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STEREOSPECIFIC SULFOXIDATION BY TOLUENE AND NAPHTHALENE DIOXYGENASES

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SUMMARY: Studies on the sulfoxidation of aryl alkyl sulfides by purified toluene dioxygenase and naphthalene dioxygenase showed that naphthalene dioxygenase produces sulfoxides of (*S*) absolute configuration in high enantiomeric purity while those formed by toluene dioxygenase are of variable enantiomeric purity depending on the *p*-substituents on the benzene ring. Oxygen uptake experiments with naphthalene dioxygenase showed that the reaction rate and degree of oxygen incorporation are affected by both aryl and alkyl substituents. $^{18}O_2$-Incorporation experiments showed that the oxygen atom of methyl phenyl sulfoxide formed by toluene dioxygenase and naphthalene dioxygenase is derived exclusively from O_2. Accompanying studies showed that chloroperoxidase produces single (*R*)-sulfoxides (>98% enantiomeric excess) from the aryl alkyl sulfides examined in the present study.

Toluene dioxygenase (TDO) from *Pseudomonas putida* F1 (1) and naphthalene dioxygenase (NDO) from *Pseudomonas* sp. NCIB 9816-4 (2) are the initial enzymes involved in the aerobic catabolism of toluene and naphthalene, respectively. In both enzyme systems the oxygenase components are iron-sulfur proteins (ISP_{TOL} and ISP_{NAP}) which have an $\alpha_2\beta_2$ subunit composition (1, 2). The large (α) subunits each contain a Rieske-type [2Fe-2S] center and mononuclear iron (3, H. Jiang and D.T. Gibson, unpublished observations). The exact function of the small (β) subunits are not known. ISP_{TOL} and ISP_{NAP}, in the presence of NAD(P)H and two other electron transport proteins, catalyze the enantiospecific addition of dioxygen (O_2) to their respective substrates to form, in most cases, homochiral *cis*-dihydrodiols. It is now known that both dioxygenases are capable of oxidizing a variety of compounds by reactions involving stereospecific *cis*-dihydroxylation (4, 5) and monohydroxylation (6, 7). Other reactions include desaturation (8, 9), *O*-dealkylation (8), dechlorination (10, 11), *N*-dealkylation, and O_2-dependent alcohol oxidation (K. Lee and D.T. Gibson, unpublished observations).

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Abbreviations: TDO, toluene dioxygenase; NDO, naphthalene dioxygenase; HPO, horseradish peroxidase; CPO, chloroperoxidase; GC-MS, gas chromatography-mass spectrometry; CSP-HPLC, chiral-stationary phase high-performance liquid chromatography; e.e., enantiomeric excess.

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We now report the stereospecific oxidation of aryl alkyl sulfides (thioanisole and its derivatives) to sulfoxides by purified TDO and NDO and compare the results obtained when the same substrates are oxidized by horseradish peroxidase (HPO) and chloroperoxidase (CPO). The highly selective formation of (*S*)-aryl alkyl sulfoxides by NDO contrasts with previous reports that racemic or (*R*)-aryl alkyl sulfoxides are produced by oxygenases (for a review see 12). Asymmetric sulfoxidations by intact cells of different bacteria expressing TDO and NDO have been recently reported (13). However, the present study with purified enzymes provides definitive proof that the oxygenase components of TDO and NDO can function as sulfoxidases.

MATERIALS AND METHODS

Materials. Aryl alkyl sulfides were obtained from Aldrich Chemical Co., Milwaukee, WI. [^{18}O]-O_2 (98 atom %) was from Icon Isotopes, Summit, NJ. Chloroperoxidase (CPO) from *Caldariomyses fumago* (RZ: approx. 0.6) and horseradish peroxidase (HPO) type II (RZ: 1.5-2.0) were from Sigma Chemical Company, St. Louis, MO. TDO and NDO components were purified to homogeneity from recombinant *E. coli* strains (14, 15). Details of the purification procedures will be published elsewhere.

Enzymatic oxidation procedures. Transformations of aryl alkyl sulfides were conducted in capped 15-ml Corning polyethylene tubes. Reaction mixtures for CPO and HPO contained in 2.0 ml of 50 mM sodium citrate buffer (pH 5.0), 1.0 mM H_2O_2 and CPO (72 µg of protein) or HPO (1 mg of protein). Reaction mixtures for TDO and NDO contained in 2 ml of 50 mM sodium 2-(*N*-morpholino)ethane sulfonate (MES) buffer (pH 6.8), 1.0 mM NADH, 0.25 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and the respective reductase (20 µg of protein), ferredoxin (35 µg of protein) and ISP (50 µg of protein) components of TDO or NDO. Reactions were initiated by the addition of 20 µl of 50 mM substrate in methanol to give a final substrate concentration of 0.5 mM. Tubes were incubated horizontally with agitation (60 rpm) at 23°C for 2 h with the exception of reaction mixtures containing methyl *p*-nitrophenyl sulfide. Due to lower transformation efficiencies this substrate was incubated with each enzyme for 5 h.

Oxygen uptake studies. Oxygen uptake by NDO was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) equipped with a temperature-controlled water bath and a magnetic stirrer. Reactions were conducted in air-saturated 50 mM MES buffer (pH 6.8). The agitation rate was approximately 200 rpm at 24°C and the dissolved oxygen concentration was taken as 250 µM (16). Each reaction mixture contained in 0.8 ml of 50 mM MES buffer (pH 6.8), 0.25 mM NADH, $reductase_{NAP}$ (6 µg of protein), $ferredoxin_{NAP}$ (15 µg of protein), and ISP_{NAP} (25 µg of protein). Reactions were initiated by the addition of 4 µl of a 25 mM solution of the substrate in methanol. $^{18}O_2$-Incorporation experiments were conducted as described previously (7).

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 previously described (7). Under these conditions the retention times of methyl phenyl sulfoxide, ethyl phenyl sulfoxide, methyl *p*-tolyl sulfoxide, *p*-methoxyphenyl methyl sulfoxide, and methyl *p*-nitrophenyl sulfoxide were 10.6, 12.3, 12.7, 14.8, and 16.2 min, respectively. Relative yields of products were determined by integration of total ion current peak areas. For example, a 100% relative yield indicates that no substrate was detected at the end of the reaction period. Derivatization with phenylboronic acid was carried out by mixing equal volumes of reaction products and phenylboronic acid in ethyl acetate at room temperature for five minutes. Products were identified by GC-MS as previously described (7).

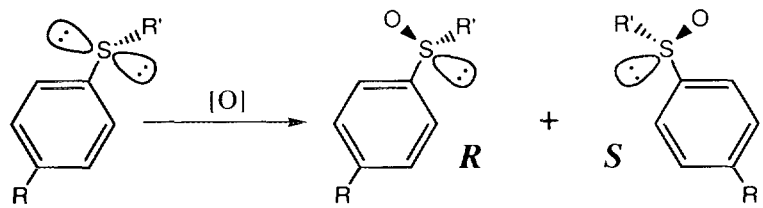
Determination of metabolite chirality. The enantiomeric compositions of the sulfoxide reaction products were determined by chiral stationary phase-high performance liquid chromatography (CSP-HPLC, 7) on a Chiralcel OB-H column (4.6 mm x 25 cm, 5 µm particle size, obtained from Chiral Technologies Inc., Exton, PA). Sulfoxides were eluted from the column with hexane and 2-propanol (9:1, vol/vol) at a flow rate of 1.0 ml/min. The column effluent was monitored at 254 nm and the areas under each peak were integrated. Analysis of the enantiomeric composition of methyl *p*-nitrophenyl sulfoxide was achieved with hexane and 2-

propanol (6:4, vol/vol) at a flow rate of 0.5 ml/min. Under these conditions, the retention volumes of the *R* and *S* enantiomers of methyl phenyl sulfoxide, ethyl phenyl sulfoxide, methyl *p*-tolyl sulfoxide, *p*-methoxyphenyl methyl sulfoxide, and methyl *p*-nitrophenyl sulfoxide were 37 and 21.8 ml, 30.8 and 14.3 ml, 39.6 and 15.6 ml, 71.3 and 29.2 ml, and 23.1 and 18.4 ml, respectively.

RESULTS

TDO and NDO, with one exception, preferentially oxidized the sulfur atoms of the aryl alkyl sulfides used in this study (Table 1). The sulfoxides were identified by comparison of their GC-MS properties with the sulfoxides formed by commercial preparations of HPO and CPO. Both hemoproteins are known to catalyze sulfoxidation of aryl alkyl sulfides (17-19). In addition, the use of CSP-HPLC to determine the enantiomeric composition of the sulfoxides formed by HPO and CPO provided a convenient procedure for determining the enantiomeric composition of the sulfoxides formed by TDO and NDO. For example, under the conditions used in the present study, HPO catalyzed the oxidation of the aryl alkyl sulfides to essentially racemic sulfoxides (Table 1). These results are in accord with previous reports (17, 18), with the exception of the stereospecific oxidation by HPO type IV which yielded (*S*)-sulfoxides in 60-70% e.e. (19). The identity of the sulfoxide enantiomers formed by HPO were confirmed by CSP-HPLC of the sulfoxides formed by CPO. Previous studies have shown that CPO oxidizes aryl alkyl sulfides to

Table 1. Stereospecific sulfoxidation of aryl alkyl sulfides by heme-containing peroxidases and Rieske [2Fe-2S] cluster, mononuclear iron-containing dioxygenases[1]



Substrate	Enantiomeric Excess (% e.e.) (Relative Yield, %)			
	Toluene Dioxygenase	Naphthalene Dioxygenase	Horseradish Peroxidase	Chloro-peroxidase
Methyl phenyl sulfide (R=H, R'=CH_3)	R > 98 (100)	S > 98 (100)	0 (100)	R > 98 (100)
Ethyl phenyl sulfide (R=H, R'=CH_2CH_3)	R > 98 (100)	S = 93 (100)	R = 16 (100)	R > 98 (100)
Methyl *p*-tolyl sulfide (R=CH_3, R'=CH_3)	S = 38 (35.3)[2]	S > 98 (100)	S = 6 (100)	R > 98 (100)
p-Methoxyphenyl methyl sulfide (R=OCH_3, R'=CH_3)	S = 32 (17)	S > 98 (100)	S = 6 (72.4)	R > 98 (100)
Methyl *p*-nitrophenyl sulfide (R=NO_2, R'=CH_3)	S = 86 (3)	S > 98 (100)	R = 10 (2.3)	R > 98 (4.8)

[1] Details of the reaction conditions and product analyses are given under Materials and Methods.

[2] The major product (62%) was identified as methyl *p*-tolyl sulfide *cis*-dihydrodiol.

(*R*)-sulfoxides (39-92% e.e.) (18). However, Table 1 shows that CPO oxidized the listed aryl alkyl sulfides almost exclusively to the (*R*)-enantiomers (>98% e.e.). The lower enantiomeric purities reported by Colonna et al. (18) may be due to longer incubation times (4 to 10 days), suboptimal incubation temperatures (4°C) or the methods used to determine enantiomeric purity (^{1}H-NMR and optical rotation).

TDO catalyzed the stereospecific oxidation of methyl phenyl sulfide and ethyl phenyl sulfide to enantiomerically pure (*R*)-sulfoxides (Table 1). In contrast, substituents at the *para*-position reduced not only reaction rate but also stereoselectivity. Thus, the electron withdrawing *p*-nitro substituent gave a low product yield with a high percentage of the (*S*)-enantiomer. GC-MS analyses of the products formed from methyl *p*-tolyl sulfide showed the presence of three compounds with molecular ions (M^+) at *m/z* 154, 154, and 172 in addition to methyl *p*-tolyl sulfoxide (M^+, *m/z* 154). The three compounds represented 62% of the total product yield and suggested that the major product was a 2,3-dihydrodiol which had undergone elimination of water, during work up or in the GC injection port, to yield phenols at the 2 and 3 positions. The formation of a *cis*-dihydrodiol was confirmed by the formation of the monobenzene boronate derivative (M^+, *m/z* 258) shown in Figure 1. This derivatization procedure led to the disappearance of the three compounds with molecular ions (M^+) at *m/z* 154, 154, and 172. These results suggest that the major product formed from methyl *p*-tolyl sulfide is the *cis*-dihydrodiol (*cis*-1,2-dihydroxy-3-methyl-6-methylthiocyclohexa-3,5-diene). The absolute stereochemistry of this reaction product was not determined.

NDO oxidized the aryl alkyl sulfides to the corresponding sulfoxides with an (*S*) absolute configuration. All of the sulfoxides were obtained in 100% yield and were of high enantiomeric

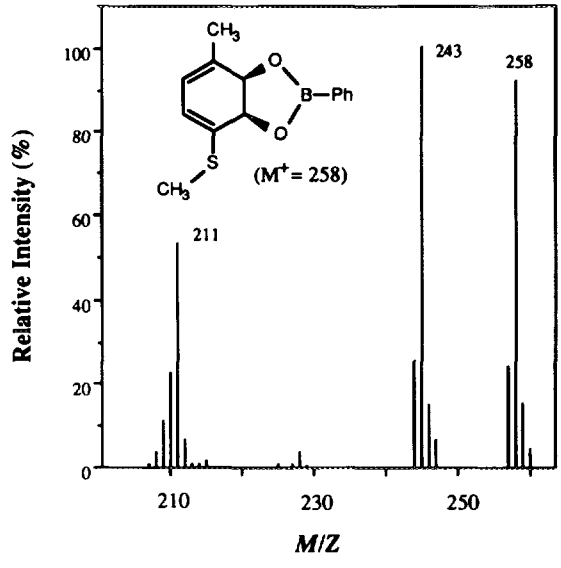


Fig. 1. Mass spectrum of the phenylboronic acid derivative of methyl *p*-tolyl sulfide *cis*-dihydrodiol formed by toluene dioxygenase (absolute stereochemistry is not intended).

purity (Table 1). Studies on the rate of formation of the sulfoxides was determined polarographically (Fig. 2). The initial rates of oxygen consumption in the presence of methyl phenyl sulfide, ethyl phenyl sulfide, and methyl *p*-tolyl sulfide were 1.7 μmol/min/mg ISP_{NAP}. The same oxidation rate was observed with naphthalene (data not shown). Oxygen uptake rates observed with *p*-methoxyphenyl methyl sulfide and methyl *p*-nitrophenyl sulfide were 1.56 and 0.90 μmol/min/mg ISP_{NAP}, respectively. In addition, the ratio of oxygen consumed to substrate added was greater than 1:1 for ethyl phenyl sulfide and methyl *p*-nitrophenyl sulfide. These observations indicate that substrate oxidation is partially uncoupled from oxygen consumption in the presence of these compounds.

When methyl phenyl sulfide was oxidized by purified TDO and NDO in the presence of $^{18}O_2$ ($^{18}O_2$:$^{16}O_2$ = 91:9) the ratios of the molecular ion ($M^+ + 2$) and (M^+) peaks at *m/z* 142 and 140 were 92:8 for both enzymes. These results show that the oxygen atom in methyl phenyl sulfoxide is derived exclusively from dioxygen.

DISCUSSION

The sulfoxidation of aryl alkyl sulfides adds to the growing list of reactions catalyzed by NDO and TDO (4-11). Other reactions include *N*-dealkylation and O_2-dependent alcohol oxidations (K. Lee and D.T. Gibson, unpublished results). The oxygenation of the pro-(*R*) or pro-(*S*) lone pair of electrons on the sulfide can be determined by the position of the substrate in the active site (regiospecificity) and the mobility of the substrate within the active site (stereospecificity) (20). Thus the sulfur atoms, rather than the aromatic or alkyl groups of the substrates shown in Table 1, appear to be in close proximity to the reactive oxygen species at the

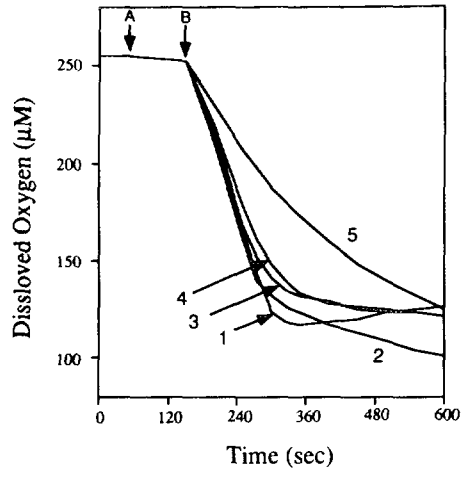


Fig. 2. Oxygen uptake by naphthalene dioxygenase determined in the presence of methyl phenyl sulfide (1), ethyl phenyl sulfide (2), methyl *p*-tolyl sulfide (3), *p*-methoxyphenyl methyl sulfide (4), and methyl *p*-nitrophenyl sulfide (5). Additions of NADH (A) and substrate (B) are indicated by arrows.

active site of both enzymes. The only exception is the formation of a sulfoxide and a *cis*-dihydrodiol from methyl *p*-tolyl sulfide by TDO. These results suggest that this substrate can adopt two orientations with respect to the reactive oxygen species responsible for the oxygenation reaction.

The results obtained with NDO suggest that the aryl alkyl substituents and the physiological substrate naphthalene, may occupy similar positions in the active site of the enzyme. This would account for the consistent formation of homochiral (*S*)-sulfoxides in high yields from the sulfides listed in Table 1. In a recent communication, naphthalene-grown cells of *Pseudomonas putida* strain NCIMB 8859 were reported to form (*S*)-sulfoxides from methyl phenyl sulfide and ethyl phenyl sulfide (13). Thus it is possible that this organism may contain a similar enzyme to the NDO purified from *Pseudomonas* sp. NCIB 9816-4. Oxygen uptake studies with purified NDO (Fig. 2) show that, with the exception of ethyl phenyl sulfide and methyl *p*-nitrophenyl sulfide, the enantiospecific oxygenation reactions are tightly coupled with respect to product formation.

TDO catalyzes the oxidation of methyl phenyl sulfide and ethyl phenyl sulfide to the corresponding (*R*)-sulfoxides (>98% e.e.). High yields of both sulfoxides were obtained (Table 1). Similar results were recently reported for TDO expressed by intact cells of *P. putida* strain UV4 and a recombinant *E. coli* strain (13). In these organisms, increasing the size of the alkyl substituent from methyl to isopropyl greatly reduced the yield of sulfoxides without significantly affecting their enantiomeric purity. The effect of larger alkyl substituents on sulfoxidation by purified TDO and NDO was not addressed in the present study. However, substituents at the *para*-position of the phenyl ring produced a remarkable change in the yields and enantiomeric purity of the sulfoxides formed by TDO. Substrates with electron-withdrawing (NO_2) and electron-donating (OCH_3) groups both yielded low amounts of sulfoxides with an excess of the (*S*)-enantiomer. Thus it appears that steric factors related to the phenyl ring may play a large role in determining the enantiospecificity of TDO. In contrast, *para*-substituents of the phenyl ring did not significantly affect the yield or the enantiomeric purity of the sulfoxides formed by NDO.

Sulfoxidation reactions are catalyzed by a diverse group of oxygenases. These include, cytochrome P-450s (21), heme-containing peroxidases (17-19), and copper-containing dopamine β-hydroxylase (22). All of these enzymes utilize free radical mechanisms to produce their respective sulfoxide products. Many of the reactions catalyzed by cytochrome P-450s including, monohydroxylation, *N*- and *O*-dealkylation, desaturation, O_2-dependent alcohol oxidation and sulfoxidation are also catalyzed by NDO and, to a lesser extent TDO. However, the oxygenase components of NDO and TDO which contain Rieske [2Fe-2S] centers and are thought to contain mononuclear iron at their respective active sites differ from cytochrome P-450s in their reactions with substrates containing π electrons. The dioxygenases produce chiral *cis*-dihydrodiols whereas cytochrome P-450s oxidize many of the same substrates to epoxides or monols. Current studies are directed towards identification of the reactive oxygen species generated by TDO and NDO which will further our understanding of the diverse oxidative reactions catalyzed by these unique dioxygenases.

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