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Studies on the Chirality of Sulfoxidation Catalyzed by Bacterial Flavoenzyme Cyclohexanone Monooxygenase and Hog Liver Flavin Adenine Dinucleotide Containing Monooxygenase†

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ABSTRACT: The stereochemical outcome of oxygen transfer to the sulfur moiety of aryl alkyl sulfides catalyzed by two flavoenzyme monooxygenases has been determined by resolution of sulfoxide product enantiomers on a high-pressure liquid chromatography column [Pirkle, W. H., Finn, J. M., Schreiner, J. L., & Hamper, B. C. (1981) *J. Am. Chem. Soc. 103*, 3964–3966] containing a 3,5-dinitrobenzoyl-D-phenylglycine chiral stationary phase. With 4-tolyl ethyl sulfide as substrate, cyclohexanone monooxygenase from *Acinetobacter* produces predominantly the (*S*)-(−)-sulfoxide (82% *S*, 18% *R*), a modest enantioselectivity. In contrast, the flavin adenine dinucleotide (FAD) containing monooxygenase purified from hog liver microsomes carries out sulfoxidation to yield the (*R*)-(+)-sulfoxide enantiomer as major product (95% *R*, 5% *S*). The presence of the minor sulfoxide enantiomer in each case appears to be due to incomplete chiral processing by each enzyme and not to a competing, achiral, nonenzymic sulfoxidation process. The mammalian FAD-containing monooxygenase also oxygenates the divalent sulfur of the antiarthritic drug sulindac sulfide to yield a single dextrorotatory isomer of the sulfoxide prodrug. Analysis of the chiral outcome of sulfoxidation catalyzed by rat liver microsomes indicated that phenobarbital treatment increases the capacity for *S*-(−)-oxygenation of 4-tolyl ethyl sulfide, suggesting that the phenobarbital-induced cytochrome P-450 isozymes catalyze formation of the (*S*)-(−)-sulfoxide preferentially, a surmise validated in the following paper [Waxman, D. J., Light, D. R., & Walsh, C. (1982) *Biochemistry* (following paper in this issue)]. With sulindac sulfide as substrate, though, both control and phenobarbital-induced microsomes catalyze sulfoxidation to yield the same (+)-sulfoxide enantiomer generated by the purified FAD-containing monoxygenase, suggesting a low degree of participation by the cytochrome P-450 isozymes in sulfoxidation of this compound.

The flavin adenine dinucleotide (FAD)[1] containing monooxygenase (EC 1.14.13.8, *N*,*N*-dimethylaniline monooxygenase), first characterized by Ziegler and co-workers in liver microsomes (Machinist et al., 1968; Ziegler, 1980), catalyzes oxygen transfer to nitrogen and sulfur atoms in many substrate types and is, along with the cytochrome P-450 mo-

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[1] Abbreviations: FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HPLC, high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; Me_3Si, trimethylsilyl.

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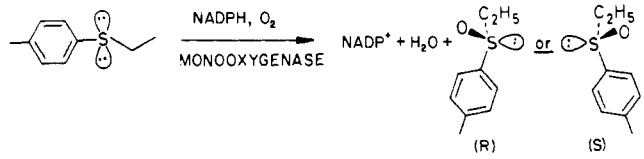
Scheme I



nooxygenase system, important in the processing of xenobiotic compounds in liver and other tissues. FAD-containing monooxygenase-catalyzed sulfur oxidations have been shown to be particularly important for the metabolism of a wide variety of insecticides (Hajjar & Hodgson, 1980). As this flavoenzyme exhibits a rather broad substrate specificity, a major question is whether it catalyzes a stereospecific transfer of oxygen to sulfur, e.g., whether chiral sulfoxides are generated.

In this paper, we report on the stereospecificity of sulfoxidation catalyzed by two enzymes, the hog liver FAD-containing monooxygenase as well as that of another FAD-containing monooxygenase, cyclohexanone monooxygenase (EC 1.14.13.?) purified from *Acinetobacter* NCIB9871 (Donoghue et al., 1976). In the accompanying paper (Waxman et al., 1982), we report on the stereoselectivity of two phenobarbital-induced P-450 isozymes purified from rat liver. To probe the stereochemistry of sulfur oxygenation, we have examined the interactions of these enzymes with aryl alkyl sulfides. 4-Tolyl ethyl sulfide was chosen as a model substrate (Scheme I) since (a) the chiral center formed at sulfur in aryl alkyl sulfoxides is stable with respect to pyramidal inversion (Rayner et al., 1968),[2] (b) preliminary experiments indicated that the sulfoxides generated are only slowly oxygenated further to achiral sulfones, and (c) the correlation of the absolute stereochemistry at sulfur with the sign of rotation of the two possible optical isomers of 4-tolyl ethyl sulfoxide has been determined by chemical conversion (Axelrod et al., 1968; Andersen, 1962) to a compound for which the absolute configuration has been determined by X-ray crystallography (Fleischer et al., 1964). In addition, the specific rotations of the purified enantiomers of 4-tolyl ethyl sulfoxide have been determined (Cope & Caress, 1966).

Whereas chiral purity determinations deduced from specific rotations (Abushanab et al., 1978) or by use of chiral NMR shift reagents [e.g., see Takata et al. (1980), Egan et al. (1981), and Whitesides & Lewis (1970)] would require the isolation of ~1–5 mg of enzymatically generated sulfoxide, use of chiral stationary phase chromatography (Pirkle et al., 1981) requires neither sulfoxide isolation nor large amounts of material. This technique permits resolution of aryl alkyl sulfoxide enantiomers while simultaneously separating out other compounds present in the incubation mixture. As product detection is $\sim 10^4$-fold more sensitive than with conventional techniques [1 nmol of racemic sulfoxide (0.1–0.2 μg) is readily resolved and quantitated], it becomes feasible to investigate the effects of a variety of experimental conditions on the chiral purity of the enzymatic product.

Using these methods, we demonstrate that whereas the bacterial cyclohexanone monooxygenase generates (*S*)-(−)-4-tolyl ethyl sulfoxide preferentially, mammalian FAD-containing monooxygenase yields the corresponding (*R*)-(+)-sulfoxide as the predominant product (~95% of the total). In addition, we have determined the chirality of oxygenation by the mammalian flavoprotein of the antiarthritic drug sulindac sulfide (Shen & Winter, 1977) which contains an aryl alkyl sulfide moiety.

Materials and Methods

General Materials. NADPH, $NADP^+$, glucose 6-phosphate, methimazole, Hepes, glycine, catalase (11 000 units/mg), and glucose-6-phosphate dehydrogenase (type XXIII) were obtained from Sigma. *n*-Octylamine, *p*-(ethylthio)-benzoic acid, and cyclohexanone were obtained from Aldrich. Phenyl methyl sulfide and benzyl methyl sulfide (Aldrich) and 4-tolyl ethyl sulfide (Sigma) were redistilled under vacuum before use. 4-Nitrophenyl allyl sulfide, 4-tolyl allyl sulfide, 4-chlorophenyl allyl sulfide, 4-methoxyphenyl allyl sulfide, sulindac sulfoxide [(*Z*)-5-fluoro-2-methyl-1-[[*p*-(methylsulfinyl)phenyl]methylene]-1*H*-indene-3-acetic acid], and sulindac sulfide [(*Z*)-5-fluoro-2-methyl-1-[[*p*-(methylthio)-phenyl]methylene]-1*H*-indene-3-acetic acid] were generous gifts of Drs. R. Firestone and T. Shen of Merck Sharp & Dohme. All other chemicals were reagent grade.

Sulfoxides. 4-Tolyl ethyl sulfoxide, phenyl methyl sulfoxide, and sulindac sulfoxide were synthesized from the parent sulfides by sodium metaperiodate oxidation in methanol (Johnson & Keiser, 1973). 4-Tolyl ethyl sulfone was prepared by oxidation of 4-tolyl ethyl sulfide (1 g of sulfide/12 mL of aqueous solution containing 17% $MgSO_4$ and 50% *tert*-butyl alcohol) with excess $KMnO_4$. These compounds were each shown to elute as a single peak upon HPLC in the solvent systems described below.

4-(Hydroxymethyl)phenyl ethyl sulfide was synthesized by a modification of a published procedure (Hoffman et al., 1960). *p*-(Ethylthio)benzoic acid (1 g) was reduced with excess lithium aluminum hydride in anhydrous ethyl ether (100 mL) to yield 4-(hydroxymethyl)phenyl ethyl sulfide (0.3 g): GC/MS *m/z* 168 (70.5, M^+), 151 (8.7, M − OH), 139 (15.9, M − CH_2CH_3), 107 (44.3, M − SCH_2CH_3), 79 (69.0), 77 (70.1), 29 (100, $CH_2CH_3^+$); GC/MS of the Me_3Si derivative [prepared from bis(trimethylsilyl)acetamide, Pierce Chemicals] *m/z* 240 (5.5, M^+), 151 [48.1, M − $OSi(CH_3)_3$], 73 [100, $Si(CH_3)_3^+$]; proton NMR (60 MHz, $CDCl_3$) δ 1.36 (t, 3 H, J = 7.2 Hz, CH_3), 2.99 (q, 2 H, J = 7.2 Hz, ethyl CH_2), 4.66 (s, 2 H, benzyl CH_2), 7.31 (s, 4 H, phenyl H_4).

Methyl ester derivatives of chemically and enzymatically oxidized sulindac sulfide were prepared by the addition of freshly distilled diazomethane in anhydrous ether. Diazomethane was generated from bis(*N*-methyl-*N*-nitroso)terephthalamide in ethylene glycol monomethyl ether by the dropwise addition of 30% aqueous NaOH. Diazomethane produced was displaced into ethyl ether on ice by a stream of argon (Fieser & Fieser, 1967).

Enzymes. Hog liver microsomal FAD-containing monooxygenase, isolated and purified to protein homogeneity by published procedures (Beaty & Ballou, 1981), was a generous gift of Dr. D. P. Ballou, University of Michigan. Cyclohexanone monooxygenase (EC 1.14.13.?), purified to homogeneity from *Acinetobacter* NCIB9871 (Donoghue et al., 1976), was a generous gift of Dr. C. C. Ryerson of this laboratory. Preparation and characterization of the control and phenobarbital-induced rat liver microsomes were as described in the following paper (Waxman et al., 1982).

HPLC Analysis. Four different HPLC systems were routinely used in conjunction with a Waters Associates instrument connected to a Micromeritics 786 variable wavelength detector and a Hewlett-Packard integrator 3390A: system A, reverse-phase chromatography on a μBondapak C-18 column (0.39 × 30 cm, Waters) with 70% methanol/30% water (v/v) as the mobile phase at a flow rate of 2 mL/min; system B,

[2] Extrapolation of the rate of pyramidal inversion of 4-tolyl methyl sulfoxide (measured by these authors between 220 and 250 °C) to 40 °C leads to an estimate of the half-life for racemization of 9×10^6 h.

normal phase chromatography on a μPorasil column (0.39 × 30 cm, Waters) with 95% hexane/5% 2-propanol (v/v) as the mobile phase at a flow rate of 4 mL/min; system C(5), enantioselective chromatography on a Pirkle type IA column containing a 3,5-dinitrobenzoyl-D-phenylglycine chiral stationary phase (Regis Chemical Co.) with 95% hexane/5% 2-propanol (v/v) as the mobile phase at a flow rate of 4 mL/min [under these conditions, the two enantiomers of 4-tolyl ethyl sulfoxide separate readily (ratio of *net* retention time (relative to void volume) of more strongly adsorbed to that of the less strongly adsorbed enantiomer ≡ α = 1.09)]; system C(10), enantioselective chromatography as in system C(5), but using 90% hexane/10% 2-propanol (v/v) as the solvent at a flow rate of 1 mL/min [under these conditions, the two enantiomers of sulindac sulfoxide methyl ester may be separated after several recyclings through the column (α = 1.022); general applications of this Pirkle IA column have been described recently (Pirkle et al., 1981)]; system D, chromatography on a Pirkle type IA column connected to the outlet of a μPorasil column with 95% hexane/5% 2-propanol (v/v) as the mobile phase at a flow rate of 4 mL/min. The enantiomers of 4-tolyl ethyl sulfoxide separated equally well in either system C(5) or system D although the retention times are longer and the peak widths somewhat broader in the latter system. System D readily separates 4-tolyl ethyl sulfone from (*R*)-(+)-4-tolyl ethyl sulfoxide, two compounds which coelute in system C(5).

Other Analytical Methods. Analytical and preparative thin-layer chromatography of sulindac sulfoxide, sulindac sulfide, and the corresponding methyl esters was performed on silica gel G in 95% chloroform/5% acetic acid (v/v) (Gund & Shen, 1977), permitting the separation of all four compounds: sulindac sulfoxide (R_f 0.35), sulindac sulfoxide methyl ester (R_f 0.45), sulindac sulfide (R_f 0.50), and sulindac sulfide methyl ester (R_f 0.76).

GC/MS of 4-(hydroxymethyl)phenyl ethyl sulfide (synthesized chemically or isolated from incubations with rat liver microsomes) and its trimethylsilyl ether derivative was performed with the kind assistance of Dr. R. DiCosimo of this department on an HP-5992 gas chromatograph–mass spectrometer utilizing a $^1/_8$ in. × 6 ft column of 3% SE-30 on 80/100 mesh Gas Chrom Q at 160 °C with He as the carrier at 30 mL/min. Mass spectrometry of 4-(hydroxymethyl)-phenyl ethyl sulfone was performed with the kind assistance of Dr. J. Leary of this department by using a Varian MAT 212 GC/MS interfaced to an SS 200 data system. Spectra were obtained by evaporation off a direct insertion probe using electron impact ionization (at 70 eV).

Standard Conditions for Enzymatic Incubations. Bacterial cyclohexanone monooxygenase was assayed at 30 pmol of enzyme/mL in 80 mM glycine, pH 9.0, at 30 °C with 0.2–0.6 mM NADPH. Cyclohexanone (97 μM) and 4-tolyl ethyl sulfide (up to 0.5 mM, the approximate solubility limit of this sulfide) were used as substrates. Rat liver microsomes were assayed (generally at 0.15 nmol of cytochromes P-450/mL) by incubation in 0.1 M KP_i, pH 7.4, 20% glycerol (v/v), and 20 μM ethylenediaminetetraacetate with 0.2 mM NADPH and 0.5 mM 4-tolyl ethyl sulfide at 37 °C. Hog liver microsomal FAD-containing monooxygenase was assayed by incubation of 88 pmol of enzyme/mL in 0.1 M Hepes, pH 7.5, with NADPH (0.2–0.6 mM), 4-tolyl ethyl sulfide (up to 0.5 mM), and *n*-octylamine (2.4 mM, when indicated). 4-Tolyl ethyl sulfide, phenyl methyl sulfide, benzyl methyl sulfide, and sulindac sulfide were added from a concentrated stock solution in methanol to give the final concentrations indicated and 1% methanol (v/v) in the assay mixture. Cyclohexanone was added from a 9.7 mM stock solution in water. All other substrates were added from concentrated stock solutions in dimethyl sulfoxide to yield a final concentration of 0.5–1% solvent.

In large-scale incubations designed to produce milligram quantities of product, an NADPH generating system consisting of 0.1 mM $NADP^+$, 2 mM glucose 6-phosphate, and up to 14 units/mL glucose-6-phosphate dehydrogenase was used. Catalase (10 μg/mL) was included in prolonged incubations as a precaution against the buildup of hydrogen peroxide.

Reaction mixtures containing volatile sulfides were incubated in tubes capped with serum stoppers to minimize sulfide volatilization (e.g., 50% loss of 0.5 mM 4-tolyl ethyl sulfide was observed during a 1-h incubation at 37 °C in an uncapped tube vs. ~10–20% loss in a capped tube, as monitored by HPLC in system A).

Activity Measurements. Purified enzymes typically were assayed by monitoring NADPH oxidation at 340 nm by using an extinction coefficient of 6220 M^{-1} cm^{-1}. Since both sulindac sulfide and 4-nitrophenyl allyl sulfide absorb at 340 nm, a different method was used for these substrates. After an extinction coefficient of 2330 M^{-1} cm^{-1} for 4-nitrophenyl allyl sulfide at 340 nm was determined, a net extinction coefficient of 8550 M^{-1} cm^{-1} was used at this wavelength, assuming stoichiometric oxidation of NADPH and sulfide (4-nitrophenyl allyl sulfoxide has negligible absorbance at 340 nm). An isosbestic point (335.5 nm) for the conversion of sulindac sulfide to sulindac sulfoxide was used to monitor NADPH oxidation of this substrate [ϵ(NADPH) = 6130 M^{-1} cm^{-1} at 335.5 nm]. NADPH oxidation in the absence of substrate did not exceed 5% of the substrate-dependent turnover with either purified monooxygenase. Microsomal activities were monitored by direct product analysis by HPLC as described above.

Enzymatic Generation of Sulindac Sulfoxide. FAD-containing monooxygenase (3.3 nmol) was incubated with sulindac sulfide (60 μM) in 0.1 M Hepes buffer, pH 7.5, 2.4 mM *n*-octylamine, and 0.5% methanol (v/v) containing an NADPH regenerating system and 10 μg/mL catalase (100 mL final volume) at 37 °C. Incubation was continued overnight, with fresh aliquots of sulindac sulfide (to yield a concentration of 60 μM) added at 90 and 180 min. Analytical thin-layer chromatography (see above) suggested approximately 50% conversion to sulindac sulfoxide. The sample was then acidified and ether extracted.

Rat liver control and phenobarbital-induced microsomes were incubated for 300 min at 37 °C with 0.25 mM sulindac sulfide (nominal concentration), 1.8% methanol (v/v), and an NADPH regenerating system in standard assay buffer (see above) at 1 mg of protein/mL. Incubations (160 mL for control and 320 mL for phenobarbital microsomes, respectively) were halted by acidification; NaCl was then added to 0.1 g/10 mL, followed by two extractions with CH_2Cl_2 (0.6 volume of each). The yield (percent conversion of sulindac sulfide to sulindac sulfoxide) for control microsomes was 33.6% and for phenobarbital microsomes was 39.4%.

Enzymatically generated sulindac sulfoxide was purified by preparative thin-layer chromatography and analyzed by polarimetry as described below. The overall recovery of sulindac (sulfide + sulfoxide) was ~60%.

Chemical oxidation of 4-tolyl ethyl sulfide by hydrogen peroxide was measured in capped tubes in 0.1 M Hepes, pH 7.5, at 37 °C with varying H_2O_2 concentrations. Loss of sulfide and formation of sulfoxide were each monitored by HPLC (system A).

Table I: Sulfoxidation Catalyzed by Bacterial Cyclohexanone Monooxygenase[a]

substrate	V_{max} (min^{-1})	K_m (μM)	product chirality
4-tolyl ethyl sulfide	470 ± 50	220 ± 50	82.0 ± 1.0% *S*
cyclohexanone	1800 ± 100	4 ± 0.5	NA[b]

[a] Assay conditions as described under Materials and Methods. Data for cyclohexanone under the same reaction conditions are taken from Ryerson (1980). [b] NA designates not applicable.

Chiral Product Analysis. Chiralities are expressed as the percent of the total sulfoxide product which is either the *S* or the *R* enantiomer, as indicated. The chiral purity of enzymatically generated sulfoxides was investigated by using two approaches. Samples to be assayed by enantioselective chromatography were extracted with hexane, and an aliquot was then injected directly onto the HPLC column [system C(5) or system D]. Sulindac sulfoxide was converted to the methyl ester before injection [system C(10)]. Samples to be analyzed polarimetrically were extracted from enzymatic incubation mixtures with hexane, ethyl ether, or methylene dichloride. Sulfoxides were subsequently purified either by HPLC (4-tolyl ethyl sulfoxide and 4-nitrophenyl allyl sulfoxide; system A) or by thin-layer chromatography (TLC) (sulindac sulfoxide). Optical rotations were measured under the conditions indicated by using a Perkin-Elmer polarimeter equipped with both mercury and hydrogen lamps and a water-thermostated 10-cm microcell (1 mL). Specific rotations were determined with samples exhibiting absolute rotations of >100 mdeg with sample concentration determined by direct HPLC analysis (4-tolyl ethyl sulfoxide and 4-nitrophenyl allyl sulfoxide) or by UV–visible spectrometry (sulindac sulfoxide).

Identification of p-(Hydroxymethyl)phenyl Ethyl Sulfide Isolated from Microsomal Incubations with 4-Tolyl Ethyl Sulfide. Control microsomes (0.42 nmol of P-450/mL) were incubated with 4-tolyl ethyl sulfide for 2 h at 37 °C with an NADPH regenerating system under standard incubation conditions with the addition of fresh aliquots of substrate (to a final concentration of 0.5 mM) every 30 min. The reaction mixture (160 mL) was extracted with hexane (5 × 100 mL), and the hexane extract was then dried under reduced pressure to an oily residue containing (as determined by HPLC analysis in system D) unreacted 4-tolyl ethyl sulfide, both of the corresponding sulfoxide enantiomers, 4-tolyl ethyl sulfone, and the unknown [4-(hydroxymethyl)phenyl ethyl sulfide]. The unknown was purified by preparative HPLC (system D) and shown to have the same retention time as authentic 4-(hydroxymethyl)phenyl ethyl sulfide in two different HPLC systems [system C(5) and system B]. The unknown and its trimethylsilyl ether derivative were also shown to comigrate with authentic 4-(hydroxymethyl)phenyl ethyl sulfide and its trimethylsilyl ether derivative, respectively, upon GC analysis on 3% SE-30. Mass spectra obtained for the unknown and its Me_3Si derivative were identical with those of authentic 4-(hydroxymethyl)phenyl ethyl sulfide: GC/MS for the unknown *m/z* 168 (66.1), 151 (9.2), 139 (13.8), 107 (41.6), 79 (71.6), 77 (68.2), 29 (100) (see above for values obtained with authentic standards); GC/MS for the trimethylsilyl ether derivative of the unknown *m/z* 240 (6.7), 151 (42.1), 73 (100) (see above for spectra of authentic standards); proton NMR (90 MHz, $CDCl_3$) of the unknown revealed resonances expected for 4-(hydroxymethyl)phenyl ethyl sulfide δ 1.31 (t, J = 7.3 Hz), 2.95 (q, J = 7.3 Hz), 4.67 (s), 7.31 (s), and additional unidentified resonances between δ 0.07 and 1.25. Attempts to further purify the sample (HPLC system D; removal of solvent under reduced pressure) resulted in nonenzymatic oxidation to 4-(hydroxymethyl)phenyl ethyl sulfone: MS *m/z* 200 (53.2, M^+), 171 (97.2, $M - CH_2CH_3$), 107 (100, $M - SO_2CH_2CH_3$), 89 (59.2), 77 (89.2); proton NMR (250 MHz, $CDCl_3$) δ 1.27 (t, 3 H, J = 7.5 Hz, CH_3), 3.12 (q, 2 H, J = 7.5 Hz, ethyl CH_2), 4.83 (s, 2 H, benzyl CH_2), 7.57 [d, 2 H, J = 7.7 Hz, ring H_2 (ortho to hydroxymethyl)], 7.90 [d, 2 H, J = 8.4 Hz, ring H_2 (ortho to sulfonyl)].

Results and Discussion

Chirality of Sulfoxidation Catalyzed by Bacterial Cyclohexanone Monooxygenase. The recent observation that bacterial cyclohexanone monooxygenase, which converts cyclohexanone to ϵ-caprolactone, catalyzes an efficient oxidation of thiane to thiane sulfoxide (Ryerson, 1980) raises the question of the chirality of the oxygenation reaction. The chirality of sulfoxidation catalyzed by this monooxygenase was studied by utilizing 4-tolyl ethyl sulfide as a model substrate (Scheme I). Pirkle column analysis indicated that sulfoxide of modest chiral purity is generated [82% *S*-(−) enantiomer; Table I and Figure 1]. In addition, the V_{max} of sulfoxide formation was found to be 26% of that observed with cyclohexanone as substrate (Table I).

Chiral Oxidation of Divalent Sulfur Catalyzed by Hog Liver FAD-Containing Monooxygenase. The FAD-containing monooxygenase isolated from liver microsomes catalyzes oxidation of a number of sulfur-containing substrates with a fairly uniform maximal turnover of about 60 min^{-1} at pH 7.5 [Table II and Ziegler (1980)]. Stop-flow studies have demonstrated that turnover is limited by the rate of breakdown of the enzyme-bound 4a-hydroxyflavin pseudobase (Beaty & Ballou, 1981; Ballou, 1981) to yield H_2O and free enzyme. Two simple aryl alkyl sulfides, 4-nitrophenyl allyl sulfide and 4-tolyl ethyl sulfide, are oxidized to a sulfoxide product with a positive sign of rotation (Table II). In the case of 4-tolyl ethyl sulf-

Table II: Oxidation of Sulfides to Sulfoxides Catalyzed by Purified FAD-Containing Monooxygenases

sulfide substrate	turnover (min^{-1})	K_m (μM)	$[\alpha]_D$ (deg) of sulfoxide product	chiral purity (%) of sulfoxide by enantioselective HPLC
4-tolyl ethyl sulfide	48[a]	13	+155[b]	95 ± 1
	27[c]	nd[j]	nd	94 ± 1
4-nitrophenyl allyl sulfide	53[d]	nd	+270[e]	
4-chlorophenyl allyl sulfide	58[f]	nd	nd	
4-tolyl allyl sulfide	65[g]	nd	nd	
4-methoxyphenyl allyl sulfide	60[f]	nd	nd	
phenyl methyl sulfide	62[a]	16.5	nd	
benzyl methyl sulfide	49[a]	~1.5	nd	
sulindac sulfide	80[a]	~3.0	+61.0[h]	>90[i]

[a] V_{max}. [b] Acetone, 25 °C. [c] Incubation in the absence of *n*-octylamine at 0.5 mM sulfide. [d] 0.2 mM sulfide. [e] Water, 37 °C. [f] 0.5 mM sulfide. [g] 0.75 mM sulfide. [h] Methanol, 27 °C. [i] Estimated from Figure 3. [j] Not determined.

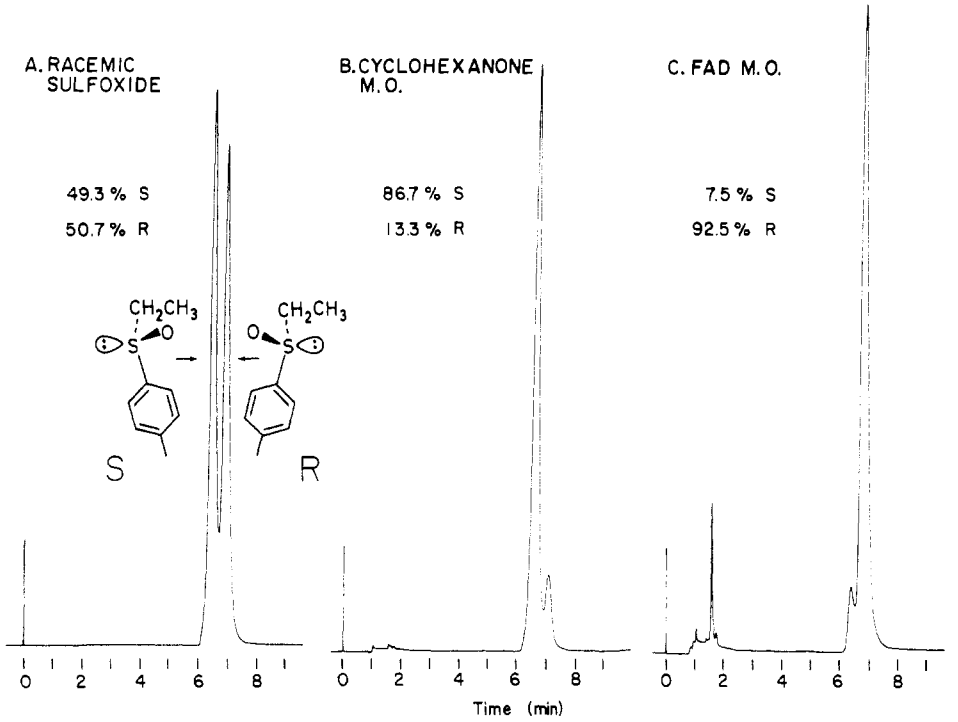


FIGURE 1: Enantioselective chromatography of 4-tolyl ethyl sulfoxide prepared chemically or enzymatically from 4-tolyl ethyl sulfide. HPLC separation on a chiral Pirkle type IA column using solvent system C(5) with detection by A_{254nm}. (A) Racemic sulfoxide (60 nmol) prepared by sodium metaperiodate oxidation; (B) sulfoxide product (10 nmol) prepared by incubation with purified bacterial cyclohexanone monooxygenase under standard incubation conditions; (C) sulfoxide (10 nmol) generated by incubation with purified hog liver microsomal FAD-containing monooxygenase as described under Materials and Methods. Sulfoxide chiralities determined in this experiment are included in the figure; representative chiralities obtained in additional experiments were as detailed in the text.

Table III: Cyclohexanone Monooxygenase Oxidation of Ethyl Tolyl Sulfide: Effects of Catalase and Extent of Reaction on Chirality of Sulfoxide and Secondary Oxidation to Sulfone

condition [initial rate (min^{-1})][a]	sulfide utilization (%)[d]	sulfoxide chirality [% (*S*)-4-tolyl ethyl sulfoxide][e]	4-tolyl ethyl sulfone formation (% total products)[e]
no additions (turnover = 260)	32	81.8	nd[b]
	64	81.4	0.84
	83	83.1	1.6
+catalase[c] (turnover = 280)	35	82.1	0.33
	64	82.6	0.74
	83	82.6	1.4

[a] 500 μM 4-tolyl ethyl sulfide/80 mM glycine, pH 9.0, at 30 °C. [b] nd = not determined. [c] 110 units/mL. [d] Determined by NADPH consumption. In independent experiments, NADPH consumption was shown to be >90% coupled to sulfoxide formation. [e] Determined by HPLC (system D).

oxide, the specific rotation of the enzymatic product, [α] +155°, can be compared to that of chirally pure *R* enantiomer, [α] +203.2° (Cope & Caress, 1966), suggesting a minimum of 88% chiral purity for the enzymatic product, in fair agreement with the chiral purity determined by chiral stationary phase chromatography (93–95% (*R*)-(+)-sulfoxide, Figure 1). Thus, liver microsomal FAD-containing monooxygenase oxidizes 4-tolyl ethyl sulfide to the corresponding sulfoxide with a stereospecificity which is opposite to that of cyclohexanone monooxygenase.

Investigation of the Lack of 100% Chiral Selectivity in Sulfur Oxidation Catalyzed by Purified Monooxygenases. One possible explanation for the lack of 100% chiral purity of the flavoenzyme-generated sulfoxides could be that two sources contribute to sulfoxide formation, direct flavoprotein-mediated sulfide oxidation, which is 100% chiral, and a second, concurrent process which is nonenzymatic and racemic. In the case of cyclohexanone monooxygenase, for example, a chiral purity of 82% could result from 64% of the total flux to enzyme-generated 100% (*S*)-(−)-sulfoxide and 36% of the flux to sulfoxide from nonenzymatically produced racemate. Efforts were made, therefore, to test this possibility.

If sulfoxide formation were due to two different processes, then alterations in the rate of one process should affect the chiral purity of the final product. However, the chiral purity of the sulfoxide formed was found to be independent of the concentration of either monooxygenase in the assay (data not shown) and remained constant over the time course of the reaction (Tables III and IV). In the case of the FAD-containing monooxygenase, inclusion of the activator *n*-octylamine (2.4 mM) (Ziegler et al., 1973) increased the rate of sulfoxidation by a factor of 1.7 without influencing the chiral purity of the enzymatic product formed (Table II). In addition, no evidence for the formation of 4-tolyl ethyl sulfoxide from 4-tolyl ethyl sulfide was seen during control incubations in the absence of enzyme, nor was 4-tolyl ethyl sulfide oxidized in air-saturated buffer upon incubation under standard assay conditions for several hours.

One potential source of racemic sulfoxide is the oxidation of 4-tolyl ethyl sulfide by hydrogen peroxide which might be generated by flavoprotein-mediated uncoupled reduction of molecular oxygen during nonproductive turnover. Since for

Scheme II

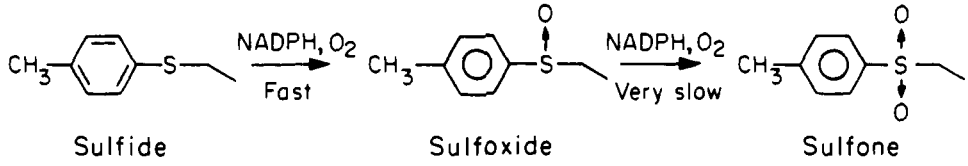


Table IV: FAD-Containing Monooxygenase Oxidation of 4-Tolyl Ethyl Sulfide: Effects of Catalase and Extent of Reaction on Chirality of Sulfoxidation

condition [initial rate (min^{-1})][a]	sulfide utilization (%)[c]	sulfoxide chirality [% (R)-4-tolyl ethyl sulfoxide][d]
no additions	32	94.9
(turnover = 37)	64	95.5
	96	95.4
+catalase[b]	32	95.0
(turnover = 39)	65	95.0
	96	95.1

[a] 500 μM 4-tolyl ethyl sulfide/0.1 M Hepes, pH 7.5 at 37 °C. [b] 110 units/mL. [c] Determined by NADPH consumption. Sulfoxide formation was shown to be fully coupled to NADPH consumption in independent experiments. Sulfone formation did not exceed 0.1% of the total product in any of these incubations. [d] Determined by HPLC (system D).

both flavoprotein monooxygenases substrate oxidation was found to be coupled to NADPH consumption by better than 95%, the possibility of adventitious oxidation by H_2O_2 is unlikely. Moreover, incubation of either flavoprotein monooxygenase in the presence of catalase (10 μg/mL) did not decrease the turnover number or the chiral purity of the sulfoxide formed (Tables III and IV). In addition, a second-order rate constant determined for the oxidation of 4-tolyl ethyl sulfide by H_2O_2 [$k = (4.9 \pm 0.7) \times 10^{-3}$ M^{-1} s^{-1} at 37 °C under standard incubation conditions] indicated that a very substantial concentration of H_2O_2 (10–100 mM) would be required to effect measurable nonenzymatic sulfide oxidation. Even if all of the NADPH utilized in the experiment shown in Table III were converted to H_2O_2 (i.e., without any substrate oxygenation), the amount of 4-tolyl ethyl sulfoxide formed by chemical oxidation would only account for <0.5% of the total observed product. Thus, the amount of product formation which can be attributed to H_2O_2 is vanishingly small.

Therefore, it is apparent that both enantiomers are generated enzymatically and that these flavoenzymes show only moderate intrinsic stereoselectivity. This suggests a partitioning of the substrate–4a-hydroperoxyflavoenzyme complex (Ballou, 1981)[3] into distinct pathways differing only slightly in activation energies. The partitioning observed with bacterial cyclohexanone monooxygenase (~82% *S*, i.e., $S/R \sim 5$) corresponds to a difference in activation energies of only ~1.0–1.1 kcal/mol (30 °C)[4] and that observed with the FAD-containing monooxygenase ($R/S \sim 19$) to ~1.7 kcal/mol (37 °C). For the achievement of 99% chiral purity (which would be essentially indistinguishable from 100% chiral purity by using our analytical methods), the enzyme need only destabilize the pathway leading to the minor enantiomer by 2.8 kcal/mol relative to the major pathway. That the FAD-containing monooxygenase can achieve such a high degree of stereoselectivity is indicated by the independent experiments of D. Ziegler and L. Poulson (unpublished experiments) with a similar sulfide substrate, 4-tolyl methyl sulfide. In that case, a ≥98% (*R*)-(+)-sulfoxide production was observed, in general agreement with the stereochemical observations reported here.

4-Tolyl Ethyl Sulfone Formation. Under standard assay conditions, cyclohexanone monooxygenase catalyzed a slow oxidation of 4-tolyl ethyl sulfide to 4-tolyl ethyl sulfone (Table III). Significantly less sulfone was formed in incubations of 4-tolyl ethyl sulfide with the FAD-containing microsomal monooxygenase (less than 0.1% of the total for the incubations shown in Table IV), in part a consequence of the >20-fold slower oxidation of 4-tolyl ethyl sulfoxide (0.5 mM) as compared to that of 4-tolyl ethyl sulfide under the same conditions (data not shown). During overnight incubations of the FAD-containing monooxygenase with racemic 4-tolyl ethyl sulfoxide, a preferential oxidation of the *S* enantiomer to the achiral sulfone was observed such that the residual sulfoxide substrate became enriched in the *R* enantiomer (10% enantiomeric excess, with quantitation by using HPLC system D) (Scheme II). This preferential utilization of the (*S*)-sulfoxide by the FAD-containing monooxygenase suggests that the near-absence of 4-tolyl ethyl sulfone in prolonged incubations of the FAD-containing monooxygenase with 4-tolyl ethyl sulfide reflects the absence of the preferred substrate [i.e., the (*S*)-(−)-sulfoxide] for the second oxidation, the (*R*)-(+)-sulfoxide being the predominant product of the initial sulfoxidation.

Chiral Sulfur Oxidation Catalyzed by Rat Liver Microsomes. Since the in vivo locale of the FAD-containing monooxygenase is the endoplasmic reticulum, we examined the stereochemical outcome of sulfoxidation catalyzed by microsomal fractions containing this enzyme. In contrast to the results obtained with purified FAD-containing monooxygenase, 4-tolyl ethyl sulfoxide of low chiral purity [~46% (*R*)-(+)-sulfoxide, 54% (*S*)-(−)-sulfoxide] was obtained upon incubation of rat liver microsomes with 4-tolyl ethyl sulfide in the absence of *n*-octylamine (Figure 2A). In cases where the microsomes were isolated from livers of phenobarbital-pretreated animals, the chiral purity of the sulfoxide formed was increased, but in this case, the (*S*)-(−)-sulfoxide was the predominant enantiomer formed (~75% of the total). These results are best explained if we assume that the chirality of sulfoxide produced by these microsomes reflects contributions both from the FAD-containing monooxygenase and from cytochrome P-450. As we have seen, the former catalyzes formation of the (*R*)-(+)-sulfoxide, and thus, the latter must then catalyze formation of the (*S*)-(−)-sulfoxide. Thus, the 2.6-fold increase in microsomal cytochrome P-450 content which we obtained upon phenobarbital induction [see Waxman et al. (1982)] correlates well with the 2.75-fold increase in (*S*)-(−)-sulfoxide formation activity (see Figure 2 legend). Conversely, just as the FAD-containing monooxygenase is not induced by phenobarbital pretreatment (Burke & Mayer, 1974), the level of *R*-(+)-sulfoxidation activity is essentially unchanged in phenobarbital-induced as compared to control

[3] $NADP^+$ is also part of this complex in both the liver microsomal FAD-containing monooxygenase and the bacterial cyclohexanone monooxygenase.

[4] If for two pathways $k_1 = 19k_2$, then $A_1e^{-\Delta G_1^*/(RT)} = 19A_2e^{-\Delta G_2^*/(RT)}$ and $\Delta G_1^* - \Delta G_2^* = -2.303RT \log 19$, assuming $\log A_1 - \log A_2$ is insignificant.

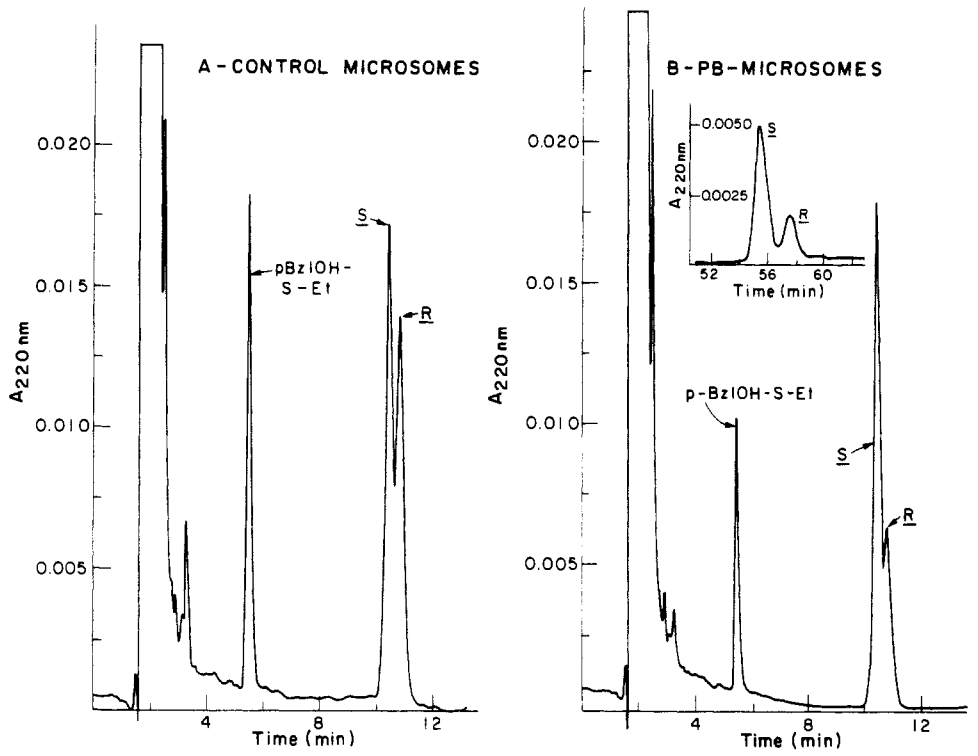
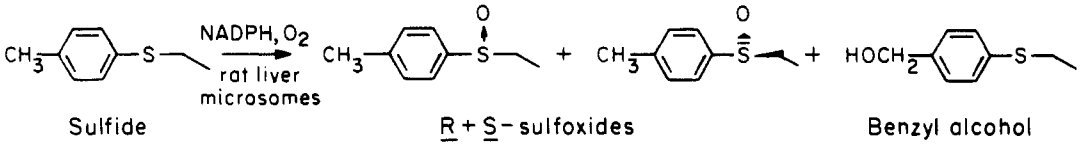


FIGURE 2: Microsomal oxidation of 4-tolyl ethyl sulfide. Liver microsomes prepared from control (A) or phenobarbital-induced rats (B) were incubated with the sulfide for 10 min at 37 °C as described under Materials and Methods. Products were analyzed by using HPLC system D after extraction into hexane with detection by A_{220nm}. The prominent peak eluting at t = 5.7 min was identified as 4-(hydroxymethyl)phenyl ethyl sulfide as described under Materials and Methods. 4-Tolyl ethyl sulfoxide generated by control microsomes integrated to 55 ± 1% S enantiomer; that of the phenobarbital microsomes integrated to 74 ± 1% S enantiomer. Molar ratio of total sulfoxide to 4-(hydroxymethyl)phenyl ethyl sulfone = 6 or 10 for the samples shown in panels A and B, respectively [quantitation as described by Waxman et al. (1982)]. Panel B insert: Near-base-line resolution of (S)- and (R)-sulfoxides obtained after four recyclings of the sulfoxide. Percent S = 75.4 and 74.2 for the first and fifth elutions, respectively.

Scheme III



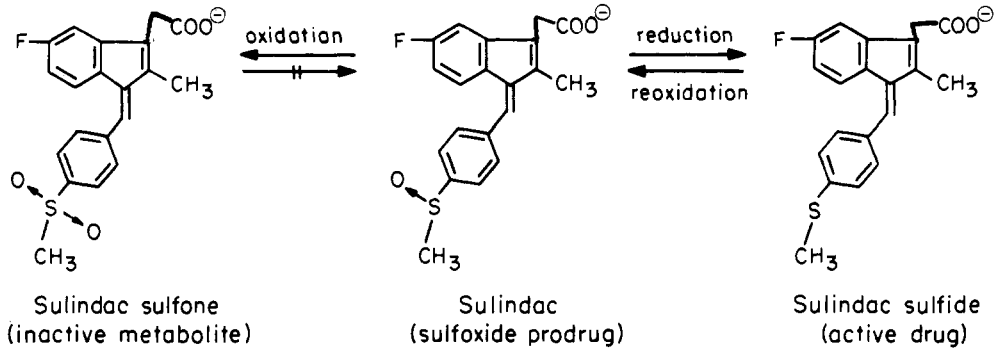
microsomes (Figure 2). These findings are supported by studies documented in the following paper (Waxman et al., 1982), which demonstrate that several of the major cytochrome P-450 isozymes purified from phenobarbital-induced rat liver microsomes do indeed catalyze conversion of 4-tolyl ethyl sulfide to (S)-(−)-4-tolyl ethyl sulfoxide as the predominant enantiomer (75–85% of the total sulfoxide, depending on the P-450 enzyme employed).

In addition to (R)-(+)- and (S)-(−)-4-tolyl ethyl sulfoxides, both control and phenobarbital-induced microsomes catalyzed conversion of 4-tolyl ethyl sulfide to an additional product which eluted at t = 5.7 min in the chromatograms shown in Figure 2. This product was prepared in milligram quantities from a large-scale microsomal incubation and subsequently identified as 4-(hydroxymethyl)phenyl ethyl sulfide (Scheme III) as described under Materials and Methods. This compound, resulting from oxygen transfer to a benzylic carbon, would not be expected to be formed in reactions catalyzed by the FAD-containing monooxygenase (Ziegler, 1980) and, indeed, was not observed in incubations of either purified flavoprotein with 4-tolyl ethyl sulfide. Studies with purified cytochrome P-450 isozyme PB-1 reported in the following paper confirm that this microsomal product is derived from a cytochrome P-450 catalyzed benzylic oxidation (Waxman et al., 1982).

Chiral Oxidation of Sulindac Sulfide by Liver Microsomes and Purified Microsomal FAD-Containing Monooxygenase. Sulindac sulfoxide is a nonsteroidal antiinflammatory agent which is delivered therapeutically as the sulfoxide prodrug and reduced in vivo to the pharmacologically active sulfide (Shen & Winter, 1977) (Scheme IV). The sulfoxidation of sulindac sulfide (as well as other sulfides) by prostaglandin cyclooxygenase in ram seminal vesicle microsomes has recently been shown to protect against autoinactivation of that key prostaglandin biosynthetic enzyme (Egan et al., 1980). The reversible metabolic equilibrium between sulfide and sulfoxide coupled to an irreversible oxidation to sulindac sulfone has also been described (Duggan et al., 1980).

Apart from the work with prostaglandin cyclooxygenase, other possible enzymatic sites for the reoxidative cycling of this sulfur-containing drug have not been elucidated. The possibility that the FAD-containing monooxygenase might participate in sulfoxidation was, therefore, examined. As shown in Table II, sulindac sulfide is an excellent substrate for the purified hog microsomal FAD-containing monooxygenase, exhibiting both a high V_{max} and a low K_m. That sulindac sulfoxide was the enzymatic product was confirmed by thin-layer chromatography (see Materials and Methods). This finding provides an exception to the general observation (Ziegler, 1980) that anionic compounds are not substrates for

Scheme IV



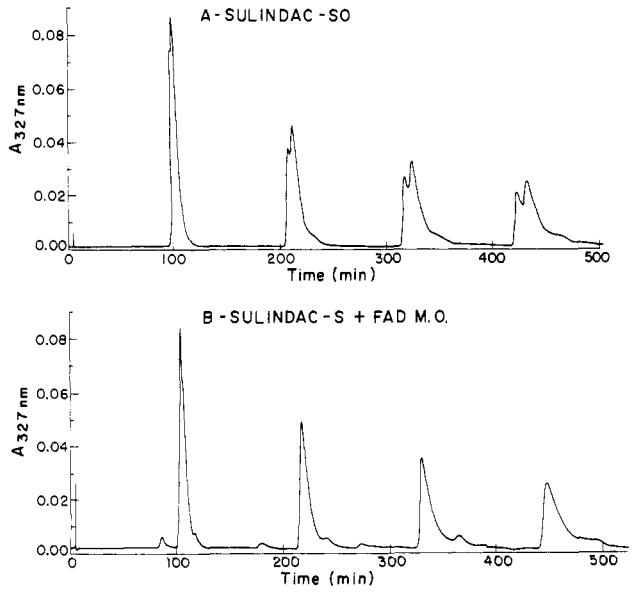


FIGURE 3: Enantioselective chromatography of sulindac sulfoxide methyl ester. Sulindac sulfide was oxidized either chemically or enzymatically and the resulting sulfoxide then methylated with diazomethane (see Materials and Methods). Shown is an HPLC separation on a chiral Pirkle type IA column obtained by using HPLC system C(10) with detection by A_{327nm}. Each sample was recycled through the column 4 times to maximize resolution. (A) Racemic sulindac sulfoxide methyl ester (43 nmol) prepared by sodium metaperiodate oxidation of sulindac sulfide followed by methylation; (B) sulindac sulfoxide methyl ester (34 nmol) isolated from an incubation with hog liver microsomal FAD-containing monooxygenase followed by methylation.

the FAD-containing monooxygenase. Chromatography of the corresponding sulindac sulfoxide methyl ester[5] on a chiral Pirkle column demonstrated that the sulfoxide product was of high chiral purity (Figure 3), estimated as >90%. Further support for the high chiral purity of the product is suggested by comparison of the measured specific rotation of enzyme-generated sulfoxide (unesterified), $[\alpha]^{27}_D$ +61.6° (methanol, 3.57 g/L; Table II), with that reported for enantiomers resolved by fractional crystallization, $[\alpha]^{27}_D$ +22.6° (methanol).[6] The higher rotation of the enzymatic product suggests a new (minimal) value for chirally pure sulindac sulfoxide enantiomers. Given the enzymatic generation of (*R*)-(+)-sulfoxide from the simple aryl sulfides, it is not unreasonable to expect that the flavoprotein-generated (+) enantiomer also corresponds to the *R* configuration, a supposition which requires experimental verification.

Sulindac sulfide is a poor substrate for sulfoxidation catalyzed by several purified rat liver microsomal cytochrome P-450 isozymes (turnover ca. ≤1–2 min^{-1}),[7] in contrast to the much more facile oxidation of 4-tolyl ethyl sulfide effected by these hemoprotein monooxygenases (Waxman et al., 1982). Consistent with this observation is the finding that both control and phenobarbital-induced rat liver microsomes oxidize sulindac sulfide to a sulfoxide of high chiral purity having the same sign of rotation as the sulfoxide generated by purified FAD-containing monooxygenase. Thus, for sulindac sulfoxide generated by control microsomes (see Materials and Methods), $[\alpha]^{27}_D$ +49 ± 2° (methanol, 2.6 g/L), and for that generated by phenobarbital-induced microsomes, $[\alpha]^{27}_D$ +43 ± 2° (methanol, 6.0 g/L). If $[\alpha]^{27}_D$ +61.6° (as determined for sulindac sulfoxide generated by FAD-containing monooxygenase) is taken to be indicative of 100% chirally pure material, then these specific rotations correspond to ~90% (+) and 85 (+) for sulfoxides generated by control and phenobarbital-induced microsomes, respectively. That the chiral purity of the sulindac sulfoxide generated by phenobarbital-induced microsomes is somewhat less that of control microsomes could reflect the presence of a small amount of (−)-sulfoxide product contributed by the phenobarbital-induced cytochrome P-450 isozymes.

Methyl phenyl sulfide (perhaps as a model for sulindac sulfide) has recently been used to study the chirality of sulfur oxidation effected by prostaglandin cyclooxygenase containing ram seminal vesicle microsomes (Egan et al., 1981). Microsomes were shown to generate sulfoxide of only marginal chiral purity [56% *S*-(−), 44% *R*-(+)]. The low chirality of the product was attributed to the low stereospecificity of the prostaglandin cyclooxygenase peroxidase carrying out the sulindac sulfide sulfur oxygenation with concomitant reduction of the 15α-peroxy group of prostaglandin H_2 to the 15α-alcohol. Given the ubiquity of the microsomal FAD-containing monooxygenase (Ziegler, 1980), an alternate explanation might be that the net chirality observed in the ram seminal vesicle microsomes reflects contributions both from prostaglandin cyclooxygenase peroxidase, which generates (*S*)-(−)-sulfoxide of a high chiral purity, and from FAD-containing monooxygenase, which would probably generate (*R*)-(+)-sulfoxide if present in this tissue. The chirality (or lack thereof) of the sulindac sulfoxide generated by the *purified* prostaglandin cyclooxygenase in this mode was not determined. It now can be determined by the methodology described in this paper.

In contrast to the FAD-containing monooxygenase, prostaglandin cyclooxygenase, acting in its peroxidase mode, is a

[5] The unmethylated sulfoxides were not readily eluted from the chiral Pirkle column. Comigration with authentic sulindac sulfoxide methyl ester provided further evidence for the enzymatic S-oxygenation of sulindac sulfide.

[6] Page 155 of Shen & Winter (1977).

[7] Sulindac sulfide turnover catalyzed by several P-450 isozymes was assayed by using purified proteins and standard conditions as described in Waxman et al. (1982).

heme protein. The nature of the prosthetic group, though, is no help in predicting the chirality of enzymatic sulfur oxygenation as evidenced by the production of sulfoxides of opposite chirality by the two flavoprotein monooxygenases described in this paper. Finally, one might be able to gauge the role of FAD-containing monooxygenase in the oxidative loop of the in vivo redox cycle that makes sulindac such a long-acting antiarthritic drug by the usual administration of a synthetic (*R*)/(*S*)-sulindac sulfoxide racemate and analysis over time to see if the *R* isomer predominates via resynthesis from sulindac sulfide.

Acknowledgments

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