in E. coli clones in appropriate constructs. The level of expression of the two subunits was very high as observed on SDS-PAGE gels of urea-solubilized cells (data not shown). However, the yield of purification of \( \alpha \) and \( \beta \) subunits was low (0.4 and 0.2 mg/g cell paste (wet weight), respectively), presumably because of the association of a por-

The purified proteins were detected on SDS-PAGE (Fig. 1). Gel filtration HPLC analysis showed that the native form of the purified His-tagged \( \alpha \) subunit was a monomer (\( M_r \) = 44,000), while the His-tagged \( \beta \) subunits had a tendency to join together to produce homodimers plus large conglomerates. The UV visible absorbance spectra of purified \( \alpha \) subunit (but not \( \beta \) subunit) was similar to the spectra of ISP\(_{BPH}\) and His-tagged ISP\(_{BPH}\) (Fig. 2). There was a slight shift of the peaks with maxima at 335 and 443 nm instead of 323 and 455 nm. After reduction with excess dithionite under aerobic conditions, the spectra resembled that of other reduced Rieske-type proteins. Assuming that the \( \varepsilon_{443} \) of this protein is equal to the \( \varepsilon_{455} \) of the ISP\(_{BPH}\) complex, we have estimated that on average 60% of the His-tagged \( \alpha \) subunit of purified preparations were carrying an intact Rieske center. This is supported by the observation that the ratio of iron and acid-labile sulfur per mol of His-tagged \( \alpha \) subunit was 1.3 ± 0.4 iron and 1.3 ± 0.1 sulfur. Therefore, part of the Rieske center was either denatured during purification of the His-tagged \( \alpha \) subunit or was not formed in the E. coli cells. The altered enzyme subunit was not restored by adding dithiothreitol or iron. Contrary to the naphthalene dioxygenase-\( \beta \) subunit (19), ISP\(_{BPH}\)-\( \beta \) subunit showed a broad peak between 300 and 500 (Fig. 2B). This peak was not modified under reduced conditions.

When preparations of individually purified subunits were combined in vitro and tested in the BPH dox assay immediately or after 18 h of preincubation at 4 °C, the activity recorded was only about 1% of the activity obtained with His-tagged ISP\(_{BPH}\)
preparation that had been assembled in vivo. However, when the crude cell extracts were mixed instead of the purified proteins, BPH dox activity was restored to a higher level (Table I). The activity increased with time when the mixture was preincubated for 18 h at 4 °C (results not shown) as it had been observed for the naphthalene dioxygenase (18). In order to find out which protein was deactivated during the purification, we mixed a crude E. coli lysate containing one His-tagged subunit with a purified preparation of the other His-tagged subunit. Using this approach, only trace amounts of activity were obtained when purified α subunit was added to the lysate but a fair level of activity was observed when purified β subunit was used (Table I). On the other hand, when the crude cell extracts containing the individual subunits were mixed and incubated for 18 h at 4 °C and then purified together on Ni-nitrilotriacetic acid resin, the resulting His-tagged ISP BPH preparation was active (Table I). These results suggested that the α subunit was denatured when it was purified alone. However, its prior association with the β subunit protects the enzyme against deactivation. These data clearly showed that α and β subunits of ISP BPH can associate in vitro to restore the active BPH dox.

**DISCUSSION**

In this work we have reported some of the major features of strain B-356 His-tagged ISP BPH and compared them to B-356 ISP BPH. Most of the data presented here, including the αβ3 arrangement of subunits are similar to the recently published properties of LB400 oxygenase component. It is not surprising because both enzymes are structurally closely related (11). At this time, however, we cannot explain the significant difference between the εAbs value of 8,300 M⁻¹ cm⁻¹ we have determined for B-356 ISP BPH and the one that was reported for strain LB400 ISP BPH.

Our data show that the main features that characterize B-356 ISP BPH carrying a His tail on the α subunit are very similar to those found for the untagged parental protein obtained from strain B-356 cells. Moreover, because the steps required for purification are milder, the His-tagged enzyme component preparations were less altered, as reflected by their UV visible spectral data, the iron and acid labile sulfur content, and the specific activity of the enzyme.

Hydroxylation dioxygenases are multicomponent enzymes that catalyze the transfer of electrons from an electron donor, usually a reduced nicotinamide dinucleotide, to the hydroxylating center of the oxygenase component. Based on the number of components and on the number and type of [2Fe-2S] centers involved in electron transfer, hydroxylation oxygenases have been subdivided into three classes (2, 3). BPH dox belongs to the class II B dioxygenases which also includes benzene dioxygenase and toluene dioxygenase. These enzyme systems comprise three components, a reductase containing a flavin cofactor, a Rieske-type ferredoxin protein, and a terminal oxygenase, which is a two-subunit iron-sulfur protein containing a Rieske-type [2Fe-2S] center. Absorbance spectra of purified B-356-BPH dox and LB400-BPH dox terminal oxygenases were typical of Rieske-type proteins (1, 4). Moreover, sequence analysis has identified a conserved sequence C-R-H-(aa)17-C-S-Y-H- of a Rieske center on the α subunit of both strains ISP BPH (11, 20, 21). However, confirmation of the presence of the Rieske center on the α subunit of class II B oxygenases has never been established. Suen and Gibson (19) have obtained a purified preparation of the class III naphthalene dioxygenase terminal oxygenase-α subunit. However, their purified protein was denatured and did not show a typical Rieske-type absorbance spectra. Therefore, use of His-tail protein provided, for the first time, spectral evidence that the Rieske center is located on the α subunit of the terminal oxygenase.

In spite of the fact that approximately 60% of the ISP BPH α subunit in purified preparations had retained an intact Rieske center with [2Fe-2S], these preparations were only weakly active when mixed with purified or crude preparations of β subunit. Structural modifications during the purification process to prevent reassociation with the β subunit cannot be excluded. This is also supported by the observation that these preparations did not show αβ3 heteromers on gel filtration (results not shown). The fact that the α subunit in crude cell extracts can combine with exogenous β subunit to generate an active complex might suggest the presence in cell extract of proteins or other constituents that interact with the α subunit to protect their folding. However, further study is needed to understand the reasons for impaired activity.

The sequence of events that occurs at the active site of aryldioxygenases to bind and activate the molecular oxygen and to attach it to the substrate is still unknown. The [2Fe-2S] Rieske-type cluster found in the terminal oxygenase component is presumed to involve at least two Cys and two His residues inside this domain (2, 13) to coordinate the iron atoms. In addition, other Tyr and His residues that are located farther away inside the protein are presumed to be involved in the coordination of a mononuclear Fe²⁺ which is required for oxygen binding to the enzyme (3, 13, 22).

Batie et al. (13) have clearly shown that Pseudomonas cepacia phthalate dioxygenase contains two iron atoms coordinated to Cys and His residues. They also demonstrated that the presence of an additional mononuclear Fe²⁺ is required for enzyme activity. Suen and Gibson (19) have also reported the presence of 6 iron and 4 acid labile sulfide atoms per mol of purified Pseudomonas sp. NCIB 9816-4 naphthalene dioxygenase oxygenase component, where this enzyme was found to be an αβ2 enzyme. Haddock and Gibson (4) have inferred the presence of a third mononuclear iron in LB400-BPH dox terminal oxygenase from the observation that Fe²⁺ stimulated the enzyme activity. Because of the good quality of the B-356 His-

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**Table I**

Specific activity of BPH dox preparations obtained by in vitro assembly of the His-tagged α and β subunits before and after purification of the subunits

| Source of α subunit | Source of β subunit | Specific activity a |
|---------------------|---------------------|--------------------|
| Crude E. coli lysate | Crude E. coli lysate | 11.5               |
| Crude E. coli lysate | Purified on Ni-NTA resin | 15.6               |
| Purified on Ni-NTA resin | Crude E. coli lysate | Trace              |
| Subunits α plus β purified together a on Ni-NTA | | 222                |

a BPH dox activity was evaluated by measuring the meta-degradation metabolite at 434 nm when excess purified B2,3D and B1,2O were added to the reaction mixture as described under "Experimental Procedures." The activity is reported per mg of protein in the preparation containing the α subunit.

b Crude lysate of E. coli cells producing either His-tagged α or His-tagged β were combined, incubated 18 h at 4 °C, then purified on Ni-nitrilotriacetic acid resin to obtain a purified preparation of a reconstituted His-tagged ISP BPH.

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tagged ISP$_{BPH}$ preparations, our data provides evidence that this enzyme contains 3 iron atoms and 2 acid labile sulfur atoms per mol of $\alpha\beta$ heterodimer.

It as been suggested that the $\beta$ subunit of the aryl dioxygenase component is involved in substrate recognition. Hirose et al. (7) have used various hybrid clones of P. pseudoalcaligenes strain KF707-BPH dox genes and P. putida strain F1-toluene dox genes, to show the importance of bphA2 (bphE) in determining substrate specificity. They made the observation that bphA2 could not be replaced by todC2 to obtain functional BPH dox, but that bphA1 could be replaced by todC1. Whatever the role of the $\beta$ component, it is certainly not carrying any Rieske center. This is confirmed by sequence analysis of the gene (11) and from the UV visible spectra of purified His-tagged ISP$_{BPH}$ $\beta$ subunit. However, its association to the $\alpha$ subunit is essential to protect the activity of the latter.

Our data clearly show that the assembly of the ISP$_{BPH}$ subunits can occur in vitro. We have reported for the first time the purification of an in vitro assembled ISP terminal oxygenase. Because the recombinant His-tagged components of the B-356-BPH dox have retained all properties of the parental protein, including the activity, the QIAGEN purification system appears, therefore, quite promising to further explore the various features of bacterial dioxygenases. Particularly, it opens the possibility of comparing reconstituted hybrid oxygenases containing subunits belonging to terminal oxygenases of different origin. Therefore, this system introduces a very useful new tool to further investigate the major features that distinguish the various aryl dioxygenase among them. Ongoing work in our laboratory is intended to exploit this.

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REFERENCES
1. Hurtubise, Y., Barriault, D., Powlowski, J., and Sylvestre, M. (1995) J. Bacteriol. 177, 6610–6618
2. Batie, C. J., Ballou, D. P., and Correll, C. J. (1990) in Chemistry and Biochemistry of Flavoenzymes (Müller, F., ed) pp. 544–554, CRC Press, Boca Raton, FL
3. Mason, J. R., and Cannard, R. (1992) Annu. Rev. Microbiol. 46, 277–305
4. Haddock, J. D., and Gibson, D. T. (1995) J. Bacteriol. 177, 5834–5839
5. Haddock, J. D., Horton, J., and Gilson, D. T. (1995) J. Bacteriol. 177, 20–26
6. Erickson, B. D., and Mondello, F. J. (1993) Appl. Environ. Microbiol. 58, 3858–3862
7. Hirose, J., Suyama, A., Hayashida, S., and Furukawa, K. (1994) Gene (Amst.) 138, 27–33
8. Ahmad, D., Messé, R., and Sylvestre, M. (1990) Gene (Amst.) 86, 53–61
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Sylvestre, M., and Fauteux, J. (1982) Gen. Appl. Microbiol. 26, 61–72
11. Sylvestre, M., Sirois, M., Hurtubise, Y., Bergeron, J., Ahmad, D., Shareck, F., Larose, A., Barriault, D., Guillemette, I., and Juteau, J. M. (1996) Gene (Amst.), in press
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Batie, C. J., LaHaie, E., and Ballou, D. P. (1987) J. Bid. Chem. 262, 1510–1518
14. Fogo, J. K., and Popowsky, M. (1949) Anal. Chem. 21, 732–734
15. King, T. E., and Morris, R. O. (1967) Methods Enzymol. 10, 634–637
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Bid. Chem. 193, 265–275
17. Bradford, M. M. (1976) Anal. Biochem. 72, 148–254
18. Suen, W.-C., and Gibson, D. T. (1994) Gene (Amst.) 143, 67–71
19. Suen, W.-C., and Gibson, D. T. (1993) J. Bacteriol. 175, 5877–5881
20. Taira, K., Hirose, J., Hayashida, S., and Furukawa, K. (1992) J. Bid. Chem. 267, 4844–4853
21. Erickson, B. D., and Mondello, F. J. (1992) J. Bacteriol. 174, 2903–2912
22. Neidle, E. L., Hartnett, C., Ornston, N., Bairouch, A., Rekik, M., and Harayama, S. (1991) J. Bacteriol. 173, 5385–5395
Characterization of Active Recombinant His-tagged Oxygenase Component of *Comamonas testosteroni* B-356 Biphenyl Dioxygenase
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