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Desaturation, Dioxygenation, and Monooxygenation Reactions Catalyzed by Naphthalene Dioxygenase from *Pseudomonas* sp. Strain 9816-4

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The stereospecific oxidation of indan and indene was examined with mutant and recombinant strains expressing naphthalene dioxygenase of *Pseudomonas* sp. strain 9816-4. *Pseudomonas* sp. strain 9816/11 and *Escherichia coli* JM109(DE3)[pDTG141] oxidized indan to (+)-(1*S*)-indanol, (+)-*cis*-(1*R*,2*S*)-indandiol, (+)-(1*S*)-indenol, and 1-indanone. The same strains oxidized indene to (+)- *cis*-(1*R*,2*S*)-indandiol and (+)-(1*S*)-indenol. Purified naphthalene dioxygenase oxidized indan to the same four products formed by strains 9816/11 and JM109(DE3)[pDTG141]. In addition, indene was identified as an intermediate in indan oxidation. The major products formed from indene by purified naphthalene dioxygenase were (+)-(1*S*)-indenol and (+)-(1*R*,2*S*)-indandiol. The results show that naphthalene dioxygenase catalyzes the enantiospecific monooxygenation of indan to (+)-(1*S*)-indanol and the desaturation of indan to indene, which then serves as a substrate for the formation of (+)-(1*R*,2*S*)-indandiol and (+)-(1*S*)-indenol. The relationship of the desaturase, monooxygenase, and dioxygenase activities of naphthalene dioxygenase is discussed with reference to reactions catalyzed by toluene dioxygenase, plant desaturases, cytochrome P-450, methane monooxygenase, and other bacterial monooxygenases.

Pseudomonas sp. strain 9816-4 grows with naphthalene as the sole source of carbon and energy (9). The initial reaction is catalyzed by a multicomponent enzyme system designated naphthalene dioxygenase (NDO) (11, 12, 23, 24). NDO catalyzes the NAD(P)H-dependent enantiospecific incorporation of dioxygen into naphthalene to form (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (26, 27) (Fig. 1). An analogous reaction is catalyzed by toluene dioxygenase (TDO) from *Pseudomonas putida* F1, where enantiomerically pure (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) is the first detectable oxidation product (17, 31, 60). TDO also catalyzes the enantiospecific oxidation of naphthalene to (+)-*cis*-naphthalene dihydrodiol (18, 39).

In addition to the enantiospecific oxidation of naphthalene and toluene, NDO and TDO from the above strains oxidize many related aromatic compounds to optically active dihydrodiols (10, 18, 28, 30). Other bacterial dioxygenases show similar properties, and more than 130 chiral arene *cis*-dihydrodiols have been produced from a small number of strains (7, 35, 48). The high enantiomeric purity of these compounds has led to their use as chiral synthons in the enantiospecific synthesis of a wide variety of biologically active natural products (7, 8, 46, 57). The present studies focus on another facet of this interesting group of dioxygenases, that is, their ability to catalyze reactions other than the formation of arene *cis*-dihydrodiols. For example, the TDO expressed by *P. putida* F39/D oxidizes indan to (1*R*)-indanol and oxidizes indene to *cis*-(1*S*,2*R*)-indandiol and (1*S*)-indenol (55). Similar reactions have been reported for TDO from *P. putida* UV4, although the 1-indenol produced by this strain is the (1*R*)-enantiomer (3, 5).

We now report the identification and absolute stereochemistry of the products formed from indan and indene by NDO from *Pseudomonas* sp. strain 9816-4 and confirm earlier observations on the desaturation of indan to indene by NDO (22).

MATERIALS AND METHODS

Organisms. *Pseudomonas* sp. strain 9816/11 is a mutant which oxidizes naphthalene stoichiometrically to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (40). This organism is a derivative of *Pseudomonas* sp. strain 9816-4 (9, 59), which harbors the genes for naphthalene catabolism on an 83-kb NAH plasmid designated pDTG1 (45). *Pseudomonas* sp. strain 9816/C84, a cured strain, was used as a control in experiments with strain 9816/11. *Escherichia coli* strain JM109 (DE3)[pDTG141] contains the structural genes (*nahAaAbAcAd*) for NDO in plasmid pT7-5 (50). Expression of NDO in this strain is inducible by the addition of isopropylthiogalactopyranoside (IPTG). *E. coli* JM109(DE3)[pT7-5] was used as a control in experiments with strain JM109(DE3)[pDTG141].

Biotransformation experiments. Strain 9816/11 was grown at 30°C in mineral salts basal medium (MSB) (49) with 0.2% (wt/vol) pyruvate as a carbon source in the presence of 0.05% (wt/vol) salicylate or anthranilate. These aromatic acids induce the synthesis of naphthalene catabolic enzymes in strain 9816 (2). Transformations of indan and indene, except where noted otherwise, were conducted with washed cell suspensions (turbidity, 2.0 to 2.5 at 600 nm) in 200 or 800 ml of 50 mM sodium-potassium phosphate buffer (pH 7.2) in 1.0-liter Erlenmeyer or 2.8-liter Fernbach flasks, respectively. Pyruvate (0.2%, wt/vol) was provided as an energy source, and indan or indene was added at a concentration of 0.05% (vol/vol). The cell suspensions were incubated on a rotary shaker (220 rpm) at 30°C, and at designated times, cells were removed by centrifugation, and the supernatant solutions were extracted three times with sodium hydroxide-washed ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure (30°C) prior to analysis of transformation products.

E. coli JM109(DE3)[pDTG141] was grown in a 5.0-liter Bioflow II Fermentor

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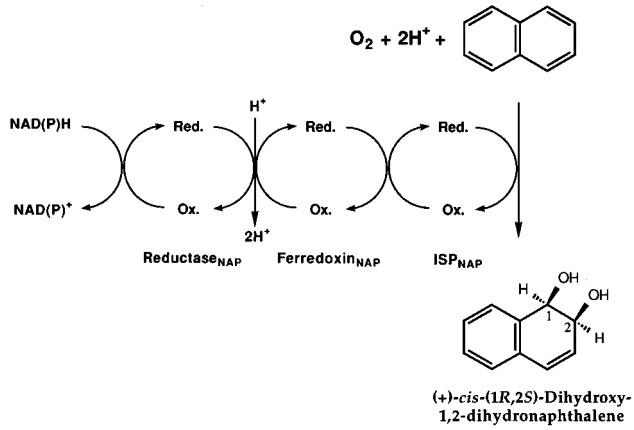

FIG. 1. Sequence of electron transfer from NAD(P)H to the oxygenase component (ISP_{NAP}) of NDO, resulting in the formation of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. The individual components $reductase_{NAP}$, $ferredoxin_{NAP}$, and ISP_{NAP} have been purified (11, 23, 24), and their structural genes have been cloned and sequenced (38, 47). The redox state of each protein is indicated as reduced (Red.) and oxidized (Ox.).

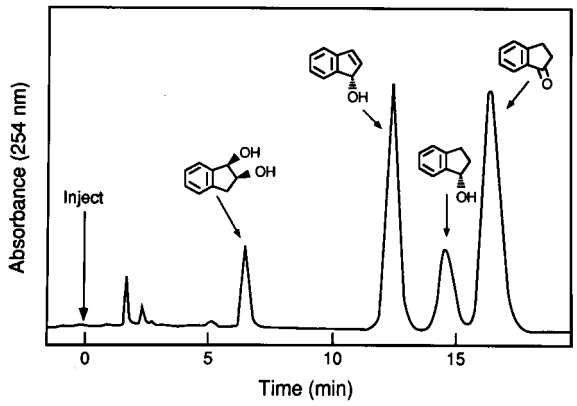

FIG. 2. HPLC separation of products formed from indan by strain 9816/11. Cells were grown as described in Materials and Methods. Products were separated by reverse-phase HPLC on a radial-compression 10-μm C-18 column. The solvent used was methanol-water (36:64) at a flow rate of 1.9 ml/min.

(New Brunswick, Inc.) as previously described (42). IPTG-induced cells were incubated with indan and indene as described above except that glucose (0.2%, wt/vol) was provided as the energy source and indan and indene were provided at 0.1 and 0.025% (vol/vol), respectively. Unused cells were stored at −70°C.

Oxidation of indan to indene by purified NDO. The individual components of NDO ($reductase_{NAP}$, $ferredoxin_{NAP}$, and ISP_{NAP}) were purified to homogeneity as described previously (11, 23, 24). Reactions were carried out in 2.34 ml of 50 mM Tris-HCl buffer, pH 7.5, in 15-ml conical glass tubes. Reaction mixtures contained $reductase_{NAP}$ (73 μg of protein), $ferredoxin_{NAP}$ (48 μg of protein), ISP_{NAP} (860 μg of protein), NADH (5.0 μmol), flavin adenine dinucleotide (5.0 nmol), and indan (3.38 μmol). Reactions were initiated by the addition of indan and were conducted at room temperature for 10 min. At this time, reaction mixtures were extracted twice with diethyl ether. The organic extract was dried over anhydrous sodium sulfate and concentrated to a small volume prior to analysis by high-pressure liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). Reverse-phase HPLC was conducted with a Beckman Ultrasphere 5-μm C-18 column, and separation of products was achieved with a programmed linear gradient of methanol-water (50 to 95% methanol) at a flow rate of 1.0 ml/min (see Fig. 3).

Oxidation of indan, indene, and (1*S*)-indanol by purified NDO. The experiments described in Table 3 were conducted with ISP_{NAP} purified from *E. coli* JM109(DE3)[pDTG121] as recently described by Suen and Gibson (52). $Reductase_{NAP}$ and $ferredoxin_{NAP}$ were purified from *E. coli* JM109(DE3)[pDTG141] (32). Transformation reactions were carried out in 15-ml conical polyethylene tubes. Reaction mixtures contained, in 2.0 ml of 50 mM 2-(4-morpholino)-ethanesulfonic acid (MES) buffer (pH 6.8), $reductase_{NAP}$ (20 μg of protein), $ferredoxin_{NAP}$ (70 μg of protein), ISP_{NAP} (50 μg of protein), ferrous ammonium sulfate (0.05 μmol), NADH (0.25 μmol), and indan, indene, or (1*S*)-indanol (0.5 μmol in 20 μl of methanol). Reactions were initiated by addition of substrate, and each tube was capped and incubated horizontally at 23°C with gentle agitation (~60 rpm) for 1 h. Oxidation products were extracted with ethyl acetate as described above prior to analysis by GC/MS.

Analytical procedures. Thin-layer chromatography (TLC) and preparative-layer chromatography (1.0- or 2.0-mm silica thickness; Merck) were conducted as described previously (43). Radial-dispersion chromatography was performed on 2.0-mm-thickness silica plates, and products were eluted with a chloroform-acetone step gradient (0 to 40% acetone; 20% steps over 1 h) at a flow rate of 7.0 ml/min. All fractions were analyzed by TLC (solvent: chloroform-acetone, 80:20, vol/vol), and those containing products were combined for further characterization. Open-column silica gel chromatography was used where indicated in the text. GC-MS was conducted as described previously (43). Relative yields of products were determined from the integration of their total ion current peak areas. Proton ^1H nuclear magnetic resonance (^1H NMR) spectra, absorption spectra, and optical rotation values were obtained as described previously (43). Reported $[\alpha]_D$ values were determined in chloroform at 25°C.

Chiral stationary-phase HPLC was conducted as described previously (43). Enantiomers of *cis*-1,2-indandiol were separated on a Chiralcel OJ column (25 cm by 4.6 mm; Chiral Technologies, Exton, Pa.) with a mobile phase of hexane and 2-propanol (9:1) at a flow rate of 0.5 ml/min. Under these conditions, the (+)-*cis*-(1*R*,2*S*)- and (−)-*cis*-(1*S*,2*R*)-enantiomers of indandiol eluted with retention times of 18.5 and 23.6 min, respectively. 1-Indenol enantiomers were separated on a Chiralcel OB-H column (25 cm by 4.6 mm) under identical conditions, with (−)-(1*R*)-indenol and (+)-(1*S*)-indenol eluting at 15.1 and 25.3 min, respectively. The Chiralcel OB-H column also resolved (−)-(1*R*)-indanol and (+)-(1*S*)-indanol, which eluted at 11.8 and 17.4 min, respectively.

Chemicals. Indan, indene, (+)-(1*S*)-indanol, (−)-(1*R*)-indanol, 1-indanone, 1,3-indandione, indole, indoline, and 2,3-dihydrobenzofuran were obtained from Aldrich Chemical Company, Milwaukee, Wis. Indan, free from contaminating indene, was obtained by reverse-phase HPLC on a semipreparative Waters C-18 column. The solvent was 70% methanol, and the flow rate was 2.5 ml/min. Under these conditions, indene elutes at 15 min and indan elutes at 22 to 23 min. The middle fractions containing pure indan were pooled to give a stock indan solution (13 mM) for enzyme experiments. Racemic *cis*-1,2-indandiol was prepared by treatment of indene with osmium tetroxide. *trans*-1,3-Indandiol was prepared by reducing 1,3-indandione with sodium borohydride. (−)-*cis*-(1*S*,2*R*)-Indandiol was prepared by reacting (±)-*cis*-indandiol with (−)-menthoxyacetyl chloride. The crude diesters were purified by silica gel chromatography and recrystallized from methanol. Direct hydrolysis of the first crystals obtained gave (−)-*cis*-(1*S*,2*R*)-indandiol (mp, 105 to 107°C; $[\alpha]_D$, 50.4°) (25).

RESULTS

Oxidation of indan by strains 9816/11 and JM109(DE3) [pDTG141]. When a suspension of anthranilate-induced cells of 9816/11 was incubated overnight with indan, several oxidation products were detected by TLC. The major product was identified as 1-indanone based on its R_f value, HPLC retention time, absorption spectrum, and the properties of its semicarbazone derivative. Minor products, tentatively identified by TLC and HPLC, were 1-indanol, 1-indenol, and 1,2-indandiol. In addition, several unidentified minor polar peaks were observed.

In order to identify primary indan oxidation products, the experiment was repeated, and product formation was determined after 1 h. Four major metabolites were detected by HPLC (Fig. 2) and identified as *cis*-1,2-indandiol, 1-indenol, 1-indanol, and 1-indanone by comparing their HPLC retention times and absorption spectra with those of authentic compounds. Sufficient material for further structural studies was obtained by incubating a 2.4-liter suspension of induced cells with indan for 1 h. Cells were removed by centrifugation, and ethyl acetate extraction of the clear supernatant solution followed by silica gel column chromatography gave crude preparations of *cis*-1,2-indandiol, 1-indanone, and a mixture of 1-indanol and 1-indenol. The monols were separated by HPLC. All four indan metabolites were crystallized and shown to have ^1H NMR and mass spectra identical to those given by authentic compounds (55). The specific rotations, $[\alpha]_D$, for *cis*-1,2-indandiol, 1-indanol, and 1-indenol were +53°, +32°, and +128°, respectively.

The formation of *cis*-1,2-indandiol and 1-indenol was unex-

TABLE 1. Yields, enantiomeric composition, and absolute configuration of hydroxylated products formed from indan by strains expressing NDO[a]

Strain[b]	1-Indanol			*cis*-1,2-Indandiol			1-Indenol		
	Yield (%)	Enantiomeric composition[c] (%)	Absolute configuration[d]	Yield (%)	Enantiomeric composition (%)	Absolute configuration	Yield (%)	Enantiomeric composition (%)	Absolute configuration
9816/11	64	79	(+)-(1*S*)-	7	86	(+)-(1*R*,2*S*)-	8	69	(+)-(1*S*)-
JM109(DE3)[pDTG141]	54	93	(+)-(1*S*)-	18	91	(+)-(1*R*,2*S*)-	19	83	(+)-(1*S*)-

[a] Products were identified by GC/MS. Yields were determined by integration of total ion current peak areas under the conditions described in Materials and Methods.
[b] Other products formed by strain 9816/11 were 1-indanone (18%) and 2-hydroxy-1-indanone (~1%). Approximately 1% indan remained at the end of the experiment. The only other product formed by strain JM109(DE3)[pDTG141] was 1-indanone (3%). Approximately 6% indan remained at the end of the experiment.
[c] Determined by integration of peak areas of enantiomers separated by chiral stationary-phase HPLC under conditions described in Materials and Methods.
[d] Absolute configuration of the major enantiomer.

pected, since previous studies showed that TDOs from *P. putida* F39/D (55) and *P. putida* UV4 (5) oxidize indan to (−)-(1*R*)-indanol and 1-indanone as the major products. In a second experiment, salicylate-induced cells of strain 9816/11 and IPTG-induced cells of JM109(DE3)[pDTG141] were incubated with indan for 5 and 24 h, respectively. Products were separated by preparative-layer chromatography (*cis*-1,2-indandiol, R_f 0.2, and indanol-indenol mixture, R_f 0.6) and identified by GC/MS. Absolute configurations were determined by chiral stationary-phase HPLC and are shown in Table 1. A significant amount of indanone (16%) was produced by strain 9816/11. We have noticed in other experiments that 1-indanone formation increases with the time of incubation of indan with strain 9816/11 and appears to be correlated with a decrease in the enantiomeric purity of (+)-(1*S*)-indanol recovered (data not shown). Indan oxidation was not observed in control experiments with strains 9816/C84 and JM109(DE3)[pT7-5].

Oxidation of indene by strains 9816/11 and JM109(DE3)[pDTG141]. Experiments analogous to those described above for indan were conducted with indene. Salicylate-induced cells of strain 9816/11 oxidized indene to two major products, which were isolated by radial-dispersion chromatography and identified as (+)-*cis*-(1*R*,2*S*)-indandiol ($[\alpha]_D$, +40°) and (+)-(1*S*)-indenol ($[\alpha]_D$, +128°). The same diol ($[\alpha]_D$, +34°) and monol ($[\alpha]_D$, +137°) products were formed by JM109(DE3)[pDTG141]. Both organisms formed small amounts of 1-indanone (Table 2).

The formation of (+)-*cis*-(1*R*,2*S*)-indandiol and (+)-(1*S*)-indenol from indan (Table 1) can be explained by the desaturation (dehydrogenation) of indan or the dehydration of 1-indanol to yield indene, which would then serve as a substrate to give the products shown in Table 2. However, indene was never detected as an intermediate in the metabolism of indan or 1-indanol by strains 9816/11 and JM109(DE3)[pDTG141]. Only minor amounts of (1*S*)-indenol were formed from (1*S*)-indanol by both strains, thus eliminating the desaturation of (1*S*)-indanol as a major source of (1*S*)-indenol.

Oxidation of indan to indene by purified NDO. In order to unequivocally establish the role of NDO in the observed dioxygenation, monooxygenation, and desaturation reactions, purified NDO components (reductase$_{NAP}$, ferredoxin$_{NAP}$, and ISP$_{NAP}$) were incubated with indan in the presence of NADH. Reaction products were extracted with ether, separated by HPLC, and identified by their retention times and absorption spectra (Fig. 3). Indan oxidation was not observed when any one of the NDO components or NADH was omitted from the reaction mixture. The identity of indene was confirmed by analyzing the ether extract by GC/MS. The reaction product had a retention time of 6.98 min, and its mass spectrum gave a molecular ion M^+ at *m/z* 116 (100%) and major fragment ions at *m/z* 115 (91%), 89 (10%), 63 (12%), and 58 (32%). These properties are identical to those given by authentic indene. When indene was oxidized by purified NDO, the only products detected were (1*S*)-indenol and *cis*-(1*R*,2*S*)-indandiol (Table 3). In contrast, the major products formed from indan were (1*S*)-indanol and (1*S*)-indenol. Smaller amounts of indene, (1*R*,2*S*)-indandiol, and 1-indanone were also detected. (1*S*)-Indanol was a poor substrate for NDO. The major oxidation product detected was a diol with the same retention time as *trans*-1,3-indandiol. Minor amounts of (1*S*)-indenol and 1-indanone were also produced.

Oxidation of indoline and dihydrobenzofuran by strain 9816/11. Previous studies have shown that NDO and TDO expressed by 9816/11 and *P. putida* F39/D, respectively, oxidize indole to indigo (13). The proposed reaction sequence involves *cis*-dihydroxylation of the heterocyclic ring followed by nonenzymatic dehydration to yield indoxyl, which autooxidizes to indigo. Salicylate-induced cells of 9816/11 oxidized indoline to

TABLE 2. Yields, enantiomeric composition, and absolute configuration of the hydroxylated products formed from indene by strains expressing NDO[a]

Strain[b]	*cis*-1,2-Indandiol			1-Indenol		
	Yield (%)	Enantiomeric composition[c] (%)	Absolute configuration[d]	Yield (%)	Enantiomeric composition (%)	Absolute configuration
9816/11	53	90	(+)-(1*R*,2*S*)-	43	94	(+)-(1*S*)-
JM109(DE3)[pDTG141]	56	86	(+)-(1*R*,2*S*)-	42	81	(+)-(1*S*)-

[a] Products were identified by ^{1}H NMR and GC/MS. Yields were determined by integration of total ion current peak areas under the conditions described in Materials and Methods.
[b] 1-Indanone (~4%) was the only other product formed by 9816-11. 1-Indanone (~2%) was the only other product formed by JM109(DE3)[pDTG141].
[c] Determined by integration of peak areas of enantiomers separated by chiral stationary-phase HPLC under conditions described in Materials and Methods.
[d] Absolute configuration of the major enantiomer.

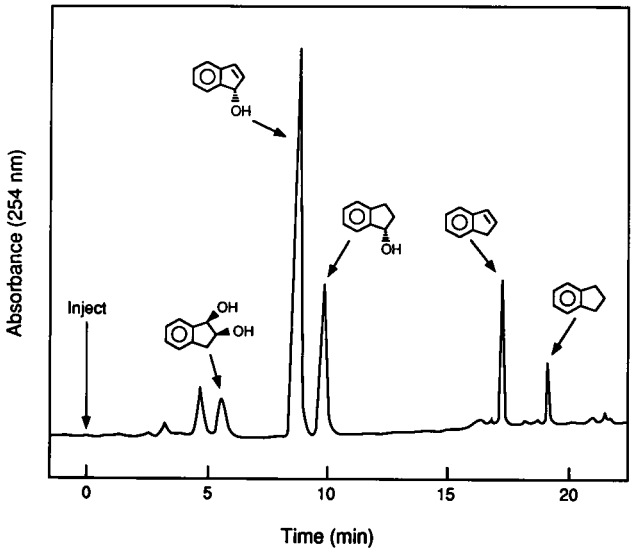

FIG. 3. HPLC separation of the products formed from indan by purified NDO. The experimental conditions and procedures used for the identification of products are described in Materials and Methods.

a blue compound, which was extracted with hot chloroform and shown to have TLC properties, absorption, and mass spectra identical to those given by authentic indigo. In contrast, toluene-induced cells of *P. putida* F39/D did not oxidize indoline to indigo.

The structural similarity of 2,3-dihydrobenzofuran to indoline suggested that it may serve as a substrate for the desaturase activity of NDO. Several products were formed from 2,3-dihydrobenzofuran by 9816/11. One of these was identified by GC/MS as 2,3-benzofuran. The identities of the other products were not determined in the present study.

DISCUSSION

The results presented show that NDO catalyzes dioxygenation, monooxygenation, and desaturation reactions with indan. The formation of (+)-(1*S*)-indenol and (+)-*cis*-(1*R*,2*S*)-indandiol from this substrate was of interest, since previous studies with TDO from *P. putida* F39/D (6, 55) and UV4 (5) showed that the major products formed from indan are (−)-(1*R*)-indanol and 1-indanone. The variable enantiomeric composition of the (1*R*)-indanol formed by these strains is due to the presence of a dehydrogenase which preferentially oxidizes (1*S*)-indanol to 1-indanone (5, 6). A similar dehydrogenase is probably responsible for the lower enantiomeric composition of the (1*S*)-indanol formed from indan by 9816/11. This conclusion is based on the higher enantiomeric composition of the (1*S*)-indanol formed by JM109(DE3)[pDTG141] and the larger amount of 1-indanone formed by 9816/11 after 5 h (Table 1). In addition to the compounds listed in Table 1, 9816/11 also produced 2-hydroxy-1-indanone as a minor product. Subsequent studies showed that NDO oxidizes 1-indanone to racemic 2-hydroxy- and (3*R*)-hydroxy-1-indanone (~80% *R*-enantiomer). In contrast, 1-indanone is not oxidized by TDO from strain F39/D (43).

Table 2 shows that NDO oxidizes indene to (+)-*cis*-(1*R*,2*S*)-indandiol and (+)-(1*S*)-indenol. The former product is of higher enantiomeric composition and of the opposite configuration than the (−)-*cis*-(1*S*,2*R*)- indandiol formed by the TDO-containing strains F39/D (55) and UV4 (5). In addition, the (+)-(1*S*)-indenol formed by NDO is of higher enantiomeric purity than the same product formed by F39/D (55) and of opposite configuration to the (−)-(1*R*)-indenol formed in high enantiomeric purity by UV4 (5).

TABLE 3. Yields, molecular weights, and retention times of products formed from indan, indene, and (1*S*)-indanol by purified NDO[a]

Product	M^+	Retention time (min)	% of product formed from:		
			Indan	Indene	(1*S*)-Indanol
Indene	116	6.98	2.4	12.5	—[b]
(1*S*)-Indenol	132	9.45	20.1	57.9	0.8
(1*S*)-Indanol	134	9.76	67.1	—	85.5
1-Indanone	132	10.4	3.5	—	2.2
(1*R*,2*S*)-Indandiol	150	12.35	7.0	29.6	—
trans-1,3-Indandiol	150	12.53	—	—	11.5

[a] Reactions were carried out for 1 h, and products were separated and identified by GC/MS as described in Materials and Methods. The absolute configurations given are based on results with intact cells (Tables 1 and 2).
[b] —, not detected.

The formation of (+)-*cis*-(1*R*,2*S*)-indandiol in high enantiomeric purity by NDO in strains 9816/11 and JM109(DE3)[pDTG141] provides a direct route to this enantiomer. Recent studies by Boyd and his associates have shown that naphthalene-grown cells of *P. putida* NCIMB 8859 oxidize indene to 1,2-indandiol, with an excess of the (1*R*,2*S*)-enantiomer. The concentration of indene used in these experiments (0.5 to 1.0 mg ml^{-1}) is critical, since the same NCIMB 8859 cells catalyze the enantiospecific removal of (1*R*,2*S*)-indandiol from a racemic mixture of *cis*-1,2-indandiol provided at lower concentrations (0.2 to 0.4 mg ml^{-1}) (1). These observations, those described in this paper, and studies currently in progress (40) indicate that NDO may provide a family of chiral synthons that differ in configuration from those formed by TDO. The explanation for these differences awaits detailed studies on the structure and mechanism of action of both dioxygenases.

The oxidation of indan and indene to (1*S*)-indenol and *cis*-(1*R*,2*S*)-indandiol by NDO suggests that the enzyme catalyzes the desaturation of indan or the dehydration of 1-indanol to yield indene, which then serves as a substrate for the monooxygenase and dioxygenase activities of the enzyme. Attempts to detect indene formation from indan during whole-cell experiments with strains 9816/11 and JM109(DE3)[pDTG141] were unsuccessful. Indene was finally detected by HPLC analysis of the products formed from indan by purified NDO (Fig. 3) and identified by showing that its retention time, absorption spectrum, and mass spectrum were identical to those of authentic indene. Additional evidence for the desaturase activity of NDO was provided by showing that strain 9816/11 oxidizes indoline (1,2-dihydroindole) to indigo. The oxidation of indole to indigo by NDO and other oxygenases, including TDO, has been reported previously (4, 13). Thus, it was of interest that the TDO expressed by strain F39/D did not oxidize indoline to indigo, a characteristic consistent with its inability to desaturate indan to indene. Other desaturation reactions catalyzed by NDO include the formation of benzofuran from dihydrobenzofuran (this study) and ethenyloxybenzene from phenetole (41).

Strains 9816/11 and JM109(DE3)[pDTG141] and purified NDO did not form detectable amounts of indene from (1*S*)-indanol, and only very low yields of 1-indenol were formed from this substrate by purified NDO (Table 3). The enzyme did, however, oxidize (1*S*)-indanol to a compound tentatively identified as *trans*-1,3-indandiol. Thus, all of the available ev-

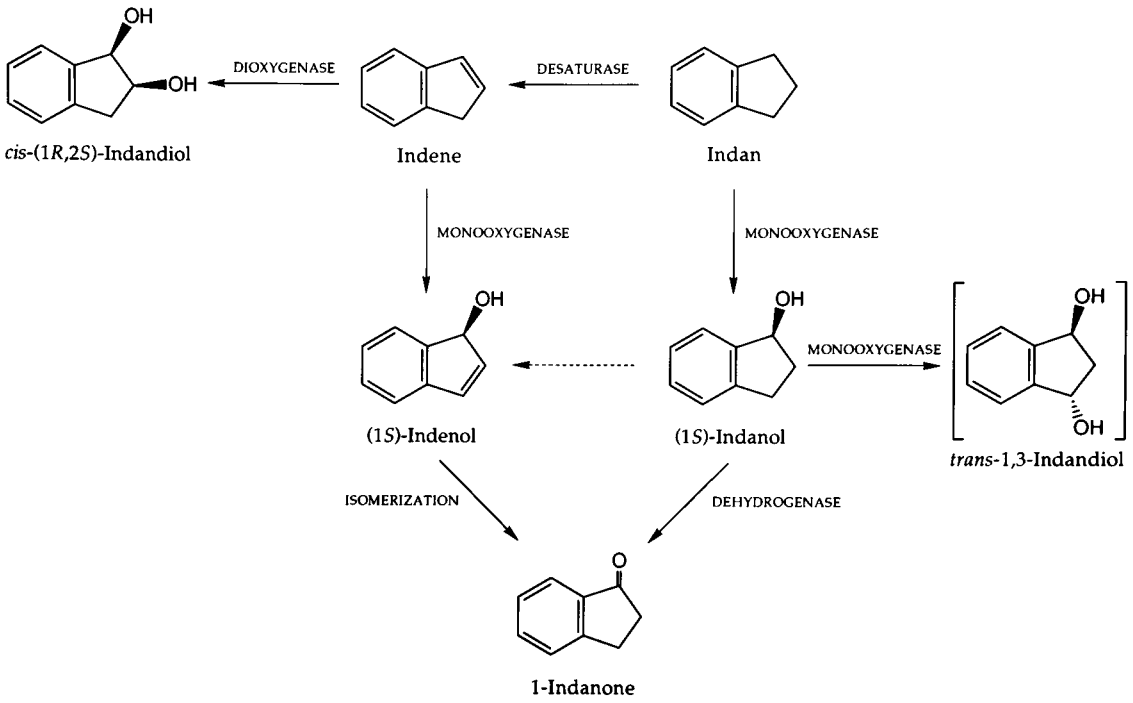

FIG. 4. Desaturase, monooxygenase, and dioxygenase reactions catalyzed by NDO. The formation of 1-indanone from (1*S*)-indenol probably occurs by nonenzymatic isomerization. The oxidation of (1*S*)-indanol to 1-indanone is catalyzed by a dehydrogenase present in cells of strain 9816/11. The desaturation of (1*S*)-indanol to (1*S*)-indenol is a minor reaction, indicated by ----➢.

idence indicates that NDO catalyzes the desaturation of indan to indene, which then serves as a substrate for the formation of *cis*-(1*R*,2*S*)-indandiol and (1*S*)-indenol, as shown in Fig. 4. The formation of 1-indanone from (1*S*)-indenol probably occurs by nonenzymatic isomerization (16), whereas 1-indanone formation from (1*S*)-indanol appears to be catalyzed by a dehydrogenase, as discussed above.

The desaturation reaction catalyzed by NDO and the structural organization of the enzyme (Fig. 1) both have features in common with plant stearoyl-acyl carrier protein (stearoyl-ACP) Δ^9 desaturase. This enzyme forms oleoyl-ACP in a reaction that requires NAD(P)H, oxygen, ferredoxin oxidoreductase, ferredoxin, and a terminal desaturase component which contains non-heme iron (34, 36). Recent studies with the cloned desaturase from the castor bean plant have shown that the iron is in the form of diiron-oxo clusters, which are involved in the generation of a high-valency iron-oxo species responsible for the desaturation reaction (14).

The desaturation and monooxygenation reactions catalyzed by NDO are also analogous to reactions catalyzed by cytochrome P-450. For example, the initial abstraction of a hydrogen atom from indan by an $(FeO)^{3+}$ species followed by oxygen rebound (hydroxylation) or removal of the β-hydrogen atom (desaturation) would account for the observed products (20, 21). In this context, it is of interest that NDO can catalyze *O*-dealkylation (41), *N*-dealkylation, and sulfoxidation reactions (32), which are also typical cytochrome P-450 reactions. Other non-heme iron oxygenases that catalyze reactions similar to those catalyzed by cytochrome P-450 are the soluble forms of methane monooxygenase (19, 33) and 4-methoxybenzoate monooxygenase (56). Ammonia monooxygenase appears to be responsible for the cytochrome P-450-type reactions catalyzed by *Nitrosomonas europaea* (29, 54). The iron at the active site of methane monooxygenase (15, 44) and plant stearoyl-ACP Δ^9 desaturase (14) is in the form of diiron clusters. In addition, deduced amino acid sequence analyses (14) show that the conserved motif proposed for the iron-binding sites in all known diiron-oxo proteins are also present in the putative oxygenase components of toluene 4-monooxygenase (58) and phenol hydroxylase (37). Lipscomb has proposed that the diiron-oxo clusters in methane monooxygenase are converted during catalysis to an [Fe(IV) · Fe(IV)]═O species, which would be a strong oxidant similar to the Fe(IV)═O species proposed for cytochrome P-450 (33). An iron-peroxo complex $[FeO_2]^+$ has been proposed for the oxygenating species in 4-methoxybenzoate *O*-demethylase (56).

The unique feature of NDO that sets is apart from the oxygenases described above is its ability to catalyze the enantiospecific incorporation of dioxygen into naphthalene and related compounds. It seems probable that this reaction is catalyzed by a strong oxidizing species generated by the interaction of oxygen with mononuclear iron present in the large (α) subunit of ISP_{NAP} (51). The monooxygenase, desaturase, and other diverse reactions catalyzed by NDO are probably catalyzed at the same site and, like cytochrome P-450, reflect differences in the "fit" of the substrate in the active site, the available oxidizable sites of the different substrates, and the rate-limiting steps in catalysis (20, 21). Current studies are directed towards understanding the role of mononuclear iron in NDO, which may involve Fenton-type reactions. In this context, it is of interest that H_2O_2 and Fe^{2+} in organic solvents can catalyze desaturation, monooxygenation, and dioxygenase reactions (53).

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REFERENCES

1. **Allen, C. C. R., D. R. Boyd, H. Dalton, N. D. Sharma, I. Brannigan, N. A. Kerley, G. N. Sheldrake, and S. C. Taylor.** 1995. Enantioselective bacterial biotransformation routes to *cis*-diol metabolites of monosubstituted benzenes, naphthalene and benzocycloalkenes of either absolute configuration. J. Chem. Soc. Chem. Commun., **1995:**117–118.
2. **Barnsley, E. A.** 1975. The induction of the enzymes of naphthalene metabolism in pseudomonads by salicylate and 2-aminobenzoate. J. Gen. Microbiol. **88:**193–196.
3. **Boyd, D. R., R. A. S. McMordie, N. D. Sharma, H. Dalton, P. Williams, and R. O. Jenkins.** 1989. Stereospecific benzylic hydroxylation of bicyclic alkenes by *Pseudomonas putida*: isolation of (+)-*R*-1-hydroxy-1,2-dihydronaphthalene, an arene hydrate of naphthalene from metabolism of 1,2-dihydronaphthalene. J. Chem. Soc. Chem. Commun. **1989:**339–340.
4. **Boyd, D. R., N. D. Sharma, R. Boyle, B. T. McMurray, T. A. Evans, J. F. Malone, H. Dalton, J. Chima, and G. N. Sheldrake.** 1993. Biotransformation of unsaturated heterocyclic rings by *Pseudomonas putida* to yield *cis*-diols. J. Chem. Soc. Chem. Commun. **1993:**49–51.
5. **Boyd, D. R., N. D. Sharma, P. J. Stevenson, J. Chima, D. J. Gray, and H. Dalton.** 1991. Bacterial oxidation of benzocycloalkenes to yield monol, diol and triol metabolites. Tetrahedron Lett. **32:**3887–3890.
6. **Brand, J. M., D. L. Cruden, G. J. Zylstra, and D. T. Gibson.** 1992. Stereospecific hydroxylation of indan by *Escherichia coli* containing the cloned toluene dioxygenase genes from *Pseudomonas putida* F1. Appl. Environ. Microbiol. **58:**3407–3409.
7. **Brown, S. M., and T. Hudlicky.** 1993. The use of arene-*cis*-diols in synthesis. Org. Synth. Theory Appl. **2:**113–176.
8. **Carless, H. A. J.** 1992. The use of cyclohexa-3,5-diene-1,2-diols in enantiospecific synthesis. Tetrahedron Asymmetry **3:**795–826.
9. **Davies, J. I., and W. C. Evans.** 1964. Oxidative metabolism of naphthalene by soil pseudomonads. Biochem. J. **91:**251–261.
10. **Deluca, M. E., and T. Hudlicky.** 1990. Microbial oxidation of naphthalene derivatives: absolute configuration of metabolites. Tetrahedron Lett. **31:**13–16.
11. **Ensley, B. D., and D. T. Gibson.** 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component. J. Bacteriol. **155:**505–511.
12. **Ensley, B. D., D. T. Gibson, and A. L. Laborde.** 1982. Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB 9816. J. Bacteriol. **149:**948–954.
13. **Ensley, B. D., B. J. Ratzkin, T. D. Osslund, M. J. Simon, L. P. Wackett, and D. T. Gibson.** 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science **222:**167–169.
14. **Fox, B. G., J. Shanklin, C. Somerville, and E. Münck.** 1993. Stearoyl-acyl carrier protein Δ^9 desaturase from *Ricinus communis* is a diiron-oxo protein. Proc. Natl. Acad. Sci. USA **90:**2486–2490.
15. **Fox, B. G., K. K. Surerus, E. Münck, and J. D. Lipscomb.** 1988. Evidence for a μ-oxo bridged binuclear iron cluster in the hydroxylase component of methane monooxygenase: Mössbauer and EPR studies. J. Biol. Chem. **263:**10553–10556.
16. **Friedrich, E. C., and D. B. Taggart.** 1975. Double-bond rearrangements of inden-1-yl derivatives. J. Org. Chem. **40:**720–723.
17. **Gibson, D. T., M. Hensley, H. Yoshioka, and T. J. Mabry.** 1970. Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. Biochemistry **9:**1626–1630.
18. **Gibson, D. T., G. J. Zylstra, and S. Chauhan.** 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1, p. 121–132. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), Pseudomonas: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
19. **Green, J., and H. Dalton.** 1989. Substrate specificity of soluble methane monooxygenase: mechanistic implications. J. Biol. Chem. **264:**17698–17703.
20. **Guengerich, F. P.** 1990. Enzymatic oxidation of xenobiotic chemicals. Crit. Rev. Biochem. Mol. Biol. **25:**97–153.
21. **Guengerich, F. P.** 1991. Reactions and significance of cytochrome P-450 enzymes. J. Biol. Chem. **266:**10019–10022.
22. **Haigler, B. E.** 1986. Ph.D. thesis, University of Texas at Austin.
23. **Haigler, B. E., and D. T. Gibson.** 1990. Purification and properties of ferredoxin$_{NAP}$, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J. Bacteriol. **172:**465–468.
24. **Haigler, B. E., and D. T. Gibson.** 1990. Purification and properties of NADH-ferredoxin$_{NAP}$ reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J. Bacteriol. **172:**457–464.
25. **Imuta, M., and H. Ziffer.** 1978. Synthesis and absolute stereochemistry of *cis*- and *trans*-1,2-indandiols: metabolites of indene and 2-indanone. J. Org. Chem. **43:**4540–4542.
26. **Jeffrey, A. M., H. J. C. Yeh, D. M. Jerina, T. R. Patel, J. F. Davey, and D. T. Gibson.** 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. Biochemistry **14:**575–584.
27. **Jerina, D. M., J. W. Daly, A. M. Jeffrey, and D. T. Gibson.** 1971. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch. Biochem. Biophys. **142:**394–396.
28. **Jerina, D. M., H. Selander, H. Yagi, M. C. Wells, J. F. Davey, V. Mahadevan, and D. T. Gibson.** 1976. Dihydrodiols from anthracene and phenanthrene. J. Am. Chem. Soc. **98:**5988–5996.
29. **Keener, W. K., and D. J. Arp.** 1994. Transformations of aromatic compounds by *Nitrosomonas europaea*. Appl. Environ. Microbiol. **60:**1914–1920.
30. **Klecka, G. M., and D. T. Gibson.** 1979. Metabolism of dibenzo[1,4]dioxan by a *Pseudomonas* species. Biochem. J. **180:**639–645.
31. **Kobal, V. M., D. T. Gibson, R. E. Davis, and A. Garza.** 1973. X-ray determination of the absolute stereochemistry of the initial oxidation product formed from toluene by *Pseudomonas putida* 39/D. J. Am. Chem. Soc. **95:**4420–4421.
32. **Lee, K., and D. T. Gibson.** Unpublished data.
33. **Lipscomb, J. D.** 1994. Biochemistry of the soluble methane monooxygenase. Annu. Rev. Microbiol. **48:**371–399.
34. **McKeon, T., and P. K. Stumpf.** 1981. Stearoyl-acyl carrier protein desaturase from safflower seeds. Methods Enzymol. **71:**275–281.
35. **McMordie, R. A. S.** 1991. Ph.D. thesis, The Queen's University, Belfast, Northern Ireland.
36. **Nagai, J., and K. Bloch.** 1968. Enzymatic desaturation of stearyl acyl carrier protein. J. Biol. Chem. **243:**4626–4633.
37. **Nordlund, I., J. Powlowski, and V. Shingler.** 1990. Complete nucleotide sequence and polypeptide analysis of multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. J. Bacteriol. **172:**6826–6833.
38. **Parales, R. E., and D. T. Gibson.** Unpublished data.
39. **Resnick, S. M.** Unpublished data.
40. **Resnick, S. M., and D. T. Gibson.** Unpublished data.
41. **Resnick, S. M., and D. T. Gibson.** 1993. Biotransformation of anisole and phenetole by aerobic hydrocarbon-oxidizing bacteria. Biodegradation **4:**195–203.
42. **Resnick, S. M., D. S. Torok, and D. T. Gibson.** 1993. Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase. FEMS Microbiol. Lett. **113:**297–302.
43. **Resnick, S. M., D. S. Torok, K. Lee, J. M. Brand, and D. T. Gibson.** 1994. Regiospecific and stereoselective hydroxylation of 1-indanone and 2-indanone by naphthalene dioxygenase and toluene dioxygenase. Appl. Environ. Microbiol. **60:**3323–3328.
44. **Rosenweig, A. C., C. A. Frederick, S. J. Lippard, and P. Nordlund.** 1993. Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. Nature (London) **366:**537–543.
45. **Serdar, C. M., and D. T. Gibson.** 1989. Isolation and characterization of altered plasmids in mutant strains of *Pseudomonas putida* NCIB 9816. Biochem. Biophys. Res. Commun. **164:**764–771.
46. **Sheldrake, G. N.** 1992. Biologically derived arene *cis*-dihydrodiols as synthetic building blocks, p. 127–166. *In* A. N. Collins, G. N. Sheldrake, and J. Crosby (ed.), Chirality in industry: the commercial manufacture and application of optically active compounds. John Wiley & Sons Ltd., Chichester, England.
47. **Simon, M. J., T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W.-C. Suen, D. L. Cruden, D. T. Gibson, and G. J. Zylstra.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene **127:**31–37.
48. **Stabile, M. R.** 1995. Ph.D. thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va.
49. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
50. **Suen, W.-C.** 1991. Ph.D. thesis, University of Iowa, Iowa City.
51. **Suen, W.-C., and D. T. Gibson.** 1993. Isolation and preliminary characterization of the subunits of the terminal component of naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816-4. J. Bacteriol. **175:**5877–5881.
52. **Suen, W.-C., and D. T. Gibson.** 1994. Recombinant *Escherichia coli* strains synthesize active forms of naphthalene dioxygenase and its individual α and β subunits. Gene **143:**67–71.
53. **Sugimoto, H., and D. T. Sawyer.** 1984. Iron(II)-induced activation of hydrogen peroxide to ferryl ion (FeO^{2+}) and singlet oxygen ($^1O_2$) in acetonitrile: monooxygenations, dehydrogenations, and dioxygenations of organic substrates. J. Am. Chem. Soc. **106:**4283–4285.
54. **Vannelli, T., M. Logan, D. M. Arciero, and A. B. Hooper.** 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. **56:**1169–1171.
55. **Wackett, L. P., L. D. Kwart, and D. T. Gibson.** 1988. Benzylic monooxygenation catalyzed by toluene dioxygenase from *Pseudomonas putida*. Biochemistry **27:**1360–1367.
56. **Wende, P., F.-H. Bernhardt, and K. Pfleger.** 1989. Substrate-modulated reactions of putidamonooxin: the nature of the active oxygen species formed

and its reaction mechanism. Eur. J. Biochem. **181:**189–197.
57. **Widdowson, D. A., and D. W. Ribbons.** 1990. The use of substituted cyclohexadiene diols as versatile chiral synthons. Janssen Chim. **8:**3–9.
58. **Yen, K.-M., M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen.** 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173:**5315–5327.
59. **Yen, K.-M., and C. M. Serdar.** 1988. Genetics of naphthalene catabolism in pseudomonads. Crit. Rev. Microbiol. **15:**247–268.
60. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940–14946.