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Substrate Specificities of Hybrid Naphthalene and 2,4-Dinitrotoluene Dioxygenase Enzyme Systems

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Bacterial three-component dioxygenase systems consist of reductase and ferredoxin components which transfer electrons from NAD(P)H to a terminal oxygenase. In most cases, the oxygenase consists of two different subunits (α and β). To assess the contributions of the α and β subunits of the oxygenase to substrate specificity, hybrid dioxygenase enzymes were formed by coexpressing genes from two compatible plasmids in *Escherichia coli*. The activities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenases containing four different β subunits were tested with four substrates (indole, naphthalene, 2,4-dinitrotoluene, and 2-nitrotoluene). In the active hybrids, replacement of small subunits affected the rate of product formation but had no effect on the substrate range, regiospecificity, or enantiomeric purity of oxidation products with the substrates tested. These studies indicate that the small subunit of the oxygenase is essential for activity but does not play a major role in determining the specificity of these enzymes.

Bacterial degradation of aromatic compounds under aerobic conditions is often initiated by multicomponent dioxygenase enzyme systems. Since many aromatic compounds are known to be toxic and/or carcinogenic, these bacterial enzymes are important for removing compounds such as benzene, toluene, naphthalene, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and nitroaromatics from the environment. Recently, there has been a great deal of interest in these broad-substrate enzymes for the production of chiral synthons used in the preparation of a wide range of biologically active chemicals and pharmaceuticals, including inositol phosphates, prostaglandins, and antitumor agents (for reviews, see references 5, 7, 24, 35, and 46). The use of these enzymatic routes for the formation of useful products from what were previously considered toxic waste materials is a driving force for the field of "green" chemistry (25).

Two enzyme systems responsible for initiating the degradation of aromatic hydrocarbons are naphthalene dioxygenase (NDO; EC 1.14.12.12) from *Pseudomonas* sp. strain NCIB 9816-4 and toluene dioxygenase (TDO; EC 1.14.12.11) from *Pseudomonas putida* F1. The oxygenase components of NDO and TDO have relaxed substrate specificities, and in addition to catalyzing stereospecific *cis*-dihydroxylation reactions, these enzymes catalyze monooxygenation, desaturation, dealkylation, and sulfoxidation reactions (3, 15, 24, 42, 44, 45). The three enzyme components from each system have been purified and characterized (10, 16, 17, 36, 50, 51), and each set of genes has been cloned, sequenced, and expressed in *Escherichia coli* (39, 47, 61, 62).

Recent studies have identified two new three-component dioxygenase systems involved in the degradation of nitroaromatic compounds: 2-nitrotoluene dioxygenase (2NTDO) from *Pseudomonas* sp. strain JS42 (1, 19, 39), and 2,4-dinitrotoluene dioxygenase (DNTDO) from *Burkholderia* (formerly *Pseudomonas*) sp. strain DNT (18, 48, 55, 56).

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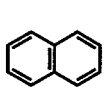
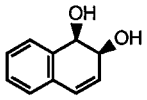
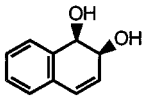
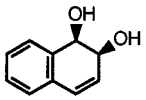
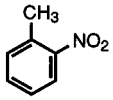
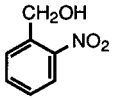
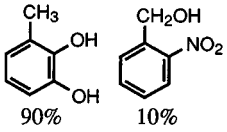
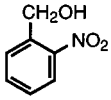
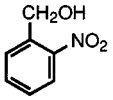
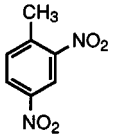
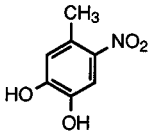
In these three-component enzyme systems, electrons are transferred from NAD(P)H to a flavoprotein reductase, a Rieske [2Fe-2S]-containing ferredoxin, and finally to the terminal oxygenase component, which is also a Rieske iron-sulfur protein. The reduced oxygenase, which was previously designated ISP, catalyzes the stereospecific addition of both atoms of molecular oxygen into the aromatic nucleus of the substrate. As the catalytic portion of the enzyme, the oxygenase determines substrate specificity. The large (α) subunit contains a Rieske [2Fe-2S] center (29, 53) and a mononuclear iron binding site, which is believed to be the site of oxygen activation (30). Several studies have implicated the α subunit in determining substrate specificity (12, 14, 32, 38, 40, 58). The function of the small (β) subunit is not known, although reports in the literature have suggested that the β subunit may be involved in substrate specificity in the toluate and biphenyl dioxygenase systems (13, 21, 23).

It is apparent from deduced amino acid sequence comparisons that the α and β subunits of the oxygenase components of the NDO, 2NTDO, and DNTDO systems are closely related to each other and distantly related to those of the TDO system (39, 55, 61). The α subunit of NDO is 84 and 80% identical to the α subunits of 2NTDO and DNTDO, respectively. The α subunit sequences of 2NTDO and DNTDO are 88% identical. The sequence of the β subunit of NDO is 76 and 78% identical to the 2NTDO and DNTDO β subunits, and the β subunits of 2NTDO and DNTDO are 92% identical. In contrast, the amino acid sequence identities of the TDO α and β subunits to the corresponding subunits of the other three enzymes are in the range of 35 and 25%, respectively. Although NDO, 2NTDO, and DNTDO oxygenase components are very similar in amino acid sequence, each enzyme forms a characteristic set of products from a series of substrates (Table 1). All four enzymes convert naphthalene to *cis*-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol), but the products have different enantiomeric compositions (Table 1). All of the enzymes oxidize 2-nitrotoluene to 2-nitrobenzyl alcohol (45, 55), but 2NTDO forms predominantly 3-methylcatechol from 2-nitrotoluene (19). DNTDO is the only enzyme capable of converting 2,4-dinitrotoluene to 4-methyl-5-nitrocatechol (40, 55). In addition, while NDO and TDO are very effective at converting indole to indigo (11, 62), DNTDO is less effective

TABLE 1. Products formed form diagnostic substrates by wild-type dioxygenases[a]

Substrate	Oxidation products formed by: NDO	2NTDO	DNTDO	TDO
Naphthalene	 ≥99% (+)-*cis*-(1*R*,2*S*)	 70% (+)-*cis*-(1*R*,2*S*)	 96% (+)-*cis*-(1*R*,2*S*)	 ≥99% (+)-*cis*-(1*R*,2*S*)
2-Nitrotoluene	 	 90% 10%		
2,4-Dinitrotoluene	 No products	No products		No products

[a] Data from references 19, 27, 28, 40, 45, 55, and 59.

(55) and 2NTDO carries out the reaction very poorly. These diagnostic substrates allowed us to assess the contributions of the oxygenase α and β subunits to regiospecificity, stereospecificity, and overall substrate range. Hybrid dioxygenases containing various β subunits were generated by using a two-plasmid expression system in *E. coli*. The genes encoding the β subunits from NDO, 2NTDO, DNTDO, and TDO were individually cloned in standard *E. coli* expression vectors. The recombinant plasmids were introduced into *E. coli* strains carrying the reductase, ferredoxin, and α subunit genes from either NDO or DNTDO on a newly constructed compatible expression vector. Following expression in *E. coli*, the hybrid enzymes were tested for the ability to oxidize the four diagnostic substrates naphthalene, 2-nitrotoluene, 2,4-dinitrotoluene, and indole.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 2. The designations for cloned dioxygenase genes are explained in the footnote to Table 2.

Media and growth conditions. *E. coli* strains were grown at 37°C in Luria-Bertani medium (9) or Terrific Broth medium (34). Antibiotics were added to the following final concentrations as appropriate: ampicillin, 150 μg/ml; chloramphenicol, 40 μg/ml; tetracycline, 25 μg/ml. To produce induced cells for biotransformation studies, JM109(DE3) strains carrying plasmids of interest were grown at room temperature (approximately 25°C) in minimal medium (MSB) (49) containing 10 mM glucose, 0.1 mM thiamine, ampicillin, and chloramphenicol. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μg/ml when the culture turbidity reached 0.6 to 0.8 at 660 nm. After a 2.5-h induction period, the cells were harvested by centrifugation. For plates, MSB was solidified with 1.8% Noble Agar (Difco Laboratories, Detroit, Mich.) and Luria-Bertani medium was solidified with 1.5% Bacto Agar (Difco Laboratories).

Molecular techniques. Plasmid DNA was purified by the method of Lee and Rasheed (34). *E. coli* strains were transformed by the method of Hanahan (20). Standard molecular biology techniques were used for the preparation and analysis of subclones (2) with *E. coli* DH5α as the host strain. DNA fragments were purified from gel slices with the GeneClean spin kit as specified by the manufacturer (Bio 101, Vista, Calif.).

Plasmid constructions. A new expression vector, pREP1 (Fig. 1), was constructed as follows. Plasmid pACYC184 was digested with *Cla*I and *Hin*cII to remove the tetracycline resistance gene, and the 0.3-kb *Cla*I-*Pvu*II fragment containing the multiple-cloning site and T7 promoter from pT7-5 was inserted. Plasmid pDTG162 contains *nahAaAbAc* under the control of the T7 promoter in pREP1. It was constructed in a two-step procedure by first deleting the *Sal*I fragment carrying the *nahAd* gene from pDTG141, forming pDTG149, and then inserting the *Sac*I-*Hin*dIII fragment of pDTG149, carrying *nahAaAbAc*, into *Sac*I-*Hin*dIII-digested pREP1 to form pDTG162. Plasmid pDTG953 was formed by insertion of the *Sac*I-*Bam*HI fragment of pJS48 carrying *dntAaAbAc* into the corresponding restriction sites in pREP1. Plasmid pDTG630, a *todC2* expression clone, was constructed in a two-step procedure. First pDTG629 was formed by inserting the 0.6-kb *Eco*RI-*Bsp*HI fragment from pDTG613 into *Eco*RI-*Nco*I-digested pUCBM21. Then the 0.7-kb *Eco*RI-*Pvu*II fragment from pDTG629 was inserted into pT7-7 to form pDTG630. Plasmid pDTG850 was constructed by inserting the 4.7-kb *Sac*I-*Eco*RI fragment of pDTG800 (carrying all of the *ntd* genes) into *Sac*I-*Eco*RI-digested pUC13. The *ntdAd* expression clone, pDTG824, was constructed by inserting the 860-bp *Mfe*I-*Eco*RI fragment of pDTG850 into *Eco*RI-digested pUC18. The *dntAd* expression clone, pDTG951, was formed by digestion of pJS48 with *Hin*dIII followed by self-ligation to delete the 4.3-kb *Hin*dIII fragment containing *dntAa*, ORF2, *dntAb*, and the first one-third of *dntAc*.

Antibody production. Two adult BALB/c AnNHsd mice (Harlan Sprague-Dawley, Indianapolis, Ind.) were immunized with the purified oxygenase component of NDO. Injections (with 115 μg each) were performed subcutaneously on day 1 with the NDO component in Freund's complete adjuvant (Difco Laboratories), subcutaneously on day 21 with the NDO component in Freund's incomplete adjuvant, and intraperitoneally on day 34 with the NDO component in phosphate-buffered saline (12 mM NaK phosphate buffer [pH 7.2], 137 mM NaCl, 2.5 mM KCl). Five days later, the mouse was sacrificed and fusion was carried out by standard procedures (22). Hybridomas were isolated and screened for the production of antibodies specific for the NDO α subunit by enzyme-

TABLE 2. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
DH5α	Δ(*lacZYA-argF*)*U169 hsdR17 relA1 supE44 endA1 recA1 thi-1 gyrA96*, ϕ80d*lacZ*Δ*M15*	Life Technologies, Gaithersburg, Md.
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* Δ(*lac-proAB*) *mcrA* [F′, *traD36 proAB*$^+$ *lacI*^{q}*Z*Δ*M15*]	60
JM109(DE3)	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* Δ(*lac-proAB*) *mcrA* [F′, *traD36*, *proAB*$^+$, *lacI*^{q}*Z*Δ*M15*], λ(DE3)	Promega Corp., Madison, Wis.
Plasmids		
pACYC184	Cm^r Tc^r, p15A origin	8
pT7-5	Ap^r, ColE1 origin, T7 promoter	57
pT7-7	Ap^r, ColE1 origin, T7 promoter, ribosome-binding site	2
pUC13	Ap^r, ColE1 origin, *lac* promoter	37
pUC18	Ap^r, ColE1 origin, *lac* promoter	60
pUCBM21	Ap^r, ColE1 origin, *lac* promoter	Boehringer Mannheim Corp.
pREP1	Cm^r, p15A origin, T7 promoter and MCS from pT7-5	This study
pDTG124	Ap^r, *nahAd* under control of the T7 promoter of pT7-7	54
pDTG141	Ap^r, *nahAaAbAcAd* under control of the T7 promoter of pT7-5	52
pDTG149	Ap^r, *nahAaAbAc* under control of the T7 promoter of pT7-5	This study
pDTG162	Cm^r, *nahAaAbAc* under control of the T7 promoter of pREP1	This study
pDTG601A	Ap^r, *todC1C2BA* in pKK223-3	62
pDTG613	Ap^r, *todC2* in pKK223-3	62
pDTG629	Ap^r, *todC2* in pUCBM21	This study
pDTG630	Ap^r, *todC2* under control of the T7 promoter of pT7-7	This study
pDTG800	Ap^r, *ntdAaAbAcAd* in pUC18	39
pDTG824	Ap^r, *ntdAd* in pUC18	This study
pDTG850	Ap^r, *ntdAaAbAcAd* in pUC13	This study
pDTG951	Ap^r, *dntAd* under control of the T7 promoter of pT7-6	This study
pDTG953	Cm^r, *dntAaAbAc* under control of the T7 promoter of pREP1	This study
pJS48	Ap^r, *dntAaAbAcAd* under control of the T7 promoter of pT7-6	55

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance. The *nah* genes encode NDO from *Pseudomonas* sp. strain NCIB 9816-4, the *ntd* genes encode 2NTDO from *Pseudomonas* sp. strain JS42, and the *dnt* genes encode DNTDO from *Burkholderia* sp. strain DNT. In each set of genes, *Aa* encodes the reductase, *Ab* encodes the ferredoxin, *Ac* encodes the oxygenase α subunit, and *Ad* encodes the oxygenase β subunit. The *tod* genes encode toluene dioxygenase from *P. putida* F1. *todA* encodes the reductase, *todB* encodes the ferredoxin, *todC1* encodes the oxygenase α subunit, and *todC2* encodes the oxygenase β subunit.

linked immunosorbent assay (22). One hybridoma that secreted a monoclonal antibody with a strong reaction in Western blot analyses with α_{NDO} was cloned twice by limiting dilution. The isotype was found to be immunoglobulin G2b by using an Isostrip kit (Boehringer Mannheim Corp., Indianapolis, Ind.). At the time of sacrifice, blood was obtained from the mouse by cardiac puncture, and a 1:10,000 dilution of the polyclonal serum showed a strong reaction by enzyme-linked immunosorbent assay with the NDO α and β subunits. Monoclonal antibody 301β, which was raised against the β subunit of TDO, was described previously (36).

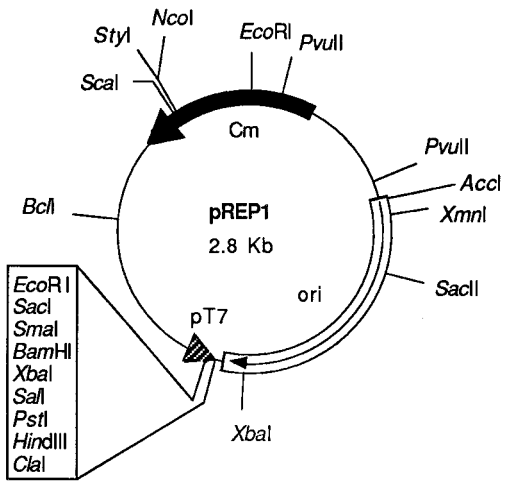


FIG. 1. Circular map of expression vector pREP1, a derivative of pACYC184. Cm, chloramphenicol resistance gene, pT7, T7 promoter from vector pT7-5.

Indigo formation. JM109(DE3) strains carrying plasmids of interest were grown overnight at 37°C on nitrocellulose filters placed on MSB agar plates containing glucose, thiamine, ampicillin, and chloramphenicol. Dried Whatman no. 1 filter papers that had been soaked in a 10% solution of indole dissolved in acetone were placed in the petri dish covers after colony formation. Production of indigo from indole vapor was observed as the colonies turned blue. No induction was carried out for these studies.

Whole-cell biotransformations. Induced cultures (1 liter) were harvested by centrifugation and resuspended in MSB containing 10 mM glucose (125 ml, final turbidity of 5.0 to 6.0 at 660 nm). Small samples of resuspended cells (four 1-ml samples) were harvested by centrifugation and stored at −20°C for protein determinations and gel electrophoresis. To initiate biotransformation reactions, cell suspensions (40 ml) were added to flasks containing 0.1% (vol/vol) 2-nitrotoluene, 0.1% (wt/vol) naphthalene, or 0.1% (wt/vol) 2,4-dinitrotoluene. Solid substrates were dissolved in acetone, and the acetone was evaporated to leave a thin coating of substrate in the flasks. Cultures were incubated at 30°C with shaking (250 rpm) for up to 24 h. Samples (0.5 ml) were taken periodically, and cells were removed by centrifugation. Culture supernatants were stored at −20°C until analyzed. After 24 h, the cells were removed from the remaining cultures by centrifugation and the culture supernatants were extracted as described below.

Separation and identification of products. High-performance liquid chromatography (HPLC) was used to quantify *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol), 2-nitrobenzyl alcohol, and 4-methyl-5-nitrocatechol in aqueous samples. HPLC analyses were performed with a Waters Corp. (Milford, Mass.) HPLC system equipped with a 600E solvent delivery system, U-6K injector, model 991 photo diode array detector, and Millennium Chromatography Manager software. Metabolites were separated on a Beckman Instruments, Inc. (Fullerton, Calif.) Ultrasphere reverse-phase column (4.6 mm by 25 cm) with a methanol-water mobile phase. Elution was carried out with a linear gradient increasing from 20 to 100% methanol over a 15-min period at a flow rate of 1 ml/min. HPLC analyses for 4-methyl-5-nitrocatechol were performed as described above, except that the water was acidified with trifluoroacetic acid (0.1%, vol/vol). Culture supernatants from 24-h incubations were extracted with sodium hydroxide-washed ethyl acetate and analyzed by thin-layer chromatography (43). All extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (44). *cis*-Naphthalene dihydrodiol was purified

TABLE 3. Two-plasmid expression system for wild-type and hybrid dioxygenase production

Dioxygenase designation	Plasmids[a] present in JM109(DE3)		Dioxygenase genes present in:	
	Plasmid A	Plasmid B	Plasmid A	Plasmid B
NDO	pDTG162	pDTG124	*nahAaAbAc*	*nahAd*
NDO-β_{2NTDO}	pDTG162	pDTG824	*nahAaAbAc*	*ntdAd*
NDO-β_{DNTDO}	pDTG162	pDTG951	*nahAaAbAc*	*dntAd*
NDO-β_{TDO}	pDTG162	pDTG630	*nahAaAbAc*	*todC2*
NDO-β_0	pDTG162	pT7-7	*nahAaAbAc*	None
DNTDO	pDTG953	pDTG951	*dntAaAbAc*	*dntAd*
DNTDO-β_{2NTDO}	pDTG953	pDTG824	*dntAaAbAc*	*ntdAd*
DNTDO-β_{NDO}	pDTG953	pDTG124	*dntAaAbAc*	*nahAd*
DNTDO-β_{TDO}	pDTG953	pDTG630	*dntAaAbAc*	*todC2*
DNTDO-β_0	pDTG953	pT7-7	*dntAaAbAc*	None
Vector control	pREP1	pT7-7	None	None

[a] In each case, plasmid A is a derivative of pREP1 (Cm^r) carrying the genes encoding reductase, ferredoxin, and oxygenase α subunit and plasmid B is a ColE1 derivative (Ap^r) carrying the oxygenase β subunit gene. See Table 2 and Materials and Methods for details of plasmid constructions and gene designations.

for chiral HPLC analysis by preparative-layer chromatography (44). Chiral stationary-phase liquid chromatography was used to resolve the two enantiomers of *cis*-naphthalene dihydrodiol with a Chirocel OJ column (Chiral Technologies, Exton, Pa.) as described previously (44). Under these conditions, the (+)-(1*R*,2*S*) and (−)-(1*S*,2*R*) enantiomers of *cis*-naphthalene dihydrodiol eluted with retention times of 30 and 33 min, respectively. Product identifications were based on comparisons to standards.

Chemicals. Naphthalene was obtained from Fisher Scientific Co., Pittsburgh, Pa. Indole, 2-nitrotoluene, 2,4-dinitrotoluene, and 2-nitrobenzyl alcohol were purchased from Aldrich Chemical Co., Milwaukee, Wis. 4-Methyl-5-nitrocatechol was a gift from Jim C. Spain. Synthetic (+/−)-*cis*-naphthalene dihydrodiol and homochiral (+)-*cis*-naphthalene dihydrodiol were prepared as previously described (26, 41).

Gel electrophoresis and Western blot analyses. Cell pellets (from 1-ml suspensions) were resuspended in 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer (2) and boiled for 10 min, and the proteins were separated on duplicate sodium dodecyl sulfate–12% polyacrylamide gels (2). One gel was stained with Coomassie blue R-250 to verify that approximately equal amounts of protein were loaded in each lane. The second gel was subjected to Western blotting as described previously (22, 36). Antigens were visualized with alkaline phosphatase conjugated goat anti-mouse immunoglobulin G (Pierce, Rockford, Ill.).

Protein determinations. Cell pellets (from 1-ml suspensions) were resuspended in 0.1 M sodium hydroxide and boiled for 1 h. Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as the standard.

RESULTS

Construction of the two plasmid expression system. A new expression vector, pREP1, was constructed for use in this study (Fig. 1). This plasmid, a derivative of pACYC184, carries a chloramphenicol resistance gene, a T7 promoter, and a multiple-cloning site and is compatible with ColE1 plasmids. In this study, the genes encoding the reductases, ferredoxins, and α subunits from the NDO and DNTDO systems were expressed from pREP1 derivatives (pDTG162 and pDTG953, respectively) in JM109(DE3). Genes encoding β subunits were coexpressed from compatible ColE1 plasmids (Table 2). All genes were inducible either directly or indirectly with IPTG. The gene encoding the NDO β subunit, *nahAd*, was directly inducible since it is under the control of the *lac* promoter in pDTG824, a pUC18 derivative. All other genes were under the control of the T7 promoter in plasmids carried in JM109 (DE3), a strain that has an IPTG-inducible T7 polymerase gene inserted in the chromosome. Six hybrid enzymes, two wild-type enzymes, and control enzymes containing no β subunit were produced with the two-plasmid expression system (Table 3).

Expression of cloned dioxygenase genes. A monoclonal antibody specific for the α subunit of NDO reacted with the α subunits of NDO, DNTDO, and 2NTDO but not TDO in crude cell extracts (Fig. 2). Similar results were obtained when purified oxygenase components of NDO, 2NTDO, and TDO were analyzed by Western blotting (data not shown). Use of this antibody demonstrated that the α subunits of NDO and DNTDO were produced by all recombinant strains except control JM109(DE3) carrying the two vectors only (Fig. 3A). Polyclonal antiserum raised against NDO reacted with the α subunits of NDO and DNTDO and also with the β subunit of NDO (Fig. 3B, lanes 1 and 8). This polyclonal antiserum was used to verify the production of the β subunit of NDO (lanes 2 and 9). The polyclonal antibody also reacted with a nonspecific *E. coli* protein present in all crude cell extracts (lanes 2 to 7 and 9 to 14). Monoclonal antibody 301β, which was raised against the β subunit of TDO (36), was used to demonstrate the presence of the TDO β subunit in extracts (Fig. 3C). These results show that all oxygenase α and β subunits were present in *E. coli* extracts with the exception of the β subunits of 2NTDO and DNTDO. Antibodies for the detection of these two β subunits were not available.

Indigo formation. One rapid qualitative measure of dioxygenase activity is the conversion of indole to indigo. The ability to form indigo by *E. coli* strains expressing wild-type and hybrid dioxygenases was tested on agar plates. While NDO and TDO have been shown to efficiently convert indole to indigo (11, 62), DNTDO does so less efficiently (55) and 2NTDO only forms

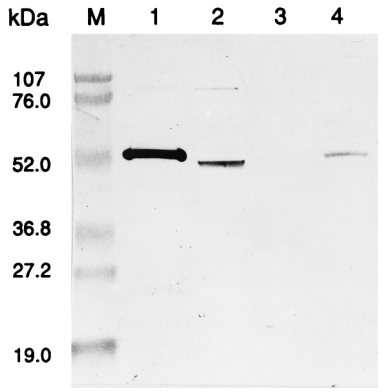


FIG. 2. Western blot analysis of crude cell extracts containing various oxygenase components (see Tables 2 and 3 for details of strains). The monoclonal antibody used was specific for α_{NDO} (see Materials and Methods). M, molecular mass standards. Lanes: 1, JM109(DE3)(pDTG162)(pDTG124) expressing NDO; 2, DH5α(pDTG800) expressing 2NTDO; 3, JM109(pDTG601A) expressing TDO; 4, JM109(DE3)(pDTG953)(pDTG951) expressing DNTDO.

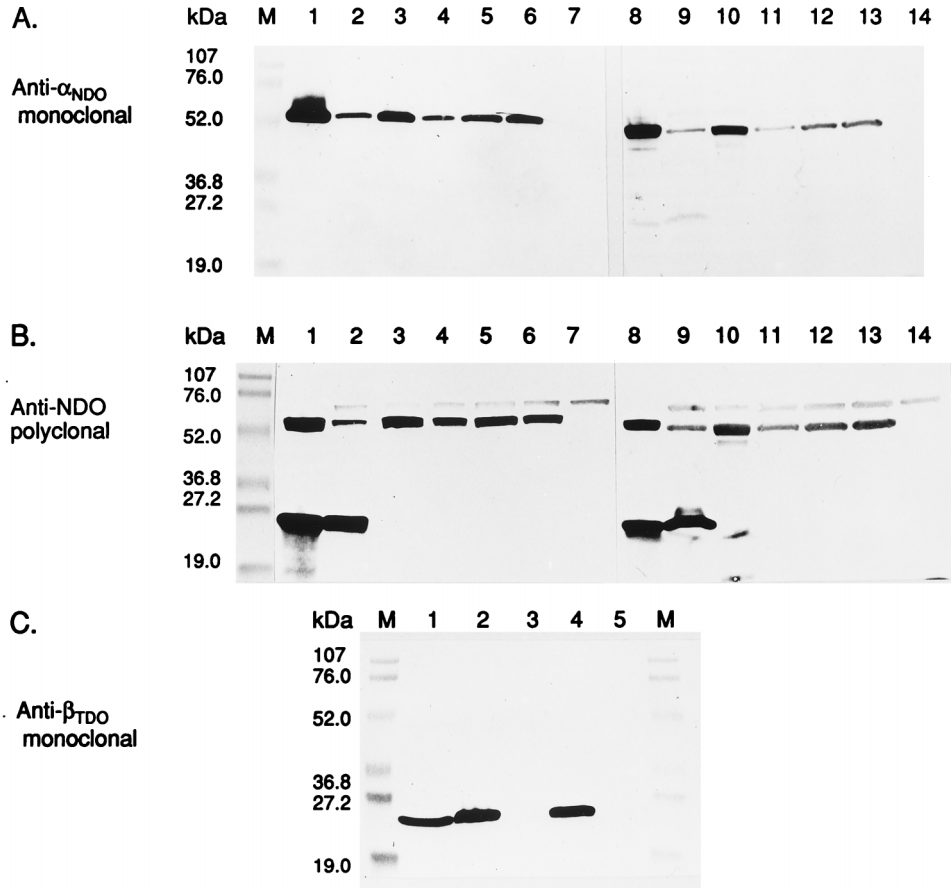


FIG. 3. Western blot analysis of crude cell extracts of strains producing hybrid dioxygenases (described in Table 3). (A) Monoclonal antibody was specific for α_{NDO}. (B) Polyclonal antibody was obtained from a mouse immunized with purified NDO. (C) Monoclonal antibody 301β was specific for β_{TDO} (36). M, molecular mass standards. (A and B) Lanes: 1 and 8, purified NDO (3.2 μg each); 2, NDO; 3, NDO-β_{2NTDO}; 4, NDO-β_{DNTDO}; 5, NDO-β_{TDO}; 6, NDO-β_0; 7, vector control; 9, DNTDO-β_{NDO}; 10, DNTDO-β_{2NTDO}; 11, DNTDO; 12, DNTDO-β_{TDO}; 13, DNTDO-β_0; 14, vector control. (C) Lanes: 1, purified TDO (5.0 μg); 2, NDO-β_{TDO}; 3, NDO-β_0; 4, DNTDO-β_{TDO}; 5, DNTDO-β_0.

trace amounts. The formation of blue colonies by JM109(DE3)(pDTG162)(pDTG824), and JM109(DE3)(pDTG162)(pDTG951), which produce NDO-β_{2NTDO} and NDO-β_{DNTDO}, respectively, was the first indication that these hybrid enzymes were active. None of the other hybrid enzyme-producing strains turned blue. Control strains expressing wild-type NDO and DNTDO formed blue colonies. Strains that lacked a small subunit or contained only vectors remained white.

***cis*-Naphthalene dihydrodiol formation.** Naphthalene was used to test hybrid enzyme activity, since NDO, TDO, 2NTDO, and DNTDO each catalyze the conversion of naphthalene to *cis*-naphthalene dihydrodiol. The specific activities of wild-type and hybrid enzymes with naphthalene as the substrate are shown in Table 4. Since 2NTDO was produced from a single plasmid, its specific activity cannot be directly compared to the specific activities of the other enzymes. These activities are consistently lower (5- to 10-fold) than are the activities in strains carrying single expression plasmids. One possible reason is that the four genes are no longer coordinately regulated from the same DNA fragment and coupled translation of the α and β subunits cannot occur. A second possibility is that differences in plasmid copy number result in the formation of unequal amounts of α and β gene products. The amounts of *cis*-naphthalene dihydrodiol formed in 5-h biotransformations are shown in Table 5. NDO-β_{TDO}, DNTDO-β_{TDO} and DNTDO-β_{NDO} did not form detectable amounts of *cis*-naphthalene dihydrodiol even after prolonged incubation (24 h) as judged by HPLC or GC-MS analyses. *cis*-Naphthalene dihydrodiol was not formed by control strains that did not contain a β subunit gene.

Stereochemistry of *cis*-naphthalene dihydrodiol formed by hybrid dioxygenases. The stereochemistry of the *cis*-naphthalene dihydrodiol formed by hybrid dioxygenases was determined by chiral stationary-phase HPLC. NDO forms enantiomerically pure (+)-(1*R*,2*S*)-*cis*-naphthalene dihydrodiol from naphthalene (27, 28). DNTDO and 2NTDO form characteristic ratios of the (+) and (−) enantiomers of *cis*-naphthalene dihydrodiol (40, 55). The results obtained with hybrid enzymes indicated that the β subunit does not play a role in determining the enantiomeric purity of the *cis*-naphthalene dihydrodiol formed (Table 4).

Products formed from 2-nitrotoluene and 2,4-dinitrotoluene. While all of the wild-type oxygenases in this study are capable of forming 2-nitrobenzyl alcohol from 2-nitrotoluene (33, 45, 55), only 2NTDO forms 3-methylcatechol from this substrate (19). Two NDO hybrid enzymes (NDO-β_{2NTDO} and NDO-β_{DNTDO}) formed 2-nitrobenzyl alcohol from 2-nitrotoluene (Table 5) but formed no 3-methylcatechol as judged by HPLC and GC-MS analyses. No oxidation products were formed from 2-nitrotoluene by the other hybrid enzymes or by the control strains carrying no β subunit gene (Table 5). From

TABLE 4. Comparison of enzyme activities and enantiomeric composition of *cis*-naphthalene dihydrodiol formed from naphthalene by wild-type and hybrid dioxygenases

Dioxygenase[a]	Sp act[b] (μg min^{-1} mg^{-1})	Enantiomeric composition [% (+)-(1*R*,2*S*)-*cis*-naphthalene dihydrodiol]
Wild-type enzymes		
NDO	2.6	≥99
2NTDO	ND[c]	70
DNTDO	0.38	96
Hybrid enzymes		
NDO-β_{2NTDO}	1.3	≥99
NDO-β_{DNTDO}	0.37	≥99
DNTDO-β_{2NTDO}	0.08	97

[a] All wild-type and hybrid enzymes except 2NTDO were generated by using the two-plasmid system (Table 3). Data presented are from one of at least two similar experiments.
[b] Micrograms of *cis*-naphthalene dihydrodiol formed per minute per milligram of protein in whole-cell biotransformations over a 2.5-h period where the rates were linear.
[c] ND, not determined.

these results, the β subunit does not appear to determine the position of attack (regiospecificity) on 2-nitrotoluene, since neither NDO-β_{2NTDO} nor DNTDO-β_{2NTDO} was capable of converting 2-nitrotoluene to 3-methylcatechol.

Of the wild-type oxygenases, only DNTDO can catalyze the conversion of 2,4-dinitrotoluene to 4-methyl-5-nitrocatechol. Of the hybrid enzymes, only DNTDO-β_{2NTDO} was capable of this conversion (Table 5). These results suggest that the α subunit confers the ability to oxidize 2,4-dinitrotoluene to 4-methyl-5-nitrocatechol, since DNTDO-β_{2NTDO} was capable of carrying out this reaction and NDO-β_{DNTDO} was not.

TABLE 5. Product formation[a] by wild-type and hybrid dioxygenases

Dioxygenase	Amt (μg/ml) of:		
	cis-Naphthalene dihydrodiol from NAP	2-Nitrobenzyl alcohol from 2NT[b]	4-Methyl-5-nitrocatechol from DNT
NDO	800	33.4	—[c]
NDO-β_{2NTDO}	279	35.4	—
NDO-β_{DNTDO}	113	8.7	—
NDO-β_{TDO}	—	ND[d]	ND
NDO-β_0	—	—	—
DNTDO	96.9	Trace[e]	18.8
DNTDO-β_{2NTDO}	32.3	—	4.9
DNTDO-β_{NDO}	—	—	—
DNTDO-β_{TDO}	—	ND	ND
DNTDO-β_0	—	—	—

[a] Products formed from naphthalene (NAP), 2-nitrotoluene (2NT) and 2,4-dinitrotoluene (DNT) were detected and quantified by HPLC after 5-h biotransformations. Data presented are from one of at least two similar experiments.
[b] No 3-methylcatechol was detected in any 2NT biotransformations, although control DH5α(pDTG800), which produces wild-type 2NTDO, formed 3-methylcatechol and 2-nitrobenzyl alcohol in a 9:1 ratio.
[c] —, no products were detected by HPLC analysis of 5-h aqueous samples or by GC-MS after extraction and concentration of 24-h biotransformation samples.
[d] ND, not determined.
[e] Detected by GC-MS after extraction and concentration of 24-h biotransformation samples. Not detected in aqueous samples by HPLC, where the limits of detection for *cis*-naphthalene dihydrodiol, 2-nitrobenzyl alcohol, and 4-methyl-5-nitrocatechol were each approximately 2 μg/ml.

DISCUSSION

The oxygenase subunits of three of the four dioxygenases used in this study are very similar in amino acid sequence, yet these enzyme systems have significant and easily measurable differences in substrate specificity. These characteristics have made the study of hybrid enzymes useful in assessing the role of the β subunit in substrate specificity. Of the six hybrids constructed, three were active. The hybrid enzymes NDO-β_{TDO} and DNTDO-β_{TDO} were not active with any of the substrates tested. This was not surprising since β_{TDO} is only 22 and 24% identical in amino acid sequence to β_{NDO} and β_{DNTDO}, respectively. It is possible that the structure of β_{TDO} differs from those of β_{NDO} and β_{DNTDO} and that for this reason it cannot interact effectively with the α subunits of either NDO or DNTDO to form an active oxygenase. It is interesting that DNTDO-β_{NDO} was not functional but DNTDO-β_{2NTDO} was quite active (Table 5), especially since β_{NDO} is slightly more similar in sequence to β_{DNTDO} than to β_{2NTDO}. Western blot analyses showed that the α and β subunits of the terminal oxygenase were present in cell extracts of strains expressing all three inactive hybrids. Therefore, the absence of enzymatic activity was not due to problems in protein production. We were unable to test for production of reductase and ferredoxin in the inactive strains, but presumably these proteins were produced, since control wild-type and active hybrid enzymes were generated with the same plasmid constructs. With the two-plasmid expression system used, the only difference between the two sets of strains for production of hybrid NDO and DNTDO enzymes was in the plasmid carrying the β subunit gene.

The α subunits of various dioxygenases are important in controlling substrate specificity. Although the oxygenase components of biphenyl dioxygenase from strains KF707 and LB400 are very similar (20 and 1 amino acid differences in the α and β subunits, respectively), the two enzymes have very different polychlorinated-biphenyl congener specificities (14). Detailed studies have indicated that the α subunits are responsible for these differences in substrate specificity (12, 32, 38).

Reports in the literature have suggested that the β subunit may play a role in determining substrate specificity (13, 21, 23). In one study, a mutation that resulted in a broad substrate toluate dioxygenase mapped to the β subunit gene (21). However, a detailed characterization of this mutant has not been reported. In another series of studies, hybrid biphenyl dioxygenase enzymes in which the biphenyl dioxygenase β subunit was replaced with the toluene dioxygenase β subunit were constructed. The resulting enzyme was reported to have an extended substrate range which included the ability to convert benzene and toluene to the corresponding *cis*-dihydrodiols (13, 23). It appeared that these hybrid enzymes formed more stable quaternary complexes than did wild-type biphenyl dioxygenase (23). In a similar study, a more sensitive assay indicated that wild-type biphenyl dioxygenase did have the ability to oxidize benzene, and the investigators concluded that the α subunit alone was critical for substrate specificity (58). Taken together, these results suggest that the hybrid enzymes might have been slightly more active due to improved enzyme stability and that the β subunit was not actually conferring new catalytic activities. Recent work in our laboratory indicated that the substrate specificity of 2NTDO was not affected when its β subunit was replaced by the β subunit from DNTDO (40). The present study indicates that the α subunit alone determines substrate specificity and the β subunit does not contribute significantly to any aspect of specificity, including substrate range, regiospecificity, and stereospecificity.

These results leave us still searching for a function for the β subunit. In a recent review article, the authors suggested that the β subunit of the benzene dioxygenase terminal component might be involved in ferredoxin docking and electron transfer, based on results of unpublished cross-linking studies (6). However, work with the closely related toluene dioxygenase system showed that the Rieske center in the purified TDO α subunit could be enzymatically reduced in the presence of NADH and catalytic amounts of reductase$_{TOL}$ and ferredoxin$_{TOL}$, indicating that the β subunit is not required for electron transfer from ferredoxin$_{TOL}$ (29). However, the β subunit was required for product formation, and active TDO could be readily reconstituted from separately purified α and β subunits.

The function of the small subunit may be primarily structural. The crystal structure of NDO (31) indicates that the β subunit does not appear to be located at the predicted active site and that only a small portion of the β subunit interacts with the Rieske center domain of the α subunit. From the crystal structure and analytical ultracentrifugation analyses, the native conformation of NDO was found to be an $\alpha_3\beta_3$ hexamer (31). The three β subunits may function to hold the three α subunits in place. This conformation is apparently essential, since the active site appears to be located at the junction between adjacent α subunits (31).

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