**Functional Analyses of Bph-Tod Hybrid Dioxygenase, Which Exhibits High Degradation Activity toward Trichloroethylene**

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Biphenyl dioxygenase (BphDox) in *Pseudomonas pseudoalcaligenes* KF707 is a multicomponent enzyme consisting of an iron–sulfur protein (ISP) that is composed of α (BphA1) and β (BphA2) subunits, a ferredoxin (FD), and a ferredoxin reductase (FDR). A recombinant *Escherichia coli* strain expressing hybrid Dox that had replaced BphA1 with TodC1 (α subunit of toluidine dioxygenase (TodDox) of *Pseudomonas putida*) exhibited high activity toward trichloroethylene (TCE) (Furukawa, K., Hirose, J., Hayashida, S., and Nakamura, K. (1994) J. Bacteriol. 176, 2121–2123). In this study, ISP, FD, and FDR were purified and characterized. Reconstitution of the dioxygenase components consisting of purified ISP, FD, and FDR exhibited oxygenation activities toward biphenyl, toluene, and TCE. Native polyacrylamide gel electrophoresis followed by the Ferguson plot analyses demonstrated that ISP, FD, and FDR were present as heteromers, whereas ISP was present as a heterotetramer. The molecular activity (k₄) of the hybrid Dox for TCE was 4.1 min⁻¹, which is comparable to that of TodDox. The Km value of the hybrid Dox for TCE was 130 μM, which was lower than 250 μM for TodDox. These results suggest that the α subunit of ISP is crucial for the determination of substrate specificity and that the α subunit conformation of ISP from α to α results in the acquisition of higher affinity to TCE, which may lead to high TCE degradation activity.

Biphenyl dioxygenase (BphDox) in *Pseudomonas pseudoalcaligenes* KF707 catalyzes the introduction of two atoms of molecular oxygen into biphenyl and some polychlorinated biphenyls (PCB). BphDox is a multicomponent enzyme encoded by the four genes, bphA1A2A3A4, where bphA1 encodes an α subunit (BphA1) of the terminal dioxygenase (an iron–sulfur protein, ISP), bphA2 encodes the β subunit (BphA2) of ISP, bphA3 encodes ferredoxin (BphA3, FD), and bphA4 encodes ferredoxin reductase (BphA4, FDR) (1). ISP requires mononuclear iron (Fe²⁺), which is likely to be the central active center for catalysis and contains a Rieske [2Fe-2S] cluster for electron transfer. Recently a classification has been presented for these types of ISP as a family of Rieske non-heme iron oxygeases (2). FD and FDR act as an electron transfer system from NADH to reduce ISP. Recent studies provided evidence that the α subunit of ISP plays a primary role in the determination of the substrate specificity for PCB (3–8). Modified BphDox constructed by subunit exchange (3), site-directed mutagenesis (4–6), DNA shuffling (7), and random priming recombinations (8) resulted in the dramatic alterations in the enzyme activities, the substrate selectivities, and the mode of oxygenation toward certain PCB congeners (9). It is also true that some BphDox from various bacteria share structural similarities to those of KF707, however, their substrate specificities toward PCB are different (10).

Toluene dioxygenase (TodDox) in *Pseudomonas putida* F1 catalyzes the conversion of toluene to cis-toluene dihydrodiol (11). Besides toluene, TodDox exhibits catalytic activity toward benzene, biphenyl, naphthalene, and trichloroethylene (TCE). The toluidine catalyzed operon is similar to the KF707 bph gene cluster in terms of gene organization and the nucleotide sequence of the corresponding genes (1, 12). TodDox is a multicomponent enzyme consisting of α (TodC1) and β (TodC2) subunits of ISP, FD (TodB), and FDR (TodA). The identities of the amino acid sequences between the corresponding components of BphDox and TodDox are in the range of 53 to 65% (1, 12).

During the course of identifying the component responsible for the substrate specificity of BphDox and TodDox, hybrid bph-tod gene clusters were constructed by replacing genes encoding the dioxygenase components from BphDox and TodDox. Among them, the recombinant *Escherichia coli* expressing the hybrid todC1-bphA3A4 genes exhibited substrate specificity similar to that of the original TodDox, but 3-fold higher activity toward TCE than TodDox (13). Chloroethylenes such as TCE have been recognized to be significant environmental pollutants in the soil, groundwater, and atmosphere (14). These compounds have been shown to persist over time in the environment and are suspected to be carcinogenic (15). In the recent past, it has been shown that TCE is degraded with a variety of oxygenases such as methane monoxygenase (16), toluene monoxygenase (17), ammonium monoxygenase (18), phenol hydroxylase (19), and TodDox (11) from aerobic bacteria. The mechanism of TCE oxygenation by TodDox has been proposed by Li and Wackett (20). TCE is converted to an iron-bound dioxygenated intermediate on the enzyme surface, and

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1 The abbreviations used are: BphDox, biphenyl dioxygenase; PCB, polychlorinated biphenyl; ISP, iron–sulfur protein; FD, ferredoxin; FDR, ferredoxin reductase; TodDox, toluene dioxygenase; TCE, trichloroethylene; PCR, polymerase chain reaction; TRX, thioredoxin gene; MOPS, 4-morpholinepropanesulfonic acid; MEQ, 50 mM MOPS buffer, pH 7.0, containing 5% ethanol and 5% glycerol; DCE, dichloroethylene; KCl, 10 mM potassium phosphate buffer, pH 7.0; PAGE, polyacrylamide gel electrophoresis.
the intermediate compound is rearranged to form formate and HCl using H₂O.

We now report the purification of the TodC1-BphA2 hybrid Dox and the kinetic analyses toward chloroethylenes.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**

*E. coli* JM109 was used for the general propagation of the plasmids and for the expression of ISP. *E. coli* BL21(DE3) was used for the expression of bphaA3 and bpha4. For the expression of bphaA1A2 and todC1BphA2, *E. coli* cells were grown at 37°C for 12 h in Luria-Bertani medium containing 0.1 mM isopropyl-β-D-thiogalactopyranoside and a concentration, 50 μg/mL, of the appropriate antibiotics. For expressions of todC1C2, bphaA3, and bpha4, *E. coli* cells were grown at 30°C for 24 h in the same medium described above.

**Plasmids for ISP Expression**

pUC-A1A2 for the expression of ISPbphaA1A2 was constructed by inserting the XhoI DNA fragment containing the bphaA1A2 genes from pKTF18 into an XhoI site of pUC119. pUC-C1A2, for the expression of the hybrid ISP todC1BphA2, was constructed by removing the PstI DNA fragment of a lambda DNA fragment clone *E. coli* todC1BphA2 and replacing it with the XhoI site around the start codon of the *bphaA2* gene. As a template DNA for site-directed mutagenesis was carried out to introduce an EcoRI enzyme for 12 h in Luria-Bertani medium containing 0.1 mM isopropyl-β-D-thiogalactopyranoside and a concentration, 50 μg/mL, of the appropriate antibiotics. For expressions of todC1C2, bphaA3, and bpha4, *E. coli* cells were grown at 30°C for 24 h in the same medium described above.

**Plasmids for FdPbphA3 and FdRbphA4 Expression**

To express FdPbphA3, the bphaA3 gene was amplified by PCR using pHF10 as the template DNA. An oligonucleotide corresponding to the 5’ sequence of the bphaA3 gene, 5’-ATTATCCATGGGATATGAAATTTACCATGACGTCG-3’ (primer #1), was used as the forward primer, where the EcoRI site is underlined. An oligonucleotide corresponding to the 3’ sequence of the bphaA3 gene, 5’-GAAGGTTCACTAAGAGAACTAGG-3’ (primer #2), was used as the reverse primer, where the KpnI site is underlined. The amplified DNA, which had been digested with EcoRI and KpnI, was inserted into the corresponding sites of pBluescriptII SK⁺ (Stratagene) to generate pBlue-C2. A BamHI/EcoRI DNA fragment containing the todC2 gene and the Shine-Dalgalno sequence of the *bphaA4* gene, 5’-AAGAATTTACCATGATTCGACCCAACA-3’ (primer #1), was used as the forward primer, where the EcoRI site is underlined. An oligonucleotide corresponding to the 3’ sequence of the todC2 gene, 5’-AAAGGTTACCCTAGAAGAAGAAACTGAGG-3’ (primer #2), was used as the reverse primer, where the KpnI site is underlined. The amplified DNA, which had been digested with EcoRI and KpnI, was inserted into the corresponding sites of pBluescriptII vector pBluescriptII region of pBlue-C1C2 by pHSG396 (Toyobo, Kyoto, Japan) at the BamHI and KpnI sites.

**Plasmids for ISP Expression**

pUC-A1A2 for the expression of ISPbphaA1A2 was constructed by inserting the XhoI DNA fragment containing the bphaA1A2 genes from pKTF18 into an XhoI site of pUC119. pUC-C1A2, for the expression of the hybrid ISP todC1BphA2, was constructed by removing the PstI DNA fragment of a lambda DNA fragment clone *E. coli* todC1BphA2 and replacing it with the XhoI site around the start codon of the *bphaA2* gene. As a template DNA for site-directed mutagenesis was carried out to introduce an EcoRI enzyme for 12 h in Luria-Bertani medium containing 0.1 mM isopropyl-β-D-thiogalactopyranoside and a concentration, 50 μg/mL, of the appropriate antibiotics. For expressions of todC1C2, bphaA3, and bpha4, *E. coli* cells were grown at 30°C for 24 h in the same medium described above.

**Protein Purification**

ISP purification was carried out according to the method described by Haddock and Gibson (23) with some modifications. All the purification steps were carried out at 4°C. Harvested recombinant *E. coli* cells were suspended in 50 mM MOPS buffer, pH 7.0, containing 5% ethanol and 5% glycerol (MEG). Cells were disrupted by a French pressure cell (Ohtake Seisakusho, Tokyo) prior to centrifugation at 17,400 x g for 10 min. The resultant viscous liquid was treated with 3% streptomycin sulfate for 30 min and centrifuged. The resulting supernatant was used as a crude enzyme. The crude enzyme was applied to a Q-Sepharose FF column (Amersham Pharmacia Biotech) equilibrated with MEG. The hybrid ISP todC1BphA2 was purified as well as the parental ISP bphaA1A2 and ISP todC1C2 were purified as follows.

ISPbphaA1A2—Proteins were eluted in stepwise fashions with 0.1 M KCl and 0.2 M KCl in MEG. A fraction showing ISP activity was eluted with 0.2 M KCl. The active fraction that had added 2.0 mM ammonium sulfate was applied to a Butyl-Toyopearl 650M column (Tohso, Tokyo, Japan) equilibrated with 2.0 mM ammonium sulfate in MEG, and the proteins were stepwise eluted with 2.0, 1.0, and 0.5 mM ammonium sulfate in MEG. The fraction showing the ISP activity was eluted with 0.5 mM ammonium sulfate and was dialyzed against 10 mM potassium phosphate buffer, pH 7.0. The dialysis was applied to a column of hydroxylapatite (Bio-Rad) equilibrated with KP, and the proteins were eluted in stepwise fashions with 0.01, 0.1, and 0.25 M KP. ISPbphaA1A2 was eluted with 0.25 M KP.

ISP todC1C2—Proteins were eluted in a linear gradient from 0.2 to 0.4 M KCl in MEG. The fraction showing ISP activity was eluted with 0.3 M KCl. The fraction in the added 1.0 mM ammonium sulfate was applied to a Butyl-Toyopearl 650M column (Tohso, Tokyo, Japan) equilibrated with 1.0 mM ammonium sulfate in MEG, and the proteins were stepwise eluted with 1.0, 0.75, and 0.5 mM ammonium sulfate in MEG. The fraction showing ISP activity was eluted with 0.5 mM ammonium sulfate. The subsequent procedure for purification of the ISP todC1C2 was the same as described above for ISPbphaA1A2.

**FDpphaA3 Fusion Protein**

The crude enzyme was prepared by extracting with 50 mM KP containing 10 mM imidazole, pH 7.0, instead of that with MEG. The crude enzyme was applied to a nickl-nitroliatriacetic acid-agarose column (Qiagen) equilibrated with 50 mM KP containing 10 mM imidazole, pH 7.0. The agarase gel was washed with washed with 50 mM KP containing 50 mM imidazole and 0.3 mM NaCl (washing buffer). The FDpphaA3 fusion protein was eluted with 50 mM KP containing 0.2 mM imidazole and 0.3 mM NaCl.

**FDPhbphA4 Fusion Protein**—Purification of the FDRpphaA4 fusion protein was done using the same method for FDpphaA3, but changing the washing buffer to 50 mM KP containing 20 mM imidazole, 0.3 mM NaCl, and 0.5% Triton X-100.

**Enzyme Assay**

An assay for dioxygenase activity was spectrophotometrically done. A total test volume of 250 μL contained 50 mM MOPS buffer, pH 7.0, 0.4 mM ferrous ammonium sulfate, 0.4 mM NADH, 5 mM biphenyl, 1 mg of cell-free extracts containing dihydrodioxide dehydrogenase, 2,3-dihydroxybiphenyl dioxygenase, and enzyme components. Incubation was done with shaking at 30°C for the appropriate time. The product catalyzed by dioxygenase from biphenyl was further converted to a yellow compound, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid by the sequential actions of dihydrodiol dehydrogenase and 2,3-dihydroxybiphenyl dioxygenase. The yellow compound formed was quantified by measuring its A443 value.

Enzymatic reactions were also detected and quantified by high performance liquid chromatography. The reaction mixture (800-μL total volume) contained 50 mM MOPS buffer, pH 7.0, 1 mM NADH, 0.4 mM FeSO₄, 1 mM biphenyl or 3 mM toluene as the substrates, and 40–840 μg of each enzyme component. The reaction was initiated by adding the substrate. 0.1 μL and the reaction mixtures were incubated at 37°C. At appropriate intervals, 150 μL of the reaction mixture was removed, then added to 300 μL of methanol in a microtube. After centrifugation for 10 min, 40 μL of the supernatant was injected into an Ultrasphere ODS column (Beckman) that had been equilibrated with water:methanol:acetoniiterol (40:30:5, v/v). The column was eluted for 6 min at 0.75 mL/min with the same solvent system, followed by elution with methanol:acetoniiterol (45:55, v/v) for 20 min. The activity was evaluated from
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### Table 1

| Plasmids          | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| pUC-A1A2          | bphA1A2 under the control of a lac promoter in pUC118, Ap<sup>+</sup> | This study          |
| pKTF183ORF3       | bphA1A2A3A4BC lacking orf3 in pUC118, Ap<sup>+</sup> | 1                   |
| pUC-C1A2          | todC1::bphA2 under the control of the lac promoter in pUC118, Ap<sup>+</sup> | This study          |
| pJEF10            | todC1::bphA2A3A4BC in pUC118, Ap<sup>+</sup> | 3                   |
| pJHF-C1C2         | todC1C2 in pUC119, Ap<sup>+</sup> | 21                  |
| pBlueScriptISK+   | Ap<sup>+</sup>          | Stratagene          |
| pBlue-C2          | todC2 in pBlueScriptII SK+, Ap<sup>+</sup> | This study          |
| pBlue-C1C2        | todC1C2 in pBlueScriptII SK+, Ap<sup>+</sup> | This study          |
| pHSG396           | Cm<sup>+</sup>          | Takara Shuzo       |
| pHSG-C1C2         | todC1C2 under the control of the lac promoter in pHSG396, Cm<sup>+</sup> | This study          |
| pET32b            | trx-6Xhis under the control of the T7 promoter, Ap<sup>+</sup> | Novagen             |
| pET32-A3         | trx-bphA3-6Xhis under the control of the T7 promoter in pET32b, Ap<sup>+</sup> | This study          |
| pJHF-A3A4BC       | bphA3A4BC in pUC119, Ap<sup>+</sup> | 21                  |
| pUC-A3A4         | bphA3A4 in pUC119, Ap<sup>+</sup> | This study          |
| pUC-A3A4N       | bphA3A4 with a Ncol site in pUC119, Ap<sup>+</sup> | This study          |
| pUC-A4          | trx-6Xhis::bphA4-6Xhis under the control of the lac promoter in pUC119, Ap<sup>+</sup> | This study          |
| pKY206          | groELC, Cm<sup>+</sup>, origin from pACYC101 | 22                  |

“Ap<sup>+</sup>,” ampicillin resistance; “Cm<sup>+</sup>,” chloramphenicol resistance.

the substrate disappearance measurement by detection at 254 nm for biphenyl and at 260 nm for toluene.

### Kinetic Analysis

Steady-state kinetic parameters for TCE, cis-DCE, trans-DCE, and 1,1-DCE were determined with purified ISP, FD BphA3, and FDR BphA4.

Two milliliters of the reaction mixture containing 2.2–3.2 μM ISP, 26–38 μM FD BphA3, 2.2–3.2 μM FDR BphA4, 20 μM ferrous ammonium sulfate, and 50 mM MOPS buffer (pH 7.0) was added to a glass vial (20 ml), which was sealed with a rubber septum and an aluminum crimp seal. Chlooroethylenes dissolved in N,N-dimethylformamide were added to the vial and preincubated at 30 °C for 30 min to allow equilibration of the chlooroethylenes between the gas and liquid phases. The reaction was initiated by the addition of 0.8 mM NADH, and incubation was carried out with shaking at 30 °C for 10 min. The amounts of the chlooroethylenes in the gas phase were measured by gas chromatographic analysis according to a previously described method (13), and those in the aqueous phase were calculated according to Henry’s Law constants for the chlooroethylenes (24). The values of V<sub>max</sub> and K<sub>m</sub> were determined using the Hanes-Woolf (S/V – S plot) analysis.

### General Analytical Procedure

Purified ISP comprised of α and β subunits was subjected to native PAGE. The mobilities of the ISP on 5%, 7.5%, 10%, and 12.5% acrylamide gels were treated with the Ferguson plot (25) to estimate the molecular mass. SDS-PAGE was carried out according to the method of Laemmli (26). The protein concentration was measured by a Bio-Rad protein assay using bovine serum albumin as the standard.

### RESULTS

**Expression of ISP, FD<sub>BphA3</sub> and FDR<sub>BphA4</sub>**—The α and β subunits of ISP were successfully coexpressed in E. coli using pUC-A1A2, pUC-C1A2, and pHSG-C1C2 (Table I). In these expression systems, a lac promoter was applied to induce the ISP and was sufficient to produce soluble ISP. ISP<sub>BphA1A2</sub> and ISP<sub>TodC1BphA2</sub> were produced as the soluble and active forms in the recombinant E. coli at 37 °C. When ISP<sub>TodC1C2</sub> was expressed in E. coli using a recombinant plasmid carrying the original todC1C2 genes, the level of expression of the β subunit was extremely low, as compared with that of the α subunit. Therefore, the Shire-Dalgarno sequence of todC2 was replaced by that of bphA1, and the pHSG-C1C2 was finally constructed. The recombinant E. coli cells carrying pHSG-C1C2 produced TodC2 with amounts equivalent to TodC1. However, the proteins were produced as prominent inclusion bodies. A reduction of the cultivation temperature from 37 °C to 30 °C resulted in an elevated yield of the soluble ISP. FD<sub>BphA3</sub> and FDR<sub>BphA4</sub> were expressed as fusion proteins to facilitate protein purification and solubilization. Recombinant E. coli cells carrying pET32-A3 under the control of a strong T7 promoter produced the soluble FD<sub>BphA3</sub> fusion protein with the N-terminal TRX, His-tag, and C-terminal His-tag with a molecular mass of 36 kDa (Fig. 1). As in the case of FD<sub>BphA1A2</sub>, the bphA4 gene was also expressed as a fusion protein with the N-terminal TRX and His-tag. However, the FDR<sub>BphA4</sub> fusion protein with a molecular mass of 63 kDa was produced in E. coli (pET32-A4) as prominent inclusion bodies. Replacement of the T7 promoter by the lac promoter resulted in increased amounts of the soluble FDR<sub>BphA4</sub> fusion protein. In addition, coexpression of a chaperone, GroELS of E. coli using pKY206, allowed the FDR<sub>BphA4</sub> fusion protein to efficiently solubilize. By using this coexpression system, the production of the FDR<sub>BphA4</sub> fusion protein was improved to 4.5-fold compared with that without pKY206.

The catalytically active components of ISPs, FD<sub>BphA3</sub> and FDR<sub>BphA4</sub>, were purified as shown in Fig. 1. The yields of the purified ISP<sub>BphA1A2</sub>, ISP<sub>TodC1BphA2</sub>, ISP<sub>TodC1C2</sub>, FD<sub>BphA3</sub>, and FDR<sub>BphA4</sub> from a 1-liter culture were 11.0, 9.4, 8.6, 22.1, and 20.4 mg, respectively.

**Reconstitution of Dox Components**—The Dox activity was observed when all purified components of ISP, FD<sub>BphA3</sub> and FDR<sub>BphA4</sub> were mixed, indicating that all components were successfully associated with one another in vitro. The maximum activity of the hybrid enzyme was observed when 12-fold excess of FD<sub>BphA3</sub> was incubated with ISP<sub>TodC1BphA2</sub> and FDR<sub>BphA4</sub>. Only 17%, 30%, and 70% of the maximum activity was observed when 3-, 6-, and 9-folds excess of FD<sub>BphA3</sub> were added, respectively (data not shown). The maximum activities of the parental BphDox and TolDox were also observed in the presence of 15- and 12-fold excesses of FD<sub>BphA3</sub>, respectively.

The specific activities of the purified ISP were determined in the presence of excess amounts of FD<sub>BphA3</sub> by high performance liquid chromatography analysis and were evaluated from the rate of substrate depletion (Table II). The specific activity of the hybrid Dox toward biphenyl was 5.3 nmol/min/nmol ISP, which was comparable to that of TolDox and was as low as 2.9% of that of BphDox. The specific activity of the hybrid Dox toward toluene was 270.3 nmol/min/nmol ISP, which was 7- and 1.5-fold higher than that of BphDox and TolDox, respectively.

**Subunit Conformation of Rieske Oxygenase**—Rieske non-heme iron oxygenase (ISP) systems that are involved in the initial dioxygenation of aromatic compounds and which comprise heteromeric subunits are generally present as heterohexamer (α2β2) or heterotetramer (α2β2) conformations (27). The α and β subunits of the parental ISP<sub>BphA1A2</sub>, ISP<sub>TodC1C2</sub>, and hybrid ISP<sub>TodC1BphA2</sub> were copurified by column chromatographies, indicating that these two subunits were tightly associated with each other and formed the catalytically active ISP.
(Fig. 1). An analysis of the purified ISP on SDS-PAGE demonstrated that the α and β subunits were associated with a 1:1 stoichiometry. To investigate how the purified subunits are organized in ISP, non-denaturing PAGE was carried out with the purified ISP. The hybrid ISP₉₀ᵈᵡ₁₋₁₈₋₂ as well as the parental ISP₉₀ᵈᵡ₁₋₂ and ISP₉₀ᵈᵡ₁₋₂₋₂ were separated on various concentrations of acrylamide gel, and the relative mobilities were determined with the various concentrations. Based on the Ferguson plot, the molecular masses of ISP₉₀ᵈᵡ₁₋₁₈₋₂ and ISP₉₀ᵈᵡ₁₋₂₋₂ were estimated to be 209, 160, and 229 kDa, respectively (Fig. 2). Because the theoretical molecular mass of ISP₉₀ᵈᵡ₁₋₁₈₋₂ is 219 kDa for α₁β₁ and 146 kDa for α₁β₂, the parental ISP₉₀ᵈᵡ₁₋₂ is present as a heterotetramer. The theoretical molecular mass of ISP₉₀ᵈᵡ₁₋₂₋₂ is 222 kDa for α₁β₁ and 148 kDa for α₁β₂, therefore, ISP₉₀ᵈᵡ₁₋₂₋₂ is present as a heterotetramer, which is in agreement with the result reported by Subramanian et al. (28). The ISP₉₀ᵈᵡ₁₋₂₋₂ hybrid has a similar conformation to ISP₉₀ᵈᵡ₁₋₁₈₋₂ and the estimated molecular mass of 229 kDa is in good agreement with the theoretical 226.5 kDa of α₁β₁₂ conformation.

**Kinetic Parameters of Hybrid Dox for Chloroethylenes**—Because the parental BphDox is inactive toward chloroethylenes, the steady-state kinetic parameters for TCE, cis-DCE, trans-DCE, and 1,1-DCE were determined with purified TolDox and hybrid Dox (Table III). The apparent molecular activity (k₀) of the hybrid Dox for TCE is comparable to that of TolDox. Because conformation of TolDox and hybrid Dox are αᵡ₁β₁₂ heterotetramer and α₁β₁₂ heterohexamer, respectively, the catalytic center activity (kₐᵡ₁) of the hybrid Dox is calculated to be 1.4 min⁻¹ site⁻¹ and 33% lower than that of TolDox. The Kᵡ value for TCE of the hybrid Dox is 130 μM, which is smaller than that of TolDox (250 μM). The resulting catalytic efficiency, kₐᵩ/Kᵡ value for TCE increased 24% for the hybrid Dox, as compared with that for TolDox. For the other chloroethylenes such as cis-DCE and 1,1-DCE, the kₐᵩ values are higher in TolDox and the Kᵡ values are smaller in the hybrid Dox. The results indicate that the hybrid Dox acquired higher affinities for a variety of chloroethylenes than the parental TolDox and that the catalytic efficiency is slightly higher in the hybrid Dox. For the trans-DCE, the hybrid Dox showed only a weak activity, and thus the kinetic parameter with a high regression coefficient could not be determined from the Hanes-Woolf plot.

**Components Responsible for Enzyme Inactivation Coupled to TCE Degradation**—It is known that TolDox is irreversibly inactivated by coupling to TCE oxygenation (20). The hybrid Dox was also gradually inactivated during TCE oxygenation and was completely inactivated after 7.5 h of incubation (Fig. 3). To identify the components responsible for being inactivated, ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂, FDB₉₀ᵈᵡ₁₋₂₋₂, or FDB₉₀ᵈᵡ₁₋₁₈₋₂ was, respectively, added to the inactivated reaction mixture. The addition of ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ permitted the immediate restoration of the enzymatic activity of the TCE oxygenation (Fig. 3). On the other hand, the addition of FDB₉₀ᵈᵡ₁₋₂₋₂ or FDB₉₀ᵈᵡ₁₋₁₈₋₂ failed to restore the activity. These results indicate that the ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ component is susceptible and involved in the inactivation of the hybrid Dox during TCE degradation.

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**FIG. 1.** SDS-PAGE of ISP, FD, and FDR fusion proteins. Proteins were separated on 10% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, mass marker proteins; lane 2, ISP₉₀ᵈᵡ₁₋₂₋₂; lane 4, ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂; lane 5, FDB₉₀ᵈᵡ₁₋₂₋₂ fusion protein; lane 6, FDR₉₀ᵈᵡ₁₋₁₈₋₂ fusion protein.

**FIG. 2.** Ferguson plots for estimation of molecular masses of ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂, ISP₉₀ᵈᵡ₁₋₂₋₂, and ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ by native PAGE using various concentrations of acrylamide gels. Purified ISP consisting of the α and β subunits was subjected to native PAGE using chicken egg white albumin monomer (45 kDa), bovine serum albumin dimer (132 kDa), urease monomer (272 kDa), and urease dimer (544 kDa) as mass standard proteins. The mobilities of ISP on 5%, 7.5%, 10%, and 12.5% acrylamide gels were treated using the Ferguson plot as follows. The relative mobilities (Rf) of the standard proteins were determined at four different gel concentrations (% T) and plotted as log(Rf) versus %T. The slope of Kr was determined using linear regression of the %T − logKr plot. The log (molecular mass of the standards) was then plotted versus −logKr. The resultant equation, log (molecular mass of the standards) = −1.9321 logKr − 7.0181 (r = 0.99479), was used to estimate the molecular mass of the ISP.

**TABLE II**

| Enzymes | Activity toward biphenyl (nmol/min/nmol ISP) | Activity toward toluene (nmol/min/nmol ISP) |
|---------|---------------------------------------------|---------------------------------------------|
| BphDox  | 183.2 ± 15                                  | 270.3 ± 6.7                                 |
| TodC₁₋₁₈₋₂ hybrid Dox | 5.3 ± 1.3                                  | 182.7 ± 0.9                                 |
| TolDox  | 4.1 ± 0.6                                   |                                             |

**TABLE III**

| Enzymes | Mol. mass estimated by Ferguson plots (α₁β₁₂) | Mol. mass estimated by SDS-PAGE (α₁β₁₂) | Predicted mol. mass (α₁β₁₂) |
|---------|-----------------------------------------------|------------------------------------------|-----------------------------|
| ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ | 209                                        | 23.0                                     | 219.0                       |
| ISP₉₀ᵈᵡ₁₋₂₋₂ | 160                                         | 21.5                                     | 222.0                       |
| ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ | 229                                        | 23.0                                     | 226.5                       |

**TABLE IV**

| Enzymes | Mol. mass estimated by Ferguson plots (α₁β₁₂) | Mol. mass estimated by SDS-PAGE (α₁β₁₂) | Predicted mol. mass (α₁β₁₂) |
|---------|-----------------------------------------------|------------------------------------------|-----------------------------|
| ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ | 209                                        | 23.0                                     | 219.0                       |
| ISP₉₀ᵈᵡ₁₋₂₋₂ | 160                                         | 21.5                                     | 222.0                       |
| ISP₉₀ᵈᵡ₁₋₁₈₋₂₋₂ | 229                                        | 23.0                                     | 226.5                       |
FIG. 3. Components responsible for enzyme inactivation coupled to TCE degradation. A, the reaction mixture containing 3.5 μM ISP_{TodC1-TodC2}, 102 μM FDBphA3, 3.5 μM FDRBphA4, 20 μM ferrous ammonium sulfate, 6.4 mM NADH, and 1 mM TCE in 50 mM Mops, pH 7.0, was incubated with shaking at 30 °C for 7.5 h to allow complete inactivation of the enzyme. 1 mM TCE and 1 mM NADH were added to the reaction mixture and further incubated for 2.5 h to confirm inactivation of the enzyme. B, ISP_{BphDox}, FDBphA3, and FDRBphA4 were then added, respectively, to the reaction mixture to recover the Dox activity. Symbols: A, residual amounts of TCE (open square); B, additions of none (open triangle), ISP_{BphDox} (closed square), FDBphA3 (closed circle), and FDRBphA4 (closed diamond).

### DISCUSSION

It is of great interest to construct microorganisms with enhanced and expanded degradation capabilities for environmental pollutants. Some attempts, including in vitro DNA shuffling, domain exchanges, and subunit exchanges have been achieved for structural remodeling of dioxygenases with minimum prior information about the enzymes (4–9, 29). We previously reported that recombinant bacteria producing hybrid TodC1-BphA2A4 dioxygenase constructed by subunit exchanges, exhibited the expanded capability of converting various aromatic compounds (21, 30) and the enhanced degradation activity toward TCE (13, 30).

To reconstitute ISP, we first mixed in vitro α and β subunits of BphDox, TodDox, and TodC1-BphA2 hybrid Dox that had been individually expressed in E. coli but failed to reconstitute ISP with high activity (data not shown). However, the ISP components of BphDox and hybrid Dox were fully active when both subunits were coexpressed in E. coli. This result is different from the previous work by Hurtubise et al. (31). They reported that His-tagged α and β subunits of ISP from *C. testosteroni* were separately expressed in E. coli and that the mixture of these subunits exhibited high activity.

It was found that the reconstituted Dox with purified ISP, FDBphA3, and FDRBphA4 are highly functional, indicating that FDBphA3 and FDRBphA4 originating from BphDox interacted with a foreign ISP that originated from TodDox and hybrid Dox, where an electron from NADH is transferred to the Rieske [2Fe-2S] center of the α subunit. The reduced ISP activates molecular oxygen and introduces it to the substrates. The maximum activity of ISP was observed when more than 12-fold equivalents of FDBphA3 were incubated with ISP and FDRBphA4. The recombinant FDBphA3 fusion protein may be less active than the native FD due to the additional fusion tags. It is also likely that relatively low activity of FDBphA3 may be associated with the instability of this protein. The FD of BphDox from *C. testosteroni* was also reported to be labile (32).

The substrate specificities of the purified hybrid Dox were similar to those of TodDox (Table II), indicating the α subunit of ISP is critically responsible for recognition of the substrates and the catalytic activity. This result also implies that, in the hybrid Dox, the structure of the functional domains such as the mononuclear iron-binding residues and substrate binding pocket can be retained even in the subunit conformation of an αβ2. Changing the subunit conformation from αβ2 to αβ3 may lead to a small change in the structure around the active site. The K_{m} values of the hybrid Dox for the chloroethylenes ranged from 130 to 370 μM (Table III). Those of the parental TodDox ranged from 250 to 824 μM (Table III). These results suggest that the hybrid Dox gains slightly higher affinity for substrates used in this study than TodDox. A binding pocket around the active center of ISP_{TodC1-BphA2} may be slightly relaxed to accommodate various chloroethylenes. Although the k_{cat} values of the hybrid Dox are comparable to those of TodDox, the k_{cat} values of the hybrid Dox are slightly lower than those of TodDox. It is likely that the conformation of the catalytic residues involved in the mononuclear iron binding in ISP_{TodC1-BphA2} is slightly changed compared with that in ISP_{TodC1-C2} (33). As previously reported (13), the recombinant

### TABLE III

| Substrates | Cl | Cl | Cl | Cl | Cl | Cl |
|------------|----|----|----|----|----|----|
| H          |     |     |     |     |     |     |
| C          |     |     |     |     |     |     |
| cis-DCE    | 0.4 | 1.3 |     |     |     |     |
| trans-DCE  |     |     | 0.1 | 1.1 | 1.3 |     |
| 1,1-DCE    | 0.2 | 0.7 | 0.4 | 0.7 | 0.7 |     |

Notes: a N.D. indicates not determined.
resting cells expressing hybrid Dox degraded TCE 3-fold faster than those expressing TolDox. The TCE used in this experiment was as low as 76 μM. Under conditions below the $K_m$ value, the hybrid Dox is able to better exert its higher activity than TolDox. Thus, the elevated activity of the recombinant bacteria expressing the hybrid Dox toward TCE reflects the elevated affinity of the hybrid Dox for TCE.

There are some reports on the irreversible inactivation of monooxygenases (34) and dioxygenases (20, 35). Lee reported that benzene dioxygenase was inactivated via the Fenton-type reaction that formed hydroxyl radicals from the uncoupled reaction of hydrogen peroxide with ferrous mononuclear iron at the catalytic center (35). Li and Wackett (20) reported that TolDox is inactivated via alkylation of the enzyme during TCE degradation. In this study, an irreversibly inactivated component of the hybrid Dox was determined to be ISP not FD or FDR (Fig. 3). Neither cleavage of the peptide bond nor dissociation of the α and β subunits occurred on ISP$_{\text{TodC1BphA2}}$ during TCE oxygenation as judged by the SDS-PAGE and native PAGE (data not shown). Some attempts to understand the mechanism for the TCE inactivation of ISP$_{\text{TodC1BphA2}}$ were carried out using chelating agents and catalase, indicating that the hydroxyl radical caused by the Fenton reaction was not implicated in the TCE inactivation of ISP$_{\text{TodC1BphA2}}$ (data not shown). Therefore, it is likely that the hybrid ISP$_{\text{TodC1BphA2}}$ is inactivated by a fashion similar to the ISP of TolDox (20).

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