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Toluene and Ethylbenzene Oxidation by Purified Naphthalene Dioxygenase from *Pseudomonas* sp. Strain NCIB 9816-4

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Purified naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4 oxidized toluene to benzyl alcohol and benzaldehyde by reactions involving benzylic monooxygenation and dioxygen-dependent alcohol oxidation, respectively. Xylene and nitrotoluene isomers were also oxidized to substituted benzyl alcohol and benzaldehyde derivatives. NDO oxidized ethylbenzene sequentially through (*S*)-1-phenethyl alcohol (77% enantiomeric excess) and acetophenone to 2-hydroxyacetophenone. In addition, NDO also oxidized ethylbenzene through styrene to (*R*)-1-phenyl-1,2-ethanediol (74% enantiomeric excess) by reactions involving desaturation and dihydroxylation, respectively. Isotope experiments with $^{18}O_2$, $H_2{}^{18}O$, and D_2O suggest that 1-phenethyl alcohol is oxidized to acetophenone by a minor reaction involving desaturation followed by tautomerization. The major reaction in the conversion of 1-phenethyl alcohol and benzyl alcohol to acetophenone and benzaldehyde, respectively, probably involves monohydroxylation to form a *gem*-diol intermediate which stereospecifically loses the incoming hydroxyl group to leave the carbonyl product. These results are compared with similar reactions catalyzed by cytochrome P-450.

Naphthalene dioxygenase (NDO) catalyzes the first reaction in the aerobic catabolism of naphthalene by *Pseudomonas* sp. strain NCIB 9816-4 (3, 46). The enzyme catalyzes the *cis*-dihydroxylation of naphthalene to form homochiral (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) in the presence of dioxygen and two reducing equivalents of NAD(P)H (18, 19). NDO is a multicomponent enzyme system in which electrons are transferred from NAD(P)H through an iron-sulfur flavoprotein ($reductase_{NAP}$) (14) and a Rieske [2Fe-2S] protein ($ferredoxin_{NAP}$) (13) to the terminal oxygenase component (ISP_{NAP}) (4). ISP_{NAP} has an $\alpha_2\beta_2$ subunit composition, and each α subunit contains a Rieske [2Fe-2S] redox center and mononuclear iron (4, 38). The latter is believed to be the site of dioxygen activation and catalysis. All of the structural genes have been cloned, and their nucleotide sequences have been determined (29, 36).

NDO has a relaxed substrate specificity that permits the enantiospecific *cis*-dihydroxylation of the aromatic nucleus in polycyclic hydrocarbons (18–20, 45) and the olefin groups of benzocycloalkenes (8, 32, 40). In addition, NDO also catalyzes benzylic monohydroxylation (1, 8, 33, 43), desaturation (8, 31, 40), O-dealkylation (31), N-dealkylation (23), and enantiospecific sulfoxidation (2, 22) reactions. With the exception of *cis*-dihydroxylation, all of the reactions described above are also catalyzed by cytochrome P-450 (P-450) (11, 12, 28, 30).

In *Pseudomonas putida* F1, toluene dioxygenase (TDO), a multicomponent enzyme system analogous to NDO, catalyzes the enantiospecific addition of dioxygen to the aromatic nucleus of toluene to form (+)-(1*S*,2*R*)-*cis*-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) (6, 47). TDO oxidizes naphthalene to homochiral (+)-*cis*-naphthalene dihydrodiol (10, 40) and styrene to (−)-*cis*-(2*R*,3*S*)-dihydroxy-2,3-dihydrostyrene (17, 42). In contrast, NDO attacks the styrene vinyl group to form (*R*)-1-phenyl-1,2-ethanediol (24). This reaction together with the O- and N-dealkylation and benzylic hydroxylation reactions catalyzed by NDO suggest that the enzyme may oxidize aromatic hydrocarbons such as toluene and ethylbenzene at the alkyl substituents rather than the aromatic nucleus. These are reactions that are also catalyzed by P-450 (5, 15, 27, 44). We now report multiple pathways for the oxidation of the methyl and ethyl groups of toluene and ethylbenzene by NDO. A preliminary report of this work has been published (26).

MATERIALS AND METHODS

Materials. Most compounds used in this study were obtained from Aldrich Chemical Co., Milwaukee, Wis. Exceptions were NADH (disodium salt, 98% purity), MES [sodium 2-(*N*-morpholino)ethanesulfonic acid], $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Sigma Chemical Co., St. Louis, Mo.), [^{18}O]-O_2 (98 atom%; Icon Isotopes, Summit, N.J.), [^{18}O]-H_2O (97.9 atom%; Isotec Inc., Miamisburg, Ohio), and 2-hydroxyacetophenone (TCI America, Portland, Oreg.) All chemicals were of analytical reagent grade. NDO components ($reductase_{NAP}$, $ferredoxin_{NAP}$, and ISP_{NAP}) were purified to homogeneity from recombinant *Escherichia coli* strains (23, 39).

Dioxygen consumption studies. Dioxygen consumption by NDO was measured at 24°C with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) as described previously (22). The dissolved dioxygen level was 250 μM. Reaction mixtures contained, in 1.0 ml of 50 mM MES buffer (pH 6.8), NADH (0.25 μmol), $reductase_{NAP}$ (4 μg), $ferredoxin_{NAP}$ (14 μg), ISP_{NAP} (25 μg), and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 μmol). Reactions were initiated by the addition of 4 μl of a 25 mM solution of the substrate (0.1 μmol) in methanol.

Enzymatic transformation studies. Procedures for the formation of transformation products by purified NDO were as described previously (22). Reaction mixtures for the results shown in Table 2 contained, in 2 ml of 50 mM MES buffer (pH 6.8), NADH (0.5 μmol), $reductase_{NAP}$ (16 μg), $ferredoxin_{NAP}$ (35 μg), ISP_{NAP} (50 μg), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 μmol), and substrate (0.25 μmol). Reaction mixtures were shaken (60 rpm) at 23°C for 2 h (enzymatic oxidation procedure I). Reaction mixtures for the results shown in Tables 1 and 3 were the same as those described for dioxygen consumption studies, and the reactions were carried out with agitation (60 rpm) at 24°C for 20 min (enzymatic oxidation procedure II).

Identification of metabolites. Reaction mixtures were extracted three times with equal volumes of NaOH-washed ethyl acetate. The organic extracts were combined, dried over anhydrous sodium sulfate, and concentrated to approximately 30 μl under nitrogen. All extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) as described previously (33). Under these conditions, the retention times of benzaldehyde (M^+ at *m/z* 106), benzyl alcohol (M^+ at *m/z* 108), styrene (M^+ at *m/z* 104), 1-phenethyl alcohol (M^+ at *m/z* 122), acetophenone (M^+ at *m/z* 120), 2-hydroxyacetophenone (M^+ at *m/z* 136), and 1-phenyl-1,2-ethanediol (M^+ at *m/z* 138) were 5.45, 6.58, 4.70, 7.15, 7.15, 9.82, and 10.72 min, respectively. All products were identified by comparing their GC-MS properties (retention times and fragmentation patterns) with those of authentic compounds. Relative yields of products were determined by integration of total ion current peak areas. 1-Phenethyl alcohol and acetophenone

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formed by NDO were first purified by thin-layer chromatography on Silica Gel 60 F_{254} plastic sheets (0.2-mm thickness; obtained from E. Merck, Gibbstown, N.J.) with a developing solvent of chloroform-acetone (8:2). R_f values for 1-phenethyl alcohol and acetophenone were 0.48 and 0.61, respectively. The products were visualized under UV light. The silica gel containing each product was scraped from the plate and extracted twice with 100-μl volumes of NaOH-washed ethyl acetate. After centrifugation, the ethyl acetate extracts were combined and concentrated under a stream of nitrogen gas. The enantiomeric composition of 1-phenethyl alcohol was determined by chiral stationary-phase high-performance liquid chromatography (HPLC) on a Chiracel OB-H column (4.6 mm by 25 cm; 5-μm particle size; obtained from Chiral Technologies Inc., Exton, Pa.) under the same conditions described previously for the determination of the enantiomeric purity of 1-phenyl-1,2-ethanediol formed from styrene by NDO (24). The (*S*)- and (*R*)-1-phenethyl alcohols eluted at 12.15 and 16.28 min, respectively.

$^{18}O_2$ incorporation studies. Reaction mixtures contained, in 0.5 ml of 50 mM MES buffer (pH 6.8), NADH (0.5 μmol), $reductase_{NAP}$ (20 μg), $ferredoxin_{NAP}$ (100 μg), ISP_{NAP} (100 μg), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 μmol), catalase (50 μg), and 1-phenethyl alcohol (0.5 μmol). Reactions were carried out with agitation (60 rpm) for 2 h at 23°C in 1-dram (3.7-ml) glass vials fitted with Teflon-lined septa and screw-on caps (Altech Associates, Inc., Deerfield, Ill.). Gas exchange procedures for introducing $^{18}O_2$ and headspace analyses were as described previously (33). Reactions were terminated by the addition of 2.0 ml of NaOH-washed ethyl acetate through the septa. Reaction mixtures were extracted and concentrated as described above. 1-Phenethyl alcohol and acetophenone were separated by thin-layer chromatography as described above, and their ^{18}O contents were determined by GC-MS.

D_2O incorporation studies. Reaction mixtures and incubation conditions were similar to those described above for $^{18}O_2$ incorporation studies, except that the reaction was carried out in air and the liquid phase contained 80% D_2O. A control experiment designed to determine the possible exchange of deuterium with the methyl hydrogens of acetophenone was conducted in the absence of $ferredoxin_{NAP}$. The reaction mixture contained 0.5 μmol of acetophenone instead of 1-phenethyl alcohol. The reactions were carried out as described above, with air replacing $^{18}O_2$. Acetophenone was isolated by thin-layer chromatography and analyzed by GC-MS. The extent of deuterium incorporated into acetophenone was determined by the change in intensity of the molecular ion (M^+ at *m/z* 120) and the formation of a $M^+ + 1$ ion. Deuterium enrichment (percent) was determined by $100 \times [(M^+ + 1) - f \times M^+]/[(M^+ + 1) - f \times M^+ + M^+]$, where f (=0.1) is a ratio of $(M^+ + 1)/M^+$ in intensity given by authentic acetophenone. Normalized enrichment (percent) was calculated from 100 × (product deuterium enrichment)/(isotope enrichment of deuterium source).

$H_2{}^{18}O$ incorporation studies. Reaction mixtures and incubation conditions were similar to those described above for D_2O incorporation studies, except that the medium contained 80% $H_2{}^{18}O$. A control reaction to determine the extent of exchange of the carbonyl oxygen of acetophenone with H_2O was carried out with 80% $H_2{}^{18}O$ as described above for the control experiment in the D_2O incorporation studies. Reactions were carried out as described above, with air replacing $^{18}O_2$. Acetophenone was isolated by thin-layer chromatography and analyzed by GC-MS.

RESULTS

Oxidation of toluene by NDO. It has been shown previously that TDO oxidizes naphthalene to homochiral (+)-*cis*-naphthalene dihydrodiol (10, 40). Thus the oxidation of toluene by NDO was of interest in terms of comparing the regio- and stereoselectivities of the two oxygenases. The oxidation of toluene by NDO (enzymatic oxidation procedure I; see Materials and Methods) yielded benzyl alcohol (73.3%) and benzaldehyde (26.7%) as determined by GC-MS analysis. This result suggested that NDO preferentially oxidizes the methyl group of toluene. In addition, the formation of benzaldehyde, presumably from benzyl alcohol, is a novel reaction for NDO.

Further biotransformations of toluene, benzyl alcohol, and benzaldehyde were conducted to determine the sequence of reactions involved in toluene oxidation by NDO. The results obtained (Table 1) show that in the presence of excess NADH, toluene was oxidized to benzyl alcohol and benzaldehyde. In contrast, when NADH was limiting, benzyl alcohol was the only detectable product. NDO did not oxidize benzyl alcohol under anaerobic conditions or under aerobic conditions in the presence of NAD^+. In control experiments, benzyl alcohol was not oxidized when $ferredoxin_{NAP}$ was omitted from the reaction mixture.

The initial rates of dioxygen consumption in the presence of toluene and benzyl alcohol were 1.84 and 0.32 μmol/min/mg of ISP_{NAP}, respectively. Under the same conditions, the initial rate of dioxygen consumption in the presence of naphthalene was 2.48 μmol/min/mg of ISP_{NAP} (Fig. 1). The amount of dioxygen consumed was almost equal to the amount of naphthalene added (>98%). In contrast, biphasic and hyperbolic rates of dioxygen consumption were observed in the presence of toluene and benzyl alcohol, respectively. Since the second phase of dioxygen consumption in the presence of toluene was similar (60 nmol/min/mg of ISP_{NAP}) to that in the presence of benzyl alcohol (80 nmol/min/mg of ISP_{NAP}), the first rapid phase of dioxygen consumption was attributed primarily to the benzylic monooxygenation of toluene to form benzyl alcohol and the second phase was attributed primarily to the oxidation of benzyl alcohol to benzaldehyde. The ratio of dioxygen consumed to toluene added was greater than 1:1 (Fig. 1), and in long-term experiments, similar results were observed with benzyl alcohol (data not shown). These observations indicate that the oxidation of both substrates is partially uncoupled from dioxygen consumption. Although dioxygen consumption was slightly increased in the presence of benzaldehyde (Fig. 1), no benzaldehyde oxidation product was detected.

TABLE 1. Yields of products formed from toluene and benzyl alcohol by purified NDO[a]

Substrate	[Substrate]/[NADH]	Product (%)
Toluene	0.4[b]	Benzyl alcohol (89.2) Benzaldehyde (10.8)
Toluene	2.5[c]	Benzyl alcohol (100)
Benzyl alcohol	2.5	Benzaldehyde (12.5)
Benzaldehyde	2.5	ND[d]

[a] Details of the reaction conditions and product analyses are given in Materials and Methods.
[b] [Substrate]/[NADH] = 100 nmol/250 nmol.
[c] [Substrate]/[NADH] = 250 nmol/100 nmol.
[d] ND, not detected.

Oxidation of nitrotoluenes and xylenes by NDO. Table 2 shows the products formed from nitrotoluene and xylene isomers by NDO. Benzyl alcohol and benzaldehyde derivatives were formed from all substrates with the exception of 2-nitro-

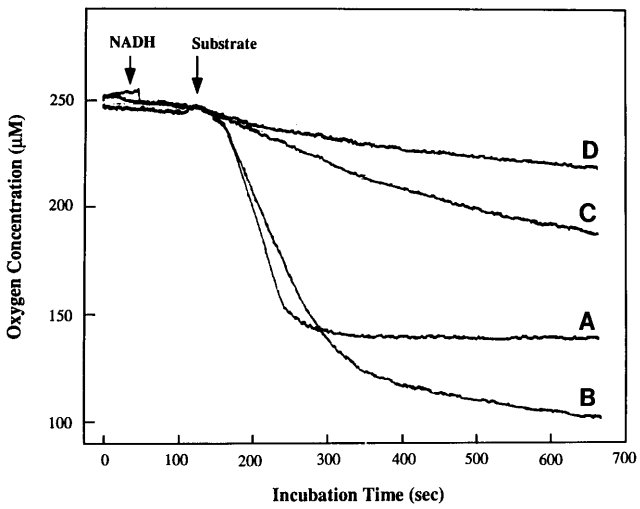

FIG. 1. Oxygen consumption by NDO in the presence of naphthalene (A), toluene (B), benzyl alcohol (C), and benzaldehyde (D). The addition of NADH and substrate are indicated by arrows. Specific experimental conditions are described in Materials and Methods.

TABLE 2. Products formed from nitrotoluenes and xylenes by purified NDO[a]

Substrate	Retention time (min)	Mass	Products (relative yield [%])
2-Nitrotoluene	12.15	153	2-Nitrobenzyl alcohol (93.3)
3-Nitrotoluene	13.60	153	3-Nitrobenzyl alcohol (96.4)
	11.14	151	3-Nitrobenzaldehyde (2.6)
4-Nitrotoluene	13.71	153	4-Nitrobenzyl alcohol (82.6)
	10.91	151	4-Nitrobenzaldehyde (3.4)
2-Methyltoluene	8.34	122	2-Methylbenzyl alcohol (95.1)
(*o*-xylene)	7.15	120	2-Tolualdehyde (4.9)
3-Methyltoluene	8.23	122	3-Methylbenzyl alcohol (86.7)
(*m*-xylene)	7.15	120	3-Tolualdehyde (13.3)
4-Methyltoluene	8.22	122	4-Methylbenzyl alcohol (72.2)
(*p*-xylene)	7.36	120	4-Tolualdehyde (21.8)

[a] Experimental conditions are described in Materials and Methods.

toluene, which did not yield 2-nitrobenzylaldehyde as a reaction product. All of the substrates tested gave the corresponding benzyl alcohols as the major products.

Ethylbenzene oxidation by NDO. Ethylbenzene was incubated with NDO for 2 h (enzymatic oxidation procedure I; see Materials and Methods), and the following products were identified by GC-MS: 1-phenethyl alcohol (70.4%), acetophenone (7.6%), 2-hydroxyacetophenone (7.0%), and 1-phenyl-1,2-ethanediol (14.4%). In addition, styrene (<0.5%) was also detected as a reaction product. The absolute stereochemistry of 1-phenethyl alcohol was determined by chiral stationary-phase HPLC and shown to be the (*S*)-enantiomer (77% enantiomeric excess [e.e.]) were obtained. In addition, the absolute stereochemistry of 1-phenyl-1,2-ethanediol was the (*R*)-enantiomer (74% e.e.).

Oxidation of ethylbenzene products by NDO. To determine the sequence of reactions leading to the formation of the products described above from ethylbenzene, each product was used as a substrate for NDO (Table 3). In this experiment, ethylbenzene was oxidized to 1-phenethyl alcohol, acetophenone, styrene, and 1-phenyl-1,2-ethanediol. (*S*)-1-phenethyl alcohol and (*R*)-1-phenethyl alcohol were both oxidized to acetophenone and 2-hydroxyacetophenone, with the (*R*)-enantiomer being the preferred substrate. However, 1-phenethyl alcohol was not oxidized to 1-phenyl-1,2-ethanediol. Acetophenone was oxidized to 2-hydroxyacetophenone,

TABLE 3. Products formed from ethylbenzene, 1-phenethyl alcohol, acetophenone, and styrene by purified NDO[a]

Substrate	Product (%)			
	1-Phenethyl alcohol	Aceto-phenone	2-Hydroxy-acetophenone	1-Phenyl-1,2-ethanediol
Ethylbenzene[b]	74.2	11.6	—[c]	13.7
(*S*)-1-Phenethyl alcohol	60.9	9.1	30.0	—
(*R*)-1-Phenethyl alcohol	47.8	17.8	34.4	—
Acetophenone	—	21.5	78.5	—
2-Hydroxyacetophenone	—	—	100.0	—
Styrene	—	—	—	100.0

[a] Details of the reaction conditions and product analyses are described in Materials and Methods.
[b] Styrene was also detected as a product (<0.5%).
[c] —, not detected.

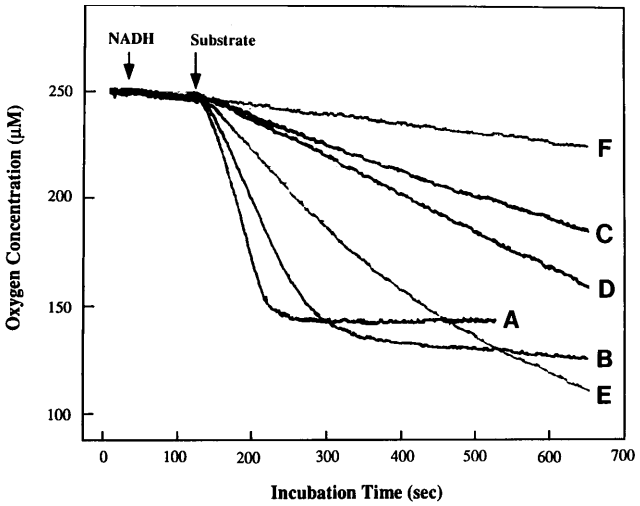

FIG. 2. Oxygen consumption by NDO in the presence of styrene (A), ethylbenzene (B), (*S*)-1-phenethyl alcohol (C), (*R*)-1-phenethyl alcohol (D), acetophenone (E), and 2-hydroxyacetophenone (F). The addition of NADH and substrate are indicated by arrows. Specific experimental conditions are described in Materials and Methods.

which was not further oxidized. These results suggest that ethylbenzene is oxidized first to 1-phenethyl alcohol, then to acetophenone, and finally to 2-hydroxyacetophenone, with a second pathway involving the formation of styrene and 1-phenyl-1,2-ethanediol.

The initial rates of dioxygen consumption in the presence of styrene, ethylbenzene, (*S*)-1-phenethyl alcohol, (*R*)-1-phenethyl alcohol, and acetophenone were 2.80, 1.76, 0.4, 0.28, and 0.8 μmol of O_2 consumed per min per mg of ISP_{NAP}, respectively (Fig. 2). The biphasic dioxygen consumption observed with ethylbenzene (Fig. 2, curve B) is similar to the result observed with toluene (Fig. 1, curve B) and can be explained in the same way. That is, the initial rapid dioxygen consumption is due primarily to the benzylic monooxygenation of ethylbenzene to 1-phenethyl alcohol; this is followed by a slower oxidation of the latter to acetophenone. The minor amount of styrene detected in ethylbenzene oxidation could also occur in the first phase of dioxygen consumption. The dioxygen consumption experiments also showed that (*R*)-1-phenethyl alcohol was a better substrate than the (*S*)-1-phenethyl alcohol. The latter is the major enantiomer formed from ethylbenzene by NDO. In addition, the ratio of dioxygen consumed to acetophenone added was greater than 1:1, and the product, 2-hydroxyacetophenone, was not oxidized further, suggesting that acetophenone hydroxylation is partially uncoupled from dioxygen consumption. The results indicate that the rate-determining step in the oxidation of ethylbenzene to 2-hydroxyacetophenone is the oxidation of 1-phenethyl alcohol to acetophenone. This accounts for the accumulation of the 1-phenethyl alcohol shown in Table 3. Furthermore, styrene was rapidly oxidized (Fig. 2, curve A), which explains why little accumulation of styrene is seen when ethylbenzene is oxidized by NDO.

Oxidation of 1-phenethyl alcohol to acetophenone in the presence of $^{18}O_2$, $H_2{}^{18}O$, and D_2O. The oxidations of 1-phenethyl alcohol and benzyl alcohol to acetophenone and benzaldehyde, respectively (Tables 1 and 3), are novel reactions that have not been reported previously for NDO. The rapid exchange of oxygen in aldehydes prevents the measurement of ^{18}O incorporation into benzaldehyde in the oxidation of benzyl

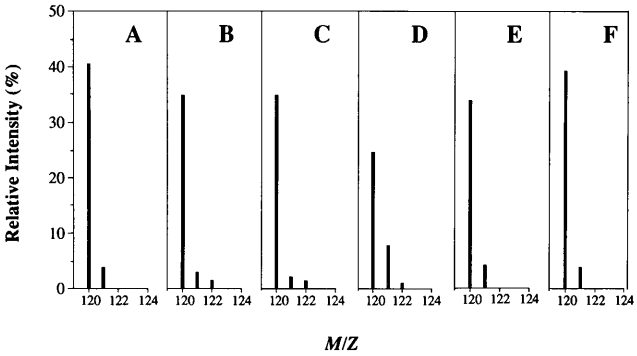

FIG. 3. Mass spectra of acetophenone in the molecular ion region. (A) Acetophenone obtained from 1-phenethyl alcohol by NDO under an atmosphere containing a mixture of $^{18}O_2$-$^{16}O_2$ (85:15); (B) acetophenone formed from 1-phenethyl alcohol by NDO in the presence of 80% $H_2{}^{18}O$; (C) acetophenone incubated in 80% $H_2{}^{18}O$ as a control for the reaction shown in panel B; (D) acetophenone produced from 1-phenethyl alcohol by NDO in the presence of 80% D_2O; (E) acetophenone incubated in 80% D_2O as a control for the reaction shown in panel D; (F) acetophenone standard. Specific experimental conditions are described in Materials and Methods.

alcohol by NDO. Consequently, 1-phenethyl alcohol was used as a substrate to study the role of $^{18}O_2$, $H_2{}^{18}O$, and D_2O in acetophenone formation by NDO.

The mass fragmentation pattern of acetophenone formed from 1-phenethyl alcohol by NDO in the presence of 85% $^{18}O_2$ did not contain a $M^+ + 2$ ion at *m/z* 122 (Fig. 3A), showing that oxygen-18 from $^{18}O_2$ is not incorporated into acetophenone. Although a small portion (3.8% enrichment) of acetophenone contained ^{18}O from $H_2{}^{18}O$ (Fig. 3B), the same amount of ^{18}O incorporation was observed in the control experiment (Fig. 3C). Thus, it can be concluded that ^{18}O from $H_2{}^{18}O$ is not incorporated into acetophenone. This result further suggests that the oxygen atom of acetophenone is not readily exchanged with solvent oxygen under the experimental conditions. In the presence of 80% D_2O, 17% of the acetophenone formed by NDO contained a deuterium atom which was determined by the increase in intensity at *m/z* 121 (Fig. 3D). The normalized enrichment value was 21%. A second experiment gave a normalized enrichment of 15%. When acetophenone was used in a control experiment (Fig. 3E), 2.0% of the acetophenone contained a deuterium atom, which can be explained by keto-enol tautomerization in the presence of 80% D_2O. This result suggests that the oxidation of 1-phenethyl alcohol to acetophenone by NDO occurs in part by desaturation (see Discussion).

DISCUSSION

TDO from *P. putida* F1 oxidizes naphthalene to homochiral *cis*-naphthalene dihydrodiol (10, 40). The reciprocal reaction is not catalyzed by NDO, which, as shown in the present study, oxidizes toluene to benzyl alcohol and benzaldehyde by reactions involving benzylic monooxygenation and dioxygen-dependent alcohol oxidation, respectively (Fig. 4). Recombinant strains expressing xylene monooxygenase encoded by the TOL plasmid pWW0 also oxidize toluene through benzyl alcohol to benzaldehyde (16), and the same reactions are catalyzed by ammonia-grown cells of *Nitrosomonas europaea* (21). Thus, xylene and ammonia monooxygenases may also catalyze dioxygen-dependent alcohol oxidations. It is of interest to note that the NDO of *P. putida* G7 catalyzes the benzylic oxidation of 1,2,4-trimethylbenzene and isomeric dimethylnaphthalenes to yield monols and diols. Although the further oxidation of the alcohols to carboxylic acids was observed, the reactions were

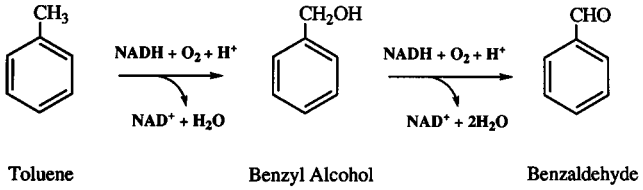

FIG. 4. Oxidation of toluene to benzyl alcohol and benzaldehyde by NDO.

attributed to dehydrogenase(s) in the host strain (35). Nevertheless, it seems likely that NDO from this strain and other pseudomonads expressing NDO activity (1) will oxidize toluene and ethylbenzene to the same products as those described in the present study.

It is of interest to note that NDO catalyzes the oxidation of isomeric xylenes and nitrotoluenes to the corresponding alcohols and aldehydes (Table 2), whereas TDO from *P. putida* F1 oxidizes *m*- and *p*-xylene to *cis*-dihydrodiols and does not oxidize *o*-xylene (7). However, TDOs from *P. putida* F1 and *Pseudomonas* sp. strain JS150 oxidize 2- and 3-nitrotoluene to the corresponding nitrobenzyl alcohols and 4-nitrotoluene to 2-methyl-5-nitrophenol and 3-methyl-6-nitrocatechol (34). The reasons for these similarities and differences between TDO and NDO are not known.

NDO oxidized ethylbenzene sequentially through (*S*)-1-phenethyl alcohol (77% e.e.) and acetophenone to 2-hydroxyacetophenone by reactions involving monooxygenation and dioxygen-dependent alcohol oxidation. In addition, ethylbenzene was oxidized through styrene to (*R*)-1-phenyl-1,2-ethanediol (74% e.e.) by reactions involving desaturation and dihydroxylation, respectively (Fig. 5). The latter pathway is supported by previous studies which have shown that NDO can catalyze the formation of double bonds (desaturation reactions) (8, 31, 40) and the dioxygenation of styrene to yield (*R*)-1-phenyl-1,2-ethanediol in 78.6% e.e. (24). These results also show that the regiospecificity of NDO differs from that of TDO since the latter oxidizes ethylbenzene to (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-ethylcyclohexa-3,5-diene as the major product (9). Ammonia-grown cells of *N. europaea* oxidize ethylbenzene by a sequence of reactions similar to that shown in Fig. 5, with the exception that styrene is oxidized to styrene 1,2-oxide (21).

Alcohol oxidation to carbonyl products is usually mediated

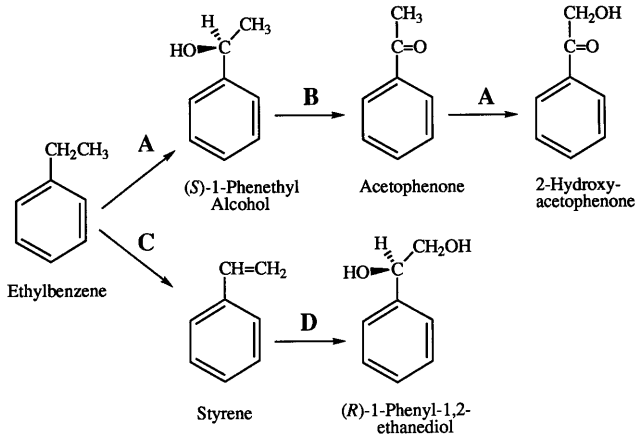

FIG. 5. NDO-catalyzed oxidation of ethylbenzene. Reactions involved are monohydroxylation (A), dioxygen-dependent alcohol oxidation (B), desaturation (C), and dihydroxylation (D).

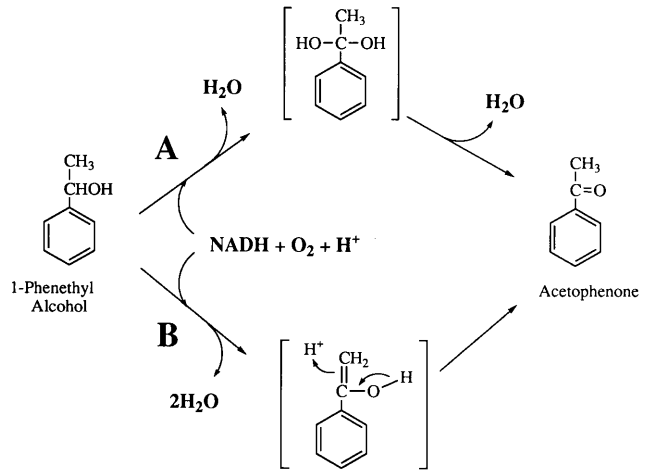

FIG. 6. Proposed pathways for the NDO-catalyzed oxidation of 1-phenethyl alcohol to acetophenone by reactions involving monohydroxylation to form a *gem*-diol intermediate (A) and the formation and subsequent tautomerization of an enol intermediate (B).

by pyridine nucleotide-dependent dehydrogenases. However, NDO catalyzes the NADH-dioxygen-dependent oxidation of benzyl alcohol and 1-phenethyl alcohol to benzaldehyde and acetophenone, respectively (Tables 1 and 3). These reactions are also catalyzed by rabbit liver P-450 (forms 2B4 and 2E1) (41). In the case of 1-phenethyl alcohol oxidation, Vaz and Coon (41) suggested that these forms of P-450 oxidize the alcohol to a carbon radical followed by oxygen rebound to form a *gem*-diol intermediate which undergoes nonstereospecific dehydration to yield acetophenone (Fig. 6A). Another possible mechanism, not utilized by either of the two P-450 forms, involves desaturation to form an enol intermediate followed by tautomerization to acetophenone (Fig. 6B). In principle, pathways A and B can be differentiated by the incorporation of one atom of dioxygen (A) or a proton from the reaction medium (B) into acetophenone. The results showed that oxygen from O_2 or H_2O was not incorporated into acetophenone (Fig. 3). Instead, solvent protons (approximately 16% of the normal enrichment) were incorporated into acetophenone, suggesting that an enol intermediate may be involved in the oxidation of 1-phenethyl alcohol by NDO. Since desaturation does not account for the total reaction, the possible involvement of both reactions (Fig. 6A and B) occurring in the oxidation of 1-phenethyl alcohol cannot be ruled out. Substrates susceptible to desaturation by NDO in most cases undergo significant monohydroxylation. Examples include ethylbenzene oxidation to styrene and 1-phenethyl alcohol (this study), indan oxidation to indene and (1*S*)-indanol (8), and (1*R*)-indanol oxidation to (1*R*)-indenol and *cis*-1,3-indandiol (25). Thus, the desaturation of 1-phenethyl alcohol could be accompanied by monohydroxylation to form a *gem*-diol intermediate. The stereospecific dehydration of putative *gem*-diol intermediates has been proposed for the oxidation of 2,5-dichlorophenol to 3,6-dichlorocatechol (37) and 2-methyl-5-nitrophenol to 3-methyl-6-nitrocatechol (34). These reactions could occur with the aid of a basic amino acid residue in the active site of the enzyme (41). The formation of a *gem*-diol intermediate could also account for the dioxygenase-dependent oxidation of benzyl alcohol to benzaldehyde.

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