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Stereoselective Sulfoxidation of a Series of Alkyl *p*-Tolyl Sulfides by Microsomal and Purified Flavin-Containing Monooxygenases

ALLAN E. RETTIE, BRIAN D. BOGUCKI, INA LIM, and G. PATRICK MEIER

Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, Washington 98195

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SUMMARY

The enantioselective sulfoxidation of a series of alkyl *p*-tolyl sulfides was compared using purified rabbit lung and mini-pig liver flavin-containing monooxygenase (FMO). Analysis was performed by chiral-phase high pressure liquid chromatography, which afforded baseline resolution of each pair of enantiomers. The extent of enantioselective sulfoxidation was found to be a function of (a) the isozyme employed, (b) the steric bulk of the alkyl substituent, and (c) pH. At pH 8.5, rabbit lung FMO catalyzed the oxidation of methyl, ethyl, propyl, and isopropyl sulfides to products with >99, 91, 85, and 63% (*R*)-(+)-stereochemistry, respectively. Corresponding values for the mini-pig liver form were 91, 82, 72, and 41% (*R*)-(+)-sulfoxide. The stereochemical profile obtained with the isolated rabbit lung form could be duplicated exactly in microsomal preparations if precautions were taken to abolish the contribution that P-450 makes to net stereochemistry. It was noted that increasing the reaction mixture pH from 8.5 to 10 led to a decrease in the stereochemical purity of products obtained from the lung form. In contrast, the stereochemical profile obtained with the isolated mini-pig liver form could not be exactly duplicated in suitably treated microsomal preparations. No evidence for multiple forms of mini-pig liver FMO was obtained, and it was concluded that discrepancies between microsomal and purified FMO metabolic profiles were most consistent with a minor modification to active site geometry occurring during purification of the mini-pig form. These data show that the active site chirality of rabbit lung and mini-pig liver FMO is largely retained following removal from microsomal membranes. Qualitative similarities in the structure-activity relationships exhibited by microsomal or purified FMO from rabbit lung and mini-pig liver suggest some conservation of active site geometry between these two otherwise distinct FMOs. Quantitative differences in the structure-activity relationships exhibited by the two FMO forms indicate that analysis of product stereochemistry may be a useful method for the discrimination of catalytically distinct FMO isozymes.

The FMO (EC 1.14.13.8) was first purified from hog liver by Ziegler and Mitchell (1). It is a membrane-bound protein that functions as an oxygenase at sulfur, nitrogen, selenium, and phosphorus centers (2). FMO and the P-450 monooxygenase, also located in the endoplasmic reticulum, are the major enzymes responsible for xenobiotic oxidation.

Recently, it has been shown that the FMO system, like the P-450 monooxygenase system, is composed of multiple forms of the enzyme (see Ref. 3 for a recent review). The rabbit lung form of the enzyme has been shown to be radically different from the hog liver form (4, 5). The two forms are immunochemically distinct, possess different substrate specificities, are differentially stable to heat and sodium cholate, and are differentially activated by octylamine. Mouse lung FMO is also distinct from the murine liver form and, like rabbit lung FMO, has a characteristic ability to utilize primary aliphatic amines as substrates (6, 7). Differences between pulmonary forms are also apparent, since the rabbit lung enzyme has some unique substrate preferences that differ from those of mouse pulmonary FMO (8). Moreover, catalytic studies suggest that the rabbit and the rat lung forms of FMO are separate enzymes (9). More recent studies (10, 11) have also raised the possibility of intratissue multiplicity. Therefore, the characteristics of the FMO appear to be tissue dependent within a species and to be species dependent within the same tissue.

Numerous studies have shown that FMO isozymes can oxidize prochiral substrates, such as ethyl *p*-tolyl sulfide and phorate, to products with high stereochemical purity (12, 13). Moreover, Light *et al.* (12) have shown that the hog liver form and its prokaryotic homolog from *Acetinobacter NCIB9871* oxidize ethyl *p*-tolyl sulfide to sulfoxide products that possess opposing stereochemistry. An extension of this approach may have promise regarding the differentiation of isozymic forms of FMO. To evaluate this possibility, we have investigated the enantioselective sulfoxidation of a series of alkyl *p*-tolyl sulfides by rabbit lung and mini-pig liver FMO. We find that the degree of enantioselective sulfoxidation is isozyme, pH, and substrate

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ABBREVIATIONS: FMO, flavin-containing monooxygenase; HPLC, high pressure liquid chromatography; P-450, cytochrome P-450; IPA, isopropyl alcohol; ABT, 1-aminobenzotriazole.

dependent and that, although the stereoselectivity observed for purified lung FMO is identical to that seen in microsomal membranes, this is not the case for our preparation of mini-pig liver FMO. The potential of this approach for discriminating between FMO isozymes and for mapping active site topography is discussed.

Materials and Methods

Chemicals. Octyl-Sepharose, DEAE-Sepharose, dye affinity columns, and 2,′5′-ADP-agarose were purchased from Sigma. Hydroxyapatite was purchased from Bio-Rad. DE52 was purchased from Whatman. The (+)- and (−)-*p*-tolyl menthyl sulfinates, dimethylaniline, and thiocresol were purchased from Aldrich. ABT was a generous gift from Dr. W. A. Garland., Hoffman-La-Roche, Nutley, NJ. Dimethylaniline *N*-oxide hydrate was a generous gift from Dr. R. F. Lawrence, University of Washington, Seattle, WA.

Synthesis. The alkyl *p*-tolyl sulfides were synthesized by the following general procedure. A solution of 10.0 mmol of *p*-thiocresol (4-methyl-thiophenol) in 10 ml of methanol was cooled to 0° under N_2 and 1.0 g of K_2CO_3, followed by 12.5 mmol of the appropriate alkyl iodide, was added. The mixture was stirred and allowed to come to room temperature overnight. The product was isolated by quenching the mixture into 40 ml of 10% NaOH and extracting with methylene chloride (three times with 10 ml each). The combined organic phase was dried over K_2CO_3 and filtered, and the solvent was evaporated to yield the sulfides. Purification by chromatography (SiO_2/hexanes) gave the following products. Methyl *p*-tolyl sulfide (99% yield): b.p. 90–95° at 30 mm Hg [literature value (14), 94° at 31 mm Hg]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.18 (2 H, d, J = 8.0 Hz), 7.10 (2 H, d, J = 8.0 Hz), 2.47 (3 H, s), and 2.32 (3 H, s). Ethyl *p*-tolyl sulfide (98% yield): b.p. 103–105° at 15 mm Hg [literature value (15), b.p. 105° at 15 mm Hg]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 (2 H, d, J = 8.0 Hz), 7.10 (2 H, d, J = 8.0 Hz), 2.90 (2 H, q, J = 7.4 Hz), 2.32 (3 H, s), and 1.28 (3 H, t, J = 7.4 Hz). Propyl *p*-tolyl sulfide (99% yield): b.p. 107–111° at 15 mm Hg [literature value (16), b.p. 242° at 243 mm Hg]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 (2 H, d, J = 8.2 Hz), 7.10 (2 H, d, J = 8.2 Hz), 2.85 (2 H, t, J = 7.3 Hz), 2.31 (3 H, s), 1.64 (2 H, hex., J = 7.3 Hz), and 1.00 (3 H, t, J = 7.3 Hz). Isopropyl *p*-tolyl sulfide (94% yield): b.p. 106–113° at 15 mm Hg [literature value (14), b.p. 110° at 14 mm Hg); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31 (2 H, d, J = 7.8 Hz), 7.11 (2 H, d, J = 7.8 Hz), 3.30 (1 H, sept., J = 6.8 Hz), 2.33 (3 H, s), and 1.27 (6 H, d, J = 6.8 Hz).

The racemic alkyl *p*-tolyl sulfoxides were prepared according to the general procedure of Leonard and Johnson (17). A solution of 1.0 mmol of the sulfide in 10 ml of methanol was cooled to 0° and a solution of 1.25 mmol of $NaIO_4$ in 2 ml of deionized water was added. The mixture was allowed to stir at 0° until all of the sulfide was consumed, as indicated by analytical thin layer chromatography. The reaction mixture was quenched into 30 ml of H_2O and extracted with methylene chloride (three times with 10 ml each) to give the pure alkyl *p*-tolyl sulfoxides [—CH_3, —CH_2CH_3, —$CH_2CH_2CH_3$, and —$CH(CH_3)_2$] in high yields (87, 92, 90, and 95%, respectively) and with the appropriate physical properties reported below for the enantiospecifically synthesized sulfoxides.

The enantiomers of the sulfoxides were generated enantiospecifically according to a modification of the procedure of Solladie *et al.* (18). A solution of the alkylmagnesium iodide was prepared from the corresponding alkyl iodide (5 mmol), magnesium (6 mmol), and 10 ml of anhydrous ether, under N_2. The solution of the Grignard reagent was slowly added to a solution of either the (−)- or the (+)-menthyl (*S*)-*p*-toluenesulfinate (4.5 mmol, 1.323 g) at −30°. After the addition, the mixture was stirred for 1 hr at −30° and then brought to room temperature and stirred until no more sulfinate was detected by analytical thin layer chromatography (10 min to 2 hr). The reaction mixture was quenched into 50 ml of 1 N HCl at 0°, extracted with methylene chloride (three times with 10 ml each), dried over K_2CO_3, and filtered, and the solvent was evaporated to give a yellow oil. The oils were isolated by chromatography (SiO_2 with 40% hexane/ethyl acetate) to give the pure (*R*)- and (*S*)-sulfoxides [from the corresponding (*S*)- or (*R*)-toluenesulfinate]. (*R*)-(+)-Methyl-*p*-tolyl sulfoxide (85% yield): m.p. 73–74°; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55 (2 H, d, J = 7.9 Hz), 7.35 (2 H, d, J = 7.9 Hz), 2.72 (3 H, s), and 2.31 (3 H, s); $[\alpha]_D^{25}$, +148° (acetone, concentration = 1.0) [literature value (19), $[\alpha]_D^{25}$, +149.5]. (*S*)-(−)-Methyl *p*-tolyl sulfoxide (79% yield): m.p. 72.5–74°; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55 (2 H, d, J = 7.9 Hz), 7.35 (2 H, d, J = 7.9 Hz), 2.72 (3 H, s), and 2.31 (3 H, s); $[\alpha]_D^{26}$, −145° (acetone, concentration = 1.0). (*R*)-(+)-Ethyl *p*-tolyl sulfoxide (82% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.54 (2 H, d, J = 7.9 Hz), 7.36 (2 H, d, J = 7.9 Hz), 2.87 (2 H, m), 2.45 (3 H, s), and 1.23 (3 H, t, J = 7.4 Hz); $[\alpha]_D^{25}$, +201.5° (acetone, concentration = 1.0) [literature value (19), $[\alpha]_D^{25}$, +202.6°. (*S*)-(−)-Ethyl *p*-tolyl sulfoxide (65% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.54 (2 H, d, J = 7.9 Hz), 7.36 (2 H, d, J = 7.9 Hz), 2.87 (2 H, m), 2.45 (3 H, s), and 1.23 (3 H, t, J = 7.4 Hz); $[\alpha]_D^{25}$, −200° (acetone, concentration = 1.0). (*R*)-(+)-Propyl *p*-tolyl sulfoxide (86% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.60 (2 H, d, J = 7.8 Hz), 7.32 (2 H, d, J = 7.8 Hz), 2.76 (2 H, m), 2.41 (3 H, s), 1.71 (2 H, m), and 1.04 (3 H, t, J = 7.1 Hz); $[\alpha]_D^{25}$, + 180.5° (acetone, concentration = 1.0). (*S*)-(−)-Propyl *p*-tolyl sulfoxide (78% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.60 (2 H, d, J = 7.8 Hz), 7.32 (2 H, d, J = 7.8 Hz), 2.76 (2 H, m), 2.41 (3 H, s), 1.71 (2 H, m), and 1.04 (3 H, t, J = 7.1 Hz); $[\alpha]_D^{25}$, −178.5° (acetone, concentration = 1.0). (*R*)-(+)-Isopropyl *p*-tolyl sulfoxide (76% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.50 (2 H, d, J = 8.0 Hz), 7.33 (2 H, d, J = 8.0 Hz), 2.81 (1 H, sept, J = 6.9 Hz), 2.43 (3 H, s), 1.22 (3 H, d, J = 6.9 Hz), and 1.17 (3 H, d, J = 6.9 Hz); $[\alpha]_D^{25}$, +193° (acetone, concentration = 1.0) [literature value (19), $[\alpha]_D^{25}$, +194°]. (*S*)-(−)-Isopropyl *p*-tolyl sulfoxide (74% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.50 (2 H, d, J = 8.0 Hz), 7.33 (2 H, d, J = 8.0 Hz), 2.81 (1 H, sept, J = 6.9 Hz), 2.43 (3 H, s), 1.22 (3 H, d, J = 6.9 Hz), and 1.17 (3 H, d, J = 6.9 Hz); $[\alpha]_D^{25}$, −190° (acetone, concentration = 1.0).

Oxidation of the sulfides (1 mmol) in CH_2Cl_2 (5 ml) with *m*-chloroperbenzoic acid (2.2 mmol), followed by crystallization (hot hexanes), gave the sulfones, which were recrystallized from hot hexane to give pure sulfones. Methyl *p*-tolyl sulfone (99% yield): m.p. 88–89° [literature value (20), m.p. 89°]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (2 H, d, J = 6.8 Hz), 7.36 (2 H, d, J = 6.8 Hz), 3.03 (3 H, s), and 2.16 (3 H, s). Ethyl *p*-tolyl sulfone (97% yield): m.p. 56.5–57° [literature value (21), m.p. 57°]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (2 H, d, J = 8.4 Hz), 7.36 (2 H, d, J = 8.4 Hz), 3.03 (2 H, q, J = 7.5 Hz), and 1.27 (3 H, t, J = 7.5 Hz). Propyl *p*-tolyl sulfone (95% yield): m.p. 52–52.5° [literature value (22), m.p. 53°]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (2 H, d, J = 8.25 Hz), 7.36 (2 H, d, J = 8.25 Hz), 3.05 (2 H, t, J = 5.3 Hz), 1.73 (2 H, qt, J = 5.3 and 7.6 Hz), and 0.99 (3 H, t, J = 7.6 Hz). Isopropyl *p*-tolyl sulfone (98% yield): m.p. 80–80.5° [literature value (23), m.p. 80°]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (2 H, d, J = 7.9 Hz), 7.36 (2 H, d, J = 7.9 Hz), 3.17 (1 H, sept., J = 6.8 Hz), 2.45 (3 H, s), and 1.39 (6 H, d, J = 6.8 Hz).

Enzyme purification. Rabbit lungs were collected from 18 adult New Zealand Whites and microsomes were prepared from 200 g of tissue. Microsomes (2 mg/ml protein) in 50 mM potassium phosphate, 25% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5 (buffer A), were solubilized by the addition of sodium cholate to a final concentration of 0.6%. The clarified solubilized material was applied to a 25-mm × 40-cm column of octyl-Sepharose equilibrated with buffer A plus 0.4% cholate. After loading and washing with equilibration buffer, elution was carried out by raising the Emulgen 911 concentration, firstly to 0.1% and then to 1%. FMO eluted with 1% Emulgen 911. This fraction was dialyzed overnight against 10 mM potassium phosphate, 25% glycerol, 0.1 mM EDTA, 0.5% Emulgen 911, pH 7.7 (buffer B), and loaded onto a DEAE-Sepharose column (25-mm × 30-mm) equilibrated with buffer B. FMO did not bind to this anion exchanger, and so it was applied directly to a small column of 2′,5′-ADP-agarose equilibrated with 10 mM potas-

sium phosphate, 0.25% Emulgen 911, pH 7.7 (buffer C). FMO concentrated at the top of this affinity column and was eluted with a gradient of 10–200 mM potassium phosphate in buffer C. A second rabbit lung FMO preparation was purified, as described above, from frozen rabbit lungs obtained from Pel-Freez Biologicals (Rogers, AR). Both FMO preparations had specific contents of 15 nmol of flavin/mg of protein. Pulmonary FMO isolated from fresh and frozen tissue catalyzed the oxidation of saturating concentrations of methimazole at rates of 580 and 600 nmol/min/mg of protein, respectively.

A whole liver, obtained from a male Yucatan mini-pig, was immediately cut into small pieces and flash-frozen in liquid nitrogen. Tissue was stored at −80° until use. Microsomes were prepared typically from 100 g of liver after the tissue was thawed at room temperature in phosphate-buffered saline. Mini-pig liver FMO was purified according to the general method of Sabourin *et al.* (24), with the modification that an initial ion-exchange chromatography step was performed. Microsomes (10 mg/ml protein) in 10 mM potassium phosphate, 0.1 mM EDTA, pH 7.6 (buffer D), were solubilized by the addition of equal volumes of buffer D plus 1% Lubrol. Following centrifugation, the clarified solubilized FMO was applied to a DE52 column equilibrated with buffer D plus 0.5% Lubrol. The unbound fraction from DE52 was then applied sequentially to Cibacron Blue and Procion Red dye affinity columns, as described previously (24). The final preparation had a specific content of 13 nmol of flavin/mg of protein and catalyzed the oxidation of methimazole in the presence of octylamine at a rate of 1280 nmol/mg/min. Detergent was removed from all preparations following binding to hydroxyapatite, extensive washing with buffer free from detergent, and elution with 400 mM potassium phosphate, 0.1 mM EDTA, pH 7.6.

Inactivation of microsomal P-450 following solubilization. Aliquots (20 ml) of liver or lung microsomal protein (10 mg/ml) in 10 mM potassium phosphate, 0.1 mM EDTA, pH 7.6, were solubilized by the addition of an equal volume of buffer containing 10 mM potassium phosphate, 0.1 mM EDTA, and 1% Lubrol, pH 7.6. After mixing at 4° for 1 hr, the solubilized material was centrifuged at 100,000 × *g* for 60 min. The supernatant was stored at −80° until use.

Mechanism-based destruction of microsomal P-450. Reaction mixtures containing lung or liver microsomal protein (10 mg), NADPH (10 μmol), $MgCl_2$ (30 μmol), and ABT (100 μmol) were incubated for 35 min at 37° in 10 ml of 0.1 M potassium phosphate buffer, pH 7.4, and the microsomal pellet was recovered by centrifugation at 100,000 × *g* for 60 min (25). The pellet was resuspended to a protein concentration of 5 mg/ml and stored at −80° until use.

Metabolism of alkyl *p*-tolyl sulfides. Sulfide metabolism was determined in incubation systems that contained 100 mM glycine, 25 mM pyrophosphate pH 8.5, 0.25 nmol of purified monooxygenase or the equivalent of 0.5 mg of microsomal protein, 0.1 mM substrate, and 0.5 mM NADPH, in a total volume of 1.0 ml. Products were extracted into the organic phase by vortex-mixing for 1 min. The pentane layer was isolated and evaporated to dryness under a stream of nitrogen. The sample was reconstituted in 0.1 ml of ethyl acetate before analysis by chiral-phase HPLC.

HPLC analysis. Analyses were performed using an SSI HPLC gradient system equipped with a controller, model 220B and 200B pumps, and a model 500 variable wavelength detector linked to a Hewlett Packard (HP) 3396A integrator. Metabolic products were resolved using two different chiral stationary phases and solvent systems, described below. System A consisted of chromatography on a Chiralcel OB column with a mobile phase of hexane/IPA (90:10), a flow rate of 1.0 ml/min, and detection at 254 nm. Under these conditions, the retention times of the chiral products were (−)- and (+)-methyl *p*-tolyl sulfoxide, 12.6 and 28.4 min; (−)- and (+)-ethyl *p*-tolyl sulfoxide, 9.4 and 24.5 min; (−)- and (+)-propyl *p*-tolyl sulfoxide, 8.8 and 23.5 min; and (−)- and (+)-isopropyl *p*-tolyl sulfoxide 6.7 and 10.8 min (10.4 and 18.8 min using a mobile phase of hexane/IPA 95:5). System B consisted of chromatography on a Chiralcel OA column with a mobile phase of hexane/IPA (95:5), a flow rate of 0.5 ml/min, and detection at 254 nm. Under these conditions, the retention times of the chiral products were (+)- and (−)-methyl *p*-tolyl sulfoxide, 23.3 and 28.5 min; (+)- and (−)-ethyl *p*-tolyl sulfoxide, 16.4 and 19.4 min; (+)- and (−)-propyl *p*-tolyl sulfoxide, 13.0 and 15.7 min; and (+)- and (−)-isopropyl sulfoxide, 12.6 and 14.1 min. Baseline resolution of all enantiomers was obtained only with system A, which was used for the majority of the analyses.

Gas chromatographic analysis. Racemic alkyl *p*-tolyl sulfoxides and each corresponding sulfone were separated using an HP model 5890 gas chromatograph interfaced to an HP model 3396A integrator. The gas chromatograph was equipped with a fused silica capillary column, coated with the bonded stationary phase DB-5 (J & W Scientific, Ventura, CA), and flame-ionization detection. Initial oven temperature was 40° and metabolites were eluted with a linear temperature program from 40° to 280° at a rate of 15°/min. Under these conditions, the methyl, ethyl, propyl, and isopropyl *p*-tolyl sulfones eluted with retention times between 11.9 and 13.2 min. Each corresponding racemic sulfoxide eluted immediately before its sulfone metabolite.

Other assays. Dimethylaniline *N*-oxidase activity was determined by the gas chromatographic method of Damani *et al.* (26). Flavin content was determined by the method of Faeder and Siegel (27). Protein content was assayed by the method of Lowry *et al.* (28). Methimazole-dependent oxygen uptake was determined with a Clark electrode as described by Poulsen and Ziegler (29), with the exception that glutathione was omitted from reaction mixtures and that reactions were carried out at pH 8.4.

Results

Chiral-phase separation of racemic alkyl *p*-tolyl sulfoxides. Baseline resolution of each pair of sulfoxide enantiomers was achieved by chromatography on an analytical Chiracel OB column, using a mobile phase consisting of hexane/IPA (90:10). In each instance, the (*S*)-(−)-enantiomer eluted first. Stereochemical assignments were made on the basis of coincident elution with enantiospecifically synthesized standards. Raw chromatograms using racemic standards are shown in Fig. 1, along with the reported stereochemical composition. Comparison of these data with the standard deviations given in Table 1 indicates that the reproducibility of this analysis is primarily dependent on the integration function of the recorder.

Enantioselective sulfoxidation of alkyl *p*-tolyl sulfides by FMO isozymes. Fig. 2 shows the high purity of the isolated FMO preparations used in this study. Both rabbit lung FMO and mini-pig liver FMO appear homogeneous when high protein loads (20 pmol; Fig. 2, *lanes 2* and *5*) are applied to the gel. Under certain circumstances (low protein load, extended electrophoresis times), the rabbit lung preparation, but not the mini-pig liver preparation, could be resolved into a doublet. In Fig. 2 this is manifested by a wider band in *lane 3* (5 pmol, rabbit lung FMO) than in *lane 4* (5 pmol, mini-pig liver FMO). The high specific content of the rabbit lung FMO preparations (15 nmol/mg) suggests that the heterogeneity of the lung preparation does not reflect a non-FMO contaminant. Table 1 shows the effect of increased alkyl substituent bulk on the rate and stereochemical outcome of sulfoxidation catalyzed by highly purified preparations of rabbit lung and mini-pig liver FMO (Fig. 2). Each of the four substrates is metabolized rapidly by both isozymes. Turnover numbers obtained with the lung enzyme varied from 40 to 64 nmol/nmol/min, whereas those for the liver enzyme varied from 30 to 46 nmol/nmol/min. Product stereochemistry, however, was highly dependent on the alkyl substituent. The formation of (*R*)-(+) sulfoxide from methyl *p*-tolyl sulfide was a stereospecific process when cata-

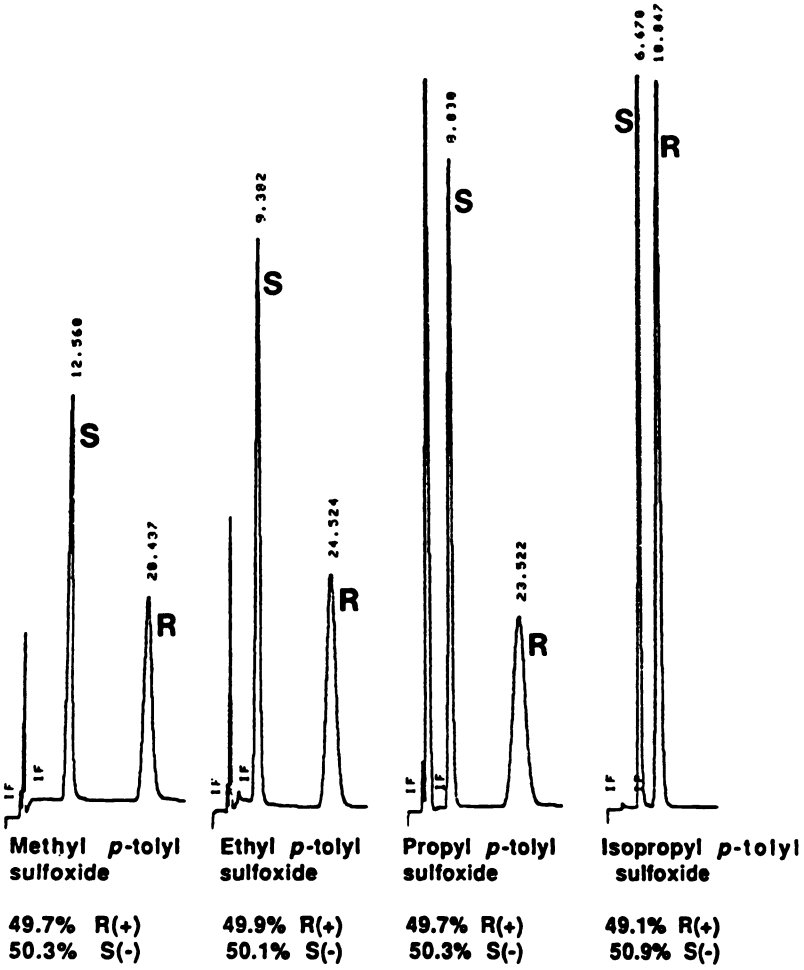

Fig. 1. Chiral-phase HPLC separation of racemic alkyl *p*-tolyl sulfoxide standards on Chiralcel OB. Chromatographic conditions shown in this figure are described in Materials and Methods. In each instance, the (*S*)-(−)-enantiomer eluted first. For each racemic sulfoxide, the enantiomeric composition determined from integrated peak areas is shown.

TABLE 1

Enantioselective sulfoxidation of a series of alkyl *p*-tolyl sulfides by purified rabbit lung and mini-pig liver FMO

Incubations and analyses were performed as described in Materials and Methods. Each stereochemical value is the mean ± standard derivation of three replicates.

Substrate	Rabbit lung FMO		Mini-pig liver FMO	
	(*R*)-(+)-Sulfoxide	Turnover	(*R*)-(+)-Sulfoxide	Turnover
	%	*nmol/nmol/min*	%	*nmol/nmol/min*
Methyl *p*-tolyl sulfide	>99	49	91 ± 2.0	46
Ethyl *p*-tolyl sulfide	91 ± 0.2	63	82 ± 0.2	30
Propyl *p*-tolyl sulfide	85 ± 0.2	64	72 ± 0.2	46
Isopropyl *p*-tolyl sulfide	63 ± 0.7	40	41 ± 0.2	36

lyzed by rabbit lung FMO but declined to 92% (*R*)-(+)-sulfoxide when catalyzed by mini-pig liver FMO. In contrast, sulfoxidation of isopropyl *p*-tolyl sulfide by these two forms of FMO gave products that showed minimal but opposing enantioselectivity. Rabbit lung FMO formed the (*R*)-(+)-enantiomer of isopropyl *p*-tolyl sulfoxide predominantly [63% (*R*)-(+)-], whereas mini-pig liver FMO favored formation of the (*S*)-(−)-enantiomer, again with modest enantioselectivity [59% (*S*)-(−)-]. For each isozyme, the extent of stereochemical purity of the (*R*)-(+)-sulfoxide formed as a function of alkyl substitution decreased in the order methyl > ethyl > propyl > isopropyl.

Effect of different conditions on the reproducibility of metabolic profiles catalyzed by rabbit lung FMO. The small standard deviations obtained in Table 1 highlight the reproducibility of this analysis under defined incubation and analytical conditions. The data summarized in Fig. 3 show that the changes in product stereoselectivity observed as a function of alkyl substituent bulk are also reproducible between different preparations of the same enzyme, under different incubation conditions (100 versus 500 μM substrate concentration), and under different analytical conditions (Chiralcel OA versus Chiralcel OB). It was particularly useful to repeat the analysis on a Chiralcel OA stationary phase, because the order of elution of the sulfoxide enantiomers was reversed, thus providing an additional check on analytical consistency.

Enantioselective sulfoxidation catalyzed by rabbit lung preparations. Initial experiments with purified P-450 LM2, a major constituent of rabbit lung microsomes (30), showed that this form of P-450 generated sulfoxides from the methyl, ethyl, propyl, and isopropyl sulfides with 81, 80, 85, and 81% (*S*)-(−)-stereochemistry, respectively. Therefore, if attempts to replicate microsomal and purified FMO stereochemical profiles were to be successful, it was obviously necessary to ensure that microsomal P-450 did not contribute to overall stereochemistry. Table 2 shows the results of experiments performed with rabbit lung microsomes that had been pretreated *in vitro* with 10 mM ABT to selectively inactivate P-450 (25). The enantioselective sulfoxidation profiles obtained with ABT-treated microsomes parallel closely those seen with the purified preparation, when incubations were carried out at

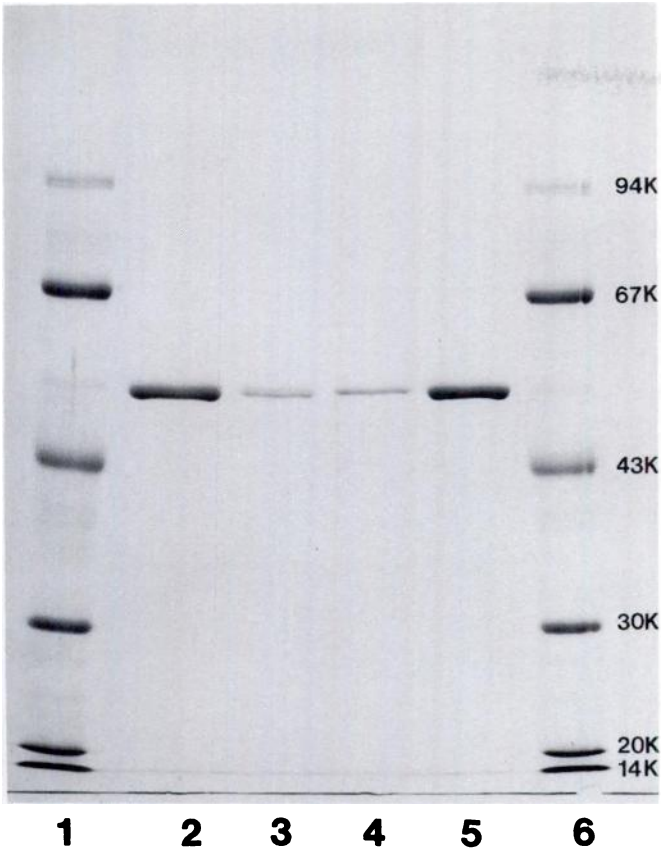

Fig. 2. Sodium dodecyl sulfate (7.5%)-Polyacrylamide gel of purified FMO. *Lanes 1* and *6*, molecular weight standards; *lanes 2* and *3*, rabbit lung FMO (20 and 5 pmol, respectively); *lanes 4* and *5*, mini-pig liver FMO (5 and 20 pmol, respectively).

pH 8.5. Minor deviations (<5%) from the profile obtained with purified isozyme are explicable on the basis of residual LM2. An even better fit of the data was obtained with microsomes that had been solubilized in the absence of glycerol. This is consistent with total abolition of P-450 activity under these circumstances. The wide pH optimum for rabbit lung FMO activity afforded us the opportunity to study these stereoselective processes at more basic pH. Because the optimum pH range for P-450 activity is narrow, relative to that for rabbit lung FMO, we expected that any residual P-450 activity in ABT-treated microsomes would be abolished at pH 10. Comparison of stereochemical profiles at the higher pH indicated that this was indeed the case. In addition, these data demonstrate that, for substrates other than the methyl sulfide, stereochemical fidelity decreased in both microsomal and purified preparations as pH increased.

Enantioselective sulfoxidation catalyzed by mini-pig liver preparations. In this series of experiments, we first treated mini-pig liver microsomes with ABT and generated stereochemical profiles for each substrate in the presence of octylamine. Octylamine, by virtue of its dual effect as an inhibitor of P-450 and a positive effector of hog liver FMO, was included to compensate for any residual P-450 activity in ABT-treated microsomes. Considerable deviations (up to 20%) were observed between the stereochemical profiles generated by purified enzyme and ABT-treated microsomes (Table 3). Microsomes solubilized in the absence of glycerol also generated stereochemical profiles similar to those of ABT-treated microsomes and at wide variance with those of the purified preparation (Table 3). Mini-pig liver microsomes that had been heated to 45° in the absence of NADPH, to selectively inactivate mini-pig liver FMO, consistently showed lower stereoselectivity for formation of each (*R*)-(+)-sulfoxide than did either ABT-treated microsomes or solubilized microsomes (Table 3).

Changes in product stereochemistry during isolation of the mini-pig liver FMO. The simplest explanation for the discrepancy between microsomal and purified enzyme stereochemical profiles is the existence of multiple catalytically distinct forms of mini-pig liver FMO and the selective purification of one (or more) of these by the methods described. We therefore examined the sulfoxidation of isopropyl *p*-tolyl sulfide at different stages of purification to determine at which step(s) product stereochemistry attributable to microsomal and purified preparations diverged (Table 4). The isopropyl substrate was chosen because, in addition to being one of the most sensitive indicators of the changes under investigation, the sulfoxide products had the shortest retention times, thus facilitating analysis. It is clear from Table 4 that the major change in product stereochemistry occurs after chromatography on DE52. Product stereochemistry generated by solubilized microsomes applied to the DE52 column was 55% (*R*)-(+)-sulfoxide, whereas the unbound fraction, which contained almost all of the recovered activity, formed sulfoxide with 39% (*R*)-(+)-stereochemistry. Subsequent chromatography steps did not markedly alter this value. No evidence was found for the existence of an additional pool(s) of FMO that possessed the requisite stereochemistry to account for the observed differences.

Discussion

A major aim of this work was to assess the utility of a series of alkyl *p*-tolyl sulfides in distinguishing between FMO isozymes, both at the level of the purified enzyme and in a crude microsomal mixture. To this end we examined product stereochemistry obtained with two distinct purified FMO enzymes and with their component microsomes that had been pretreated with ABT or detergents in the absence of glycerol to destroy P-450.

Rabbit lung microsomes solubilized in the absence of glycerol produced enantioselective sulfoxidation profiles that were identical to those obtained with the purified preparation. Rabbit lung microsomes pretreated with ABT closely mimicked the metabolic profile seen with the purified preparation; however, the degree of enantioselectivity obtained with each substrate was consistently lower with the microsomal preparation. Because any residual P-450 activity is most likely due to the pulmonary LM2 isozyme (25), we assessed the enantioselectivity of sulfoxidation by this form of P-450 and found that each substrate in the series formed principally the (*S*)-(−)-sulfoxide (80–85%). Therefore, the minor deviations observed could be attributed to the activity of residual P-450 LM2. We conclude that, as expected, the isolated rabbit lung FMO preparation retains the active site geometry of the membrane-bound form(s).

A similar approach was taken with the mini-pig liver preparation. We expected that solubilization of mini-pig liver microsomes in the absence of glycerol would permit duplication of enantioselective sulfoxidation profiles obtained with the isolated form. This did not prove to be the case, and deviations of

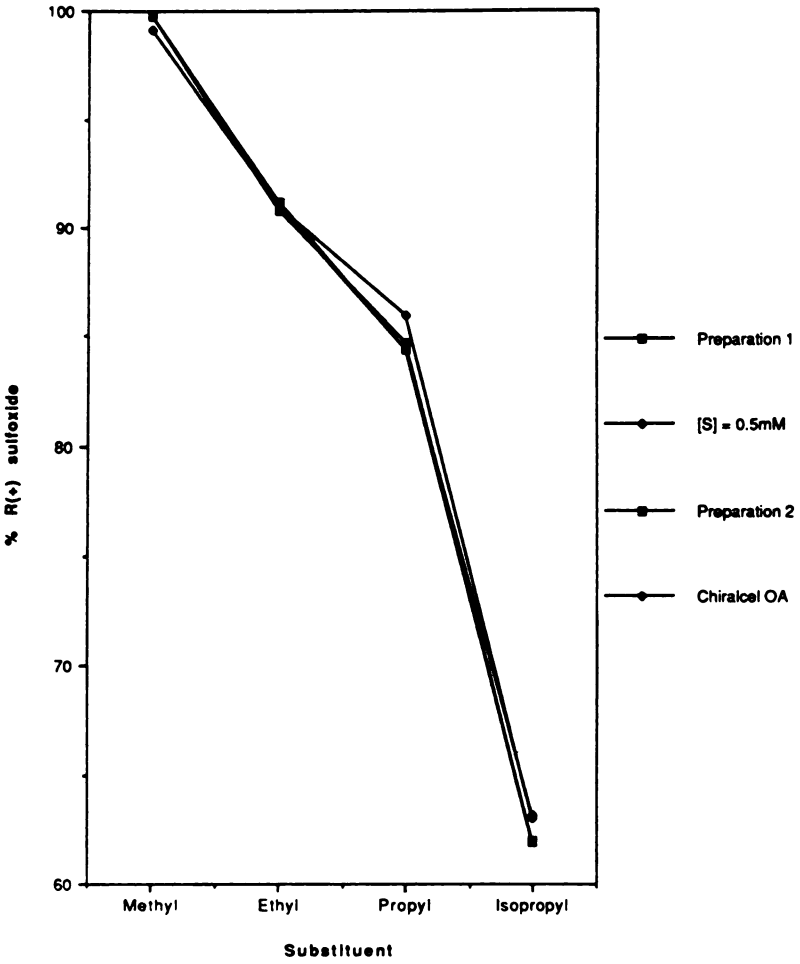

Fig. 3. Enantioselective sulfoxidation of alkyl *p*-tolyl sulfides by rabbit lung FMO under different conditions. Incubations and analyses were performed with different FMO preparations, different substrate concentrations, and different chiral stationary phases, as described in Materials and Methods. Each value is the mean of at least duplicate determinations. Replicates did not deviate by more than 1% from the mean value.

TABLE 2

Enantioselective sulfoxidation of a series of alkyl *p*-tolyl sulfides by rabbit lung preparations

Incubations and analyses were performed as described in Materials and Methods. Each value is the mean of two or three determinations.

Enzyme source	pH	Ethyl (*R*)-(+)-sulfoxide formed			
		Methyl	Ethyl	Propyl	Isopropyl
		%			
ABT-treated microsomes	8.5	96	87	82	62
Solubilized microsomes	8.5	>99	91	85	61
Purified FMO	8.5	>99	91	85	61
ABT-treated microsomes	10	>99	87	74	56
Purified FMO	10	>99	84	71	59

TABLE 3

Enantioselective sulfoxidation of a series of alkyl *p*-tolyl sulfides by mini-pig liver preparations

Incubations and analyses were performed as described in Materials and Methods. Each value is the mean of two or three determinations.

Enzyme source	Ethyl(*R*)-(+)-sulfoxide formed			
	Methyl	Ethyl	Propyl	Isopropyl
	%			
ABT-treated microsomes + octylamine	99	95	92	57
Heat-inactivated microsomes	61	45	30	ND[a]
Solubilized microsomes	96	93	88	56
Purified FMO	91	82	72	40

[a] ND, not determined due to low turnover.

up to 20% were observed. We, therefore, assessed metabolic profiles using mini-pig liver microsomes that had been pretreated with ABT. As an added precaution against residual P-450 activity, we performed incubations in the presence of octylamine. These treatments, however, did not provide an improved fit of the data. A final attempt to rationalize the data on the basis of residual P-450 activity involved determining metabolic profiles in mini-pig liver microsomes in which the thermolability of the hog liver FMO had been exploited. The metabolic profile obtained with heat-inactivated mini-pig liver microsomes was considered to reflect only the P-450 component. If the mini-pig liver data could be rationalized on the basis of residual P-450 activity, sulfoxide products would have to exhibit (*R*)-(+)-stereochemistry to an even greater degree than the purified preparation. This proved not to be the case.

TABLE 4

Enantioselectivity and recovery of isopropyl *p*-tolyl sulfide *S*-oxygenase activity of mini-pig liver FMO during purification

Chromatography, incubations, and analyses were performed as described in Materials and Methods. Each enzyme preparation was dialyzed against 10 mM potassium phosphate, 0.1 mM EDTA, pH 7.6, before analysis. Values for enantioselectivity are the mean ± standard deviation of three replicates. The yield is expressed as a percentage of total dimethylaniline *N*-oxidase activity found in the solubilized microsomal preparation. Values for the yield are the mean of duplicate determinations.

Enzyme preparation	(*R*)-(+)-Sulfoxide	Total activity	Yield
	%	*nmol of dimethylaniline N-oxide formed/min*	%
Solubilized microsomes	55 ± 0.9	46,090	100
DE52-unbound fraction	39 ± 0.2	36,420	79
DE52-bound fraction	35 ± 0.2	1,650	3.6
Cibacron Blue fraction	36 ± 0.4	16,260	35
Procion Red fraction	39 ± 0.2	ND[a]	ND

[a] ND, not determined.

It is possible that secondary biotransformation of primary sulfoxide metabolites could obscure correlations between crude tissue preparations and purified enzyme or serendipitously provide correlations where none exist. We should, therefore, assess the potential for further metabolism of primary products in both crude tissue and purified preparations. Both oxidation to sulfone and reduction to parent sulfide must be considered. Sulfone formation from several alkaryl sulfides is catalyzed by FMO, but K_m values for this process are usually orders of magnitude greater than for sulfoxide formation (2). In this study we found no evidence for formation of sulfone metabolites during FMO-catalyzed oxidation of alkyl *p*-tolyl sulfides. These data are in agreement with existing literature data (12). P-450, however, readily oxidizes alkaryl sulfoxides to the corresponding sulfones (31). Because P-450 was inactivated in microsomal experiments performed herein, it seems unlikely that secondary oxidation would confound interpretation of these data. Several enzyme systems are known that have the capacity to reduce metabolic products of FMO oxidation in crude tissue preparations (32). These include P-450 (33), aldehyde oxidase (34), and cytosolic NADPH-dependent *N*-oxide reductase (35). The latter two may be dismissed in the context of these studies, due to an inappropriate subcellular location. P-450 involvement may also be discounted on the basis of its prior inactivation.

Unlike the rabbit lung form, the hog liver form of the enzyme has a pronounced thermolability and is inactivated by ionic detergents (3). The enzyme can also undergo irreversible loss of catalytic activity during purification, and multiple investigators have documented that the final catalytic activity of hog liver FMO is dependent on the initial specific activity of the microsomes (1, 24). It is important to stress that rates of catalysis observed with the mini-pig liver microsomes and purified FMO used in this study compared favorably with specific activities reported by others and that the altered stereochemical profiles obtained with this isozyme do not appear to be secondary to enzyme inactivation.

It is interesting to note that Light *et al.* (12) have reported data for the sulfoxidation of ethyl *p*-tolyl sulfide by hog liver FMO to products with 95% (*R*)-(+)-stereochemistry. The authors also indicated that an even higher degree of stereoselectivity had been observed with methyl *p*-tolyl sulfide. These values are exactly in agreement with the stereochemistry observed following sulfoxidation of these substrates by mini-pig liver microsomes after either ABT treatment or solubilization (Table 3). The values reported by Light *et al.* (12) were obtained using an enzyme purified from hog liver by a procedure different than that reported herein. Although we could not exclude the possibility that mini-pig and hog liver FMO represent distinct flavoproteins, it seemed that it would be beneficial to examine the discrepancies in stereochemistry as a function of the purification procedure.

Studies to determine where, during purification, product stereochemistry was altered indicated that the critical step was initial chromatography on DE52. The stereochemical composition of isopropyl *p*-tolyl sulfoxide obtained from solubilized mini-pig liver microsomes applied to DE52 was 55% (*R*)-(+)-sulfoxide, whereas passage through DE52 provided an enzyme source that catalyzed this reaction to product with 39% (*R*)-(+)-stereochemistry. The latter value did not alter greatly at subsequent stages of purification and was close to that obtained with the final detergent-free preparation [41% (*R*)-(+)-]. A second population of mini-pig liver FMO could be eluted from the DE52 column with high salt, but it represented a minor portion of the total activity (<5%) and possessed a similar stereochemical profile to that of the unbound fraction. Seventeen percent of the total FMO activity applied to DE52 could not be recovered. If this apparently labile pool of mini-pig liver FMO had the capability to form isopropyl *p*-tolyl sulfoxide with 100% *R*-(+)-stereochemistry, we would still be unable to account for the observed discrepancies. Therefore, these data do not support the existence of multiple catalytically distinct forms of mini-pig liver FMO. It has been shown that solubilization of microsomally bound enzymes can result in the formation of different lipid populations of the same gene product that possess distinct chromatographic properties and catalytic activities (36). In support of a role for phospholipid in hog liver FMO catalysis, Ziegler *et al.* (37) reported that cycling of the hog liver enzyme through an anion exchange column led to significant decreases in the rate of catalysis. This could be offset by the incorporation of phosphatidylserine into incubation mixtures. We have carried out preliminary experiments to assess the role of phospholipid in influencing product stereochemistry. To date, we have not been successful in modifying product stereochemistry by the addition of phospholipids to purified preparations, but we have obtained significant changes in product stereochemistry when mini-pig liver FMO is purified in the presence of phospholipid.[1] Therefore, we conclude that our preparation of mini-pig liver FMO is not representative of the form present in microsomal membranes. We suggest that minor modifications to active site topography have occurred and may be related to changes in lipid composition that are a consequence of the purification procedure.

The mechanism of action determined for hog liver FMO catalysis dictates that V_{max} is a constant for all substrates (3).

[1] A. E. Rettie and G. P. Meier, unpublished data.

Therefore, obtaining information beyond the demonstration that a putative substrate gains access to the active site of the enzyme requires the estimation of apparent K_m values. However, little correlation appears to exist between the apparent K_m and the structural features of a given substrate. There is an extreme paucity of information regarding factors that determine substrate orientation once access to the active site of the enzyme has been attained. A recent report has indicated that hepatic forms of FMO from hog and rat liver can be distinguished on the basis of different degrees of diastereoselectivity in the oxidation of 2-aryl-1,3-oxathiolanes (38). The data reported herein concerning differences in enantioselective sulfoxidation by hepatic and pulmonary FMO forms underline the utility of a stereochemical approach to the characterization of FMO enzymes. It is now well established that hepatic FMO forms can perform oxidations at sulfur (12), phosphorus (13), and nitrogen centers (39, 40) with a high degree of stereoselectivity. Therefore, the most significant aspect of this work is the finding that FMO-dependent sulfoxidation need not produce products with high stereochemical purity and that the degree of enantioselectivity associated with the biotransformation of alkaryl sulfides is largely a function of the steric bulk of the alkyl substituent. It is clear from our data that the stereochemical purity of alkaryl (*R*)-(+)-sulfoxides generated from mammalian FMO decreases as the chain length and steric bulk of the alkyl moiety increase. In this regard, we can see that chain length is of less consequence than steric bulk (compare propyl versus isopropyl substitution). Such structure-activity relationships can provide useful insights into the orientation of substrates in the active site if partial inversion about the chiral center during catalysis can be excluded. Because, in contrast to P-450 monooxygenation, current concepts of FMO catalysis support a nucleophilic rather than a radical mechanism for the transfer of oxygen to substrate, we suggest that this type of product analysis is a direct indicator of substrate orientation and, therefore, provides a means to map the active site topography of mammalian FMO.

The progressive decrease in the purity of alkaryl (*R*)-(+)-sulfoxides generated by mammalian FMO as a function of increases in alkyl chain steric bulk has a parallel in a previous report concerning the stereoselective hydrolysis of 3-alkyl-substituted glutarate diesters (41). Hydrolysis was pro-*S* selective for diesters with small (methyl, ethyl, and propyl) alkyl substituents but pro-*R* selective with isopropyl, phenyl, and benzyl substituents. As with our data, these changes represented a continuum. By analogy to the twin binding site model proposed for active site orientation of glutarate diesters (41), we suggest that a similar model is consistent with the data reported herein. One binding site is capacious and capable of accommodating either the *p*-tolyl moiety or highly substituted alkyl groups. A second site is much more restricted and is capable of binding planar substituents or small alkyl groups. Loss of stereochemical fidelity as a function of increasing alkyl chain bulk is then a consequence of the extent to which the alkyl moiety competes with the aryl moiety for the less restricted site. This admittedly speculative model is currently being tested with a more extensive array of substrates.

In summary, we have described the enantioselective sulfoxidation of a series of alkyl *p*-tolyl sulfides by two distinct forms of FMO. Comparison of structure-activity profiles derived from microsomal and purified FMO demonstrates unambiguously that no alterations to active site geometry occur during isolation of the rabbit lung form. That such modification is possible without noticeable inactivation is shown by the discrepancies in the structure-activity profiles generated by microsomes, solubilized microsomes, and purified mini-pig liver FMO. We could find no evidence for the selective purification of multiple distinct mini-pig liver FMOs, and so we conclude that our purification protocol results in minor modification(s) to the mini-pig liver form that influence substrate orientation in the active site. Qualitative similarities between structure-activity profiles obtained with the two FMO forms are consistent with a high degree of conservation of active site topography in these two otherwise distinct isozymes. Quantitative differences suggest the utility of this approach in the discrimination of catalytically distinct forms of FMO.

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Send reprint requests to: Allan E. Rettie, Ph.D., Department of Medicinal Chemistry, BG-20, Seattle, WA 98195.