

# Stereospecific Dihydroxylation of the Styrene Vinyl Group by Purified Naphthalene Dioxygenase from *Pseudomonas* sp. Strain NCIB 9816-4

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**Naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4 adds both atoms of the dioxygen molecule to styrene to form (*R*)-1-phenyl-1,2-ethanediol. Product formation is tightly coupled to dioxygen consumption and NADH oxidation. NDO oxidizes styrene- $d_8$  at almost the same initial rate as styrene. The results indicate that dioxygen activation by NDO is different from that by cytochrome P-450 and other monooxygenases, which oxidize styrene to styrene 1,2-oxide.**

Bacterial dioxygenases which contain non-heme iron and Rieske-type (2Fe-2S) redox clusters play a crucial role in the initiation of the degradation of many aromatic hydrocarbons. These enzymes add both atoms of the dioxygen molecule to the aromatic ring of the substrate to form *cis*-dihydrodiols (10). One well-known example is the three-component naphthalene dioxygenase (NDO) from *Pseudomonas* sp. strain NCIB 9816-4 (6, 7, 11, 12, 26, 27), which catalyzes the homochiral dihydroxylation of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) in the presence of dioxygen and NAD(P)H (15, 16) (Fig. 1). Recent studies have shown that NDO exhibits a relaxed substrate specificity and catalyzes multiple oxidative reactions which lead to stereospecific monohydroxylation (9, 24, 31), desaturation (9, 23, 29), stereospecific sulfoxidation (18), O dealkylation (23), and N dealkylation (19) with appropriate substrates.

Almost all of the reactions catalyzed by NDO are also catalyzed by cytochrome P-450 (hereafter called P-450). However, P-450 has not been reported to form *cis*-dihydrodiols and NDO has not been reported to form epoxides or catalyze National Institutes of Health shift reactions (5). We used styrene as a substrate for NDO to probe for epoxide formation, since P-450 monooxygenases are known to oxidize styrene to styrene 1,2-oxide (8, 34).

**Identification of reaction products.** The oxidation of styrene by purified NDO components (reductase<sub>NAP</sub>, ferredoxin<sub>NAP</sub>, and ISP<sub>NAP</sub>) (19, 28) was conducted in 2 ml of 50 mM sodium 2-(*N*-morpholino)ethanesulfonate (MES) buffer, pH 6.8, containing NADH (0.5 μmol), Reductase<sub>NAP</sub> (16 μg), Ferredoxin<sub>NAP</sub> (35 μg), ISP<sub>NAP</sub> (50 μg), Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O (0.1 μmol), and styrene (0.25 μmol). After 2 h, an internal standard (25 μl of a 10 mM methanol stock solution of 1-phenethyl alcohol) was added to the reaction mixture, which was then extracted with ethyl acetate, concentrated, and analyzed by gas chromatography-mass spectrometry as described previously (24). Only one product, which eluted at 10.72 min and gave a mass spectrum identical to that given by authentic 1-phenyl-1,2-ethanediol, was detected. This product was purified by thin-layer chromatography (solvent, chloroform-acetone = 8:2, *R<sub>f</sub>* = 0.19), and its enantiomeric composition was determined by chiral stationary-phase-high-performance liquid chromatography (HPLC) on a Chiralcel OB-H column (4.6

mm by 25 cm, 5-μm particle size; Chiral Technologies Inc., Exton, Pa.) (24). Enantiomers were eluted with hexane and 2-propanol (9:1) at a flow rate of 0.5 ml/min, the effluent was monitored at 254 nm, and the areas under each peak were integrated. (*R*)-1- and (*S*)-1-phenyl-1,2-ethanediol eluted at 15.8 and 19.4 min, respectively. Chiral stationary-phase-HPLC analysis showed the product to be (*R*)-1-phenyl-1,2-ethanediol in 78.6% enantiomeric excess. This value was obtained from the average of three independent measurements.

**Reaction rate and stoichiometry.** The rate of formation of 1-phenyl-1,2-ethanediol was determined by dioxygen consumption and NADH oxidation. Dioxygen consumption was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) as previously described (18). Reaction mixtures contained, in 1.0 ml of 50 mM MES buffer, pH 6.8, NADH (0.25 μmol), Reductase<sub>NAP</sub> (4 μg), Ferredoxin<sub>NAP</sub> (14 μg), ISP<sub>NAP</sub> (25 μg), and Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O (0.1 μmol). Reactions were initiated by the addition of 0.1 μmol of styrene. NADH oxidation was determined by measuring the decrease in *A*<sub>340</sub>. The reaction mixtures were the same as those used for dioxygen consumption. An extinction coefficient of 6.22 mM<sup>-1</sup>

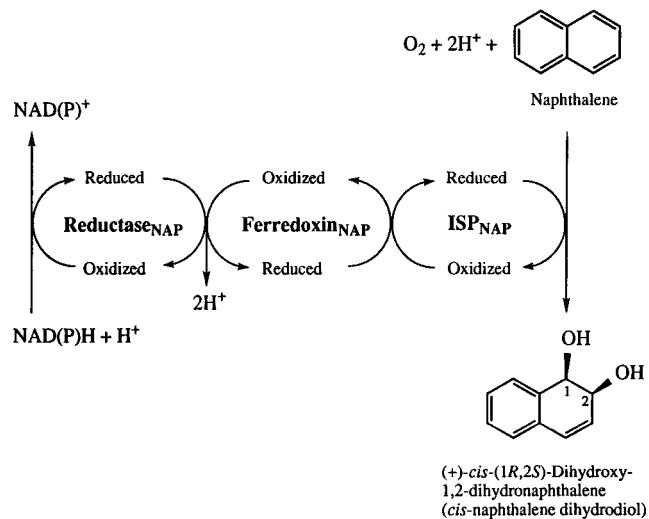


FIG. 1. Proposed electron transport chain and its role in the oxidation of naphthalene by NDO.

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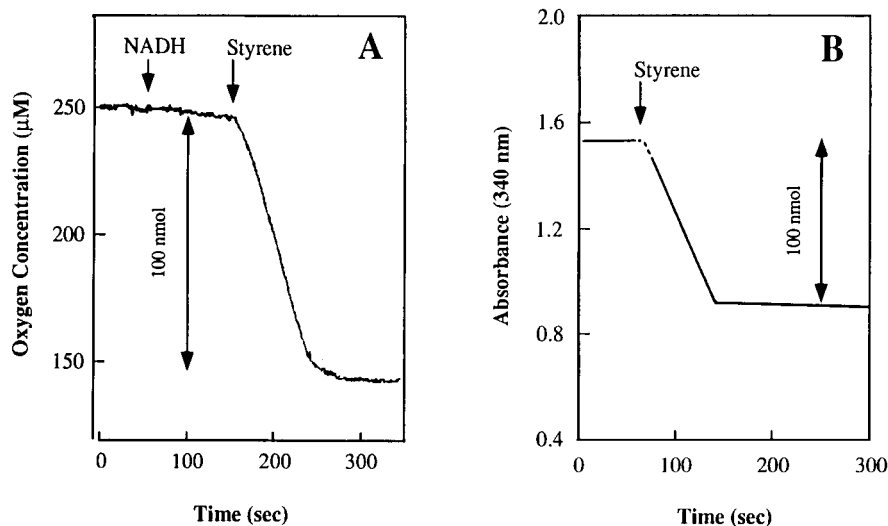


FIG. 2. Oxygen consumption (A) and NADH oxidation (B) by NDO determined in the presence of styrene. Additions of reactants are indicated by arrows.

$\text{cm}^{-1}$  was used to quantitate the amount of NADH oxidized. The initial rates of dioxygen consumption and NADH oxidation were  $2.8 \mu\text{mol}$  of dioxygen utilized per min per mg of  $\text{ISP}_{\text{NAP}}$  (Fig. 2A) and  $3.2 \mu\text{mol}$  of NADH utilized per min per mg of  $\text{ISP}_{\text{NAP}}$  (Fig. 2B), respectively. In both experiments, dioxygen consumption and NADH oxidation were tightly coupled to styrene oxidation. Stoichiometric measurements showed that 1 mol each of styrene, NADH, and dioxygen are required to form 1 mol of 1-phenyl-1,2-ethanediol.

**Oxidation of deuterated styrene.** When styrene- $d_8$  (98% atom% D; Aldrich Chemical Co., Milwaukee, Wis.) was used as a substrate for NDO, only one product was detected by gas chromatography-mass spectrometry. The product eluted at 10.66 min and gave a molecular ion,  $\text{M}^+$  (% relative intensity), at  $m/z$  146 (9.3), with major fragment ions at  $m/z$  113 (100, loss of  $\text{CD}_2\text{OH}$ ) and  $m/z$  85 (83, loss of CO from  $m/z$  113 species). The spectrum is identical to that of 1-phenyl-1,2-ethanediol

except for the increase in mass due to deuterium atoms. The initial rate of formation of deuterio 1-phenyl-1,2-ethanediol was determined by dioxygen consumption and NADH oxidation, and values of  $2.9 \mu\text{mol}$  of dioxygen utilized per min per mg of  $\text{ISP}_{\text{NAP}}$  and  $3.1 \mu\text{mol}$  of NADH utilized per min per mg of  $\text{ISP}_{\text{NAP}}$ , respectively, were obtained. Stoichiometric amounts of dioxygen and NADH were utilized in the oxidation of styrene- $d_8$  to deuterio 1-phenyl-1,2-ethanediol.

**$^{18}\text{O}_2$  incorporation.**  $^{18}\text{O}_2$  incorporation experiments were conducted as previously described (24) in an atmosphere containing  $^{16}\text{O}_2$  and  $^{18}\text{O}_2$  in a ratio of 34:66. Gas chromatography-mass spectrometry analysis of the 1-phenyl-1,2-ethanediol formed showed a ratio of  $\text{M}^+$  to  $(\text{M}^+ + 4)$  of 38:62. A  $\text{M}^+ + 2$  ion was not detected (Fig. 3). This result, together with the results obtained from dioxygen consumption experiments, indicates that both atoms of oxygen in the product originate from a single dioxygen molecule. Thus, the direct formation of

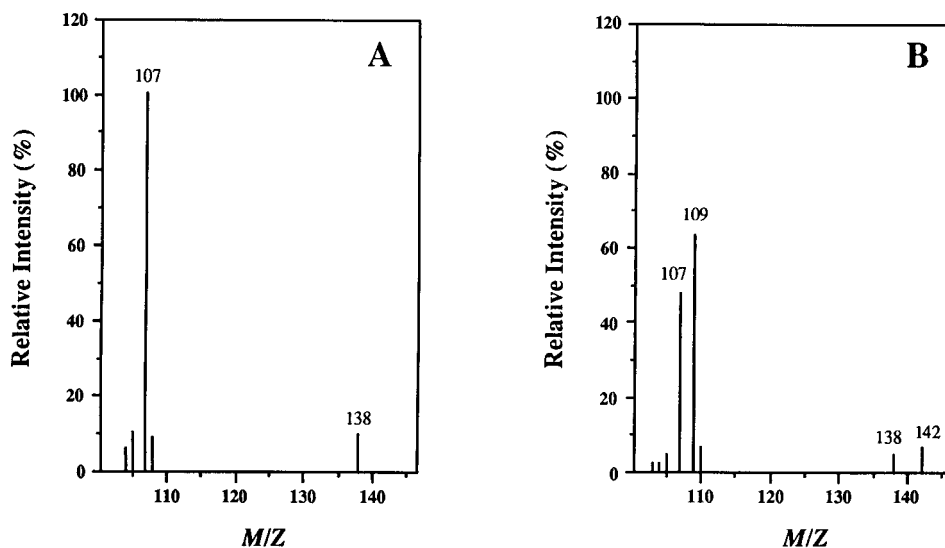


FIG. 3. Mass spectra of 1-phenyl-1,2-ethanediol formed from styrene by purified NDO under air (A) and an atmosphere containing a mixture of  $^{16}\text{O}_2$  and  $^{18}\text{O}_2$  (34:66) (B).

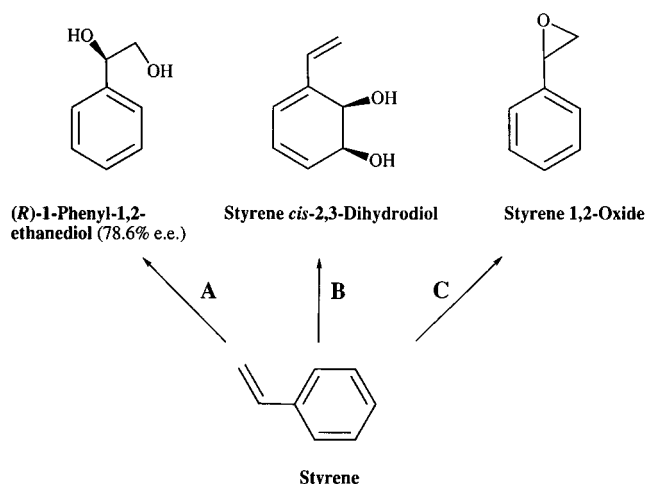


FIG. 4. Oxidation of styrene by dioxygenases, monooxygenases, and peroxidases. (A) Vinyl *cis* dihydroxylation by NDO (this study); (B) benzene ring *cis* dihydroxylation by toluene (14, 30) and styrene (1, 32) dioxygenases; (C) vinyl epoxidation by P-450 monooxygenases (8, 34), cytochrome *c* peroxidase (21), chloroperoxidase (4, 22), bacterial styrene monooxygenases (13, 20), and soluble methane (2, 3, 36) and ammonia (17) monooxygenases. e.e., enantiomeric excess.

1-phenyl-1,2-ethanediol from styrene by NDO can be distinguished from the formation of the same compound by mammalian liver. In hepatic microsomes, styrene is oxidized by P-450 monooxygenases to styrene 1,2-oxide, which serves as a substrate for epoxide hydrolase to yield 1-phenyl-1,2-ethanediol (34). In the latter reaction, oxygen from  $H_2O$  yields one of the hydroxyl groups in the product. Styrene 1,2-oxide was not a substrate for NDO (data not shown).

Figure 4 shows the products formed from styrene oxidation catalyzed by dioxygenases, monooxygenases, and peroxidases. NDO catalyzes the oxidation of styrene by direct addition of activated dioxygen to the vinyl  $\pi$ -bond (Fig. 4, reaction A). A similar result was previously reported for the oxidation of 4-vinylbenzoate to 4-glycylbenzoate by 4-methoxybenzoate monooxygenase (35). A recent thorough review on the microbial metabolism and biotransformation of styrene (33) cites only one report on the oxidation of styrene to 1-phenyl-1,2-ethanediol (25). In this case, the diol is thought to arise from the hydrolysis of styrene 1,2-oxide. *P. putida* (1) and *Rhodococcus rhodochrous* NCIMB 13259 (32) both initiate styrene oxidation at the aromatic nucleus to form (–)-*cis*-(2*R*,3*S*)-dihydroxy-2,3-dihydrostyrene (Fig. 4, reaction B). *P. putida* 39/D also catalyzes the formation of a styrene *cis*-dihydrodiol (30), and Hudlicky et al. observed changes in the regioselectivity and stereoselectivity of the toluene dioxygenase in this organism when chlorinated styrenes substituted at different ring positions were used as substrates (14). These results show that this family of dioxygenases catalyzes the dihydroxylation of  $\pi$ -bond systems, with regioselectivity being determined by the position of the  $\pi$ -bond. Oxygenases that form styrene 1,2-oxide include hepatic and bacterial P-450 isozymes (8, 34), cytochrome *c* peroxidase (21), chloroperoxidase (4, 22), bacterial styrene monooxygenases (13, 20), soluble methane monooxygenases from *Methylococcus capsulatus* (Bath) (3, 36) and *Methylosinus trichosporium* OB3b (2), and ammonia monooxygenase from *Nitrosomonas europaea* (17) (Fig. 4, reaction C). Thus, the mechanism of dioxygen activation by NDO and related dioxygenases is quite different from that of the enzymes catalyzing reaction C in Fig. 4 and should account for *cis* dihydroxylation, as well as typical P-450 reactions (monohy-

droxylation, desaturation, O and N dealkylation, and sulfoxidation).

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