Pergamon

Tetrahedron: Asymmetry, Vol. 8, No. 4, pp. 613–618, 1997
© 1997 Elsevier Science Ltd. All rights reserved.
Printed in Great Britain
0957-4166/97 $17.00 + 0.00

PII: S0957-4166(97)00033-5

Prochiral sulfide probes for the active-site topography of rabbit flavin-containing monooxygenase 2 (FMO2)

Michael B. Fisher and Allan E. Rettie *

Department of Medicinal Chemistry, University of Washington, Seattle, Washington 98195, USA

Abstract: Mammalian flavin-containing monooxygenases are known to oxygenate prochiral sulfides often with a high degree of stereoselectivity. In the present study we used two homologous series of *n*-alkyl aryl sulfides, Ar–S–$(CH_2)_nH$, (where Ar=p-tolyl or 2-naphthyl and n=1–7), to probe the active site topography of rabbit lung FMO2. Purified FMO2 was incubated with each member of the the p-tolyl or 2-naphthyl series and the stereochemistry of NADPH-dependent enzymatic sulfoxidation was determined. Within the p-tolyl series each member was a substrate for FMO2 and a complete reversal of stereospecificity was observed as the *n*-alkyl chain length increased from the methyl homolog [99% (*R*)] to the heptyl derivative [97% (*S*)]. In contrast, FMO2 generated metabolites from only the short-chain 2-naphthyl sulfides and in each case only the (*R*)-sulfoxides were formed. The data are discussed in terms of an active-site model for FMO2 which contains two binding determinants. © 1997 Elsevier Science Ltd. All rights reserved.

Introduction

The mammalian FMOs are a family of membrane-bound enzymes which catalyze the oxidation and oxygenation of a wide variety of heteroatom-containing substrates. Oxygenation is reported to occur at nitrogen, sulfur, iodine, and phosphorus.[1a,b,c] Recently, the metabolism of several therapeutic agents such as albendazole,[2a] cimetidine,[2b] nicotine,[2c] verapamil,[2d] and sulindac[5a] has been linked to mammalian forms of the enzyme. However, since no FMOs have yet been crystallized, information regarding their active site topography must be obtained by indirect means, such as pharmacophore modelling. In this regard, FMO isoforms which possess distinctive and limited substrate specificities would be expected to yield the most useful information.

Structure–activity experiments conducted with a series of phenothiazine analogs have determined that the substrate specificity of the mammalian FMOs vary considerably,[3a] and that substrate access to the active-site of rabbit FMO2 is relatively restricted.[3b,c] Rabbit FMO2, in common with the other members of this mammalian gene family, can oxygenate short-chain alkyl p-tolyl sulfides with a high degree of stereoselectivity.[4] In the present report, we synthesized two homologous series of prochiral *n*-alkyl aryl sulfide substrates and determined the configuration of the resulting sulfoxide metabolites in order to systematically probe active-site constraints for FMO2. The data derived from both series of substrates are rationalized in terms of an active site model which contains two principal binding determinants.

Results

Metabolism of the p-tolyl series by FMO2

Purified rabbit FMO2 efficiently metabolized all seven members of the p-tolyl series of compounds in an oxygen and NADPH-dependent manner, with apparent Km values <10 μM. However, the stereochemical outcome of these reactions varied dramatically. Sulfoxidation of the methyl, ethyl,

* Corresponding author.

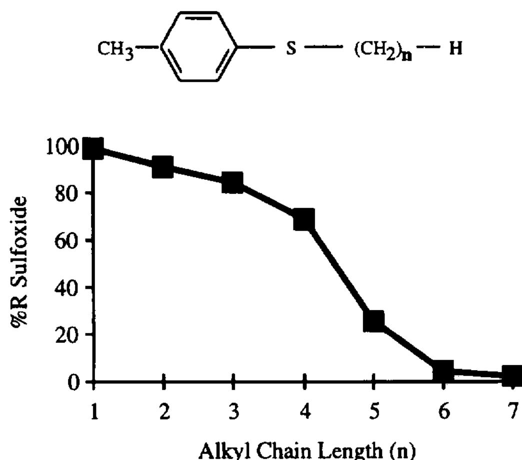

Figure 1. Stereochemical profile from alkyl p-tolyl series with FMO2.

n-propyl and *n*-butyl derivatives was selective for the (*R*)-sulfoxide as noted previously,[4d] whereas sulfoxidation of the *n*-pentyl, *n*-hexyl and *n*-heptyl homologs was increasingly stereoselective for formation of the (*S*)-sulfoxide. As shown in Figure 1, this variability represented a stereochemical continuum in which metabolism of the methyl and heptyl derivatives by FMO2 proceeded with inverse stereospecificities.

To confirm that this phenomenon was the property of a single enzyme active site, cDNA-expressed FMO2[4d] was used to generate sulfoxides from the methyl and hexyl analogs of alkyl p-tolyl sulfide. The cDNA expressed FMO2 produced sulfoxides from methyl- and hexyl p-tolyl sulfide in a highly enantioselective fashion, yielding 99%R and 96%S, respectively, identical to the data obtained with the purified enzyme.

Metabolism of the 2-naphthyl series by FMO2

In contrast to the p-tolyl series, only the methyl-, ethyl-, and *n*-propyl 2-naphthyl sulfides were substrates for FMO2. No sulfoxide metabolites were detected with the longer chain analogs, even with substrate concentrations up to 300 μM. Apparent Km values for the three short-chain 2-naphthyl sulfides were less than or equal to 10 μM. Importantly, unlike the p-tolyl analogs, (*R*)-sulfoxides were generated *stereospecifically* (>97%) by FMO2 from methyl, ethyl and *n*-propyl 2-naphthyl sulfide (Table 1). To test whether active-site constraints were responsible for the limited range of interactions between FMO2 and the 2-naphthyl substrates, we analyzed for the respective sulfoxides which could be generated by minipig liver FMO1. This isozyme is reported to have a much broader substrate specificity compared to FMO2.[3] Minipig liver FMO1 formed predominantly (*R*)-sulfoxides from the members of the 2-naphthyl series (Table 1), consistent with a more open active-site for this isoform.

Discussion

We found previously that FMO2-mediated sulfoxidation of the first four compounds (methyl to *n*-butyl) in the p-tolyl series was stereoselective for the (*R*)-enantiomer, but that enantioselectivity decreased progressively with increasing *n*-alkyl chain length.[4a,d] This structure–activity relationship for FMO2 proved to be a highly distinctive "chiral fingerprint" when comparisons were made with three other rabbit isoforms, FMO1, FMO3 and FMO5.[4d] FMO2, therefore, appeared to hold the most promise for more detailed structure–function studies aimed at elucidating features of this active-site architecture. In the present study we found that longer chain homologs of the p-tolyl series of substrates progressively form sulfoxides with the (*S*)-configuration, and so FMO2 catalyzes a reversal

Table 1. Kinetic and stereochemical data for the sulfoxidation of alkyl 2-naphthyl sulfides by FMO2 and FMO1

Analog	FMO2 Vmax[a]	FMO2 (*R*:*S*)[b]	FMO1 Vmax[a]	FMO1 (*R*:*S*)[b]
Methyl	66.5	>98:2	36.9	92:8
Ethyl	57.2	>98:2	31.3	87:13
Propyl	18.9	>97:3	11.7	80:20
Butyl	<0.2	ND[c]	12.8	83:17
Hexyl	<0.2	ND	1.4	ND

[a] nmol/nmol/min at 100 μM substrate concentration.
[b] Configuration was determined as described in Experimental.
[c] ND=Not determined due to insufficient metabolite formation.

of product stereochemistry through this simple series of alkyl p-tolyl sulfides. Chiral sulfoxidations of aryl alkyl sulfides have been described, such as with the cytochromes P450,[5a] chloroperoxidase[5b] and horseradish peroxidase,[5c] but the stereochemistry of sulfoxidation was generally independent of substrate structure.[5a–h] Flavoenzyme reactions, such as those of the bacterial cyclohexanone monooxygenase, which demonstrate substrate-dependent stereoselectivity in the formation of aryl alkyl sulfoxides have been reported.[5d–h] However, we are not aware of any precedent for the smooth inversion of stereochemistry displayed by FMO2 which provides the striking sigmoidal structure–function relationship shown in Figure 1.

We feel that these data are consistent with an active-site model which has two principal substrate binding pockets, a model similar to that proposed for pig liver esterase.[10] One site is large and prefers to bind large, planar, aromatic residues; the other pocket can accommodate short *n*-alkyl chains or a p-tolyl moiety. With this topography, short-chain alkyl p-tolyl sulfides could be oriented such that the *pro-R* lone pair of electrons, exclusively, are directed towards the oxygenating species (Figure 2A). The smaller pocket cannot accommodate the longer *n*-alkyl chains and so when the p-tolyl moiety occupies this site, the *pro-S* lone pair are oriented towards the hydroperoxide (Figure 2B). Competition between the two sites produces products with intermediate stereochemistry. The observation that FMO2 S-oxygenates only the short-chain naphthyl sulfides is consistent with this model, if the putative smaller pocket excludes a 2-naphthyl susbtituent. This *requires* that product is exclusively of the (*R*)-configuration, again consistent with the experimental findings. Recently, an ortholog of FMO2 has been identified in human brain which appears able to metabolize anti-depressant drugs.[6] Further work is required with other rigid substrate probes in order to refine this preliminary model and to determine how the information presented here for rabbit FMO2 applies to the human ortholog.

Experimental

Instrumentation

NMR spectra were obtained with a Bruker 300 MHz spectrometer. Optical rotations were measured with a Jasco DIP-4 digital polarimeter with a sodium lamp (589 nm) light source in cylindrical 10 cm × 3.5 cm quartz cells. HPLC was performed with an SSI gradient system equipped with a controller, Model 200B and 220B pumps and a model 500 variable wavelength detector linked to a Hewlett–Packard 3396A integrator. GC analysis was performed on an HP 5890 chromatograph fitted with a 30m DB-5 column and a flame-ionization detector (FID).

Materials

p-Tolyl methyl sulfide and (*R*)-(+)-p-tolyl methyl sulfoxide, 2-naphthalene thiol, alkyliodides, alkyl Grignards and menthyl sulfinates were purchased from Aldrich. p-Thiocresol and sodium m-periodate

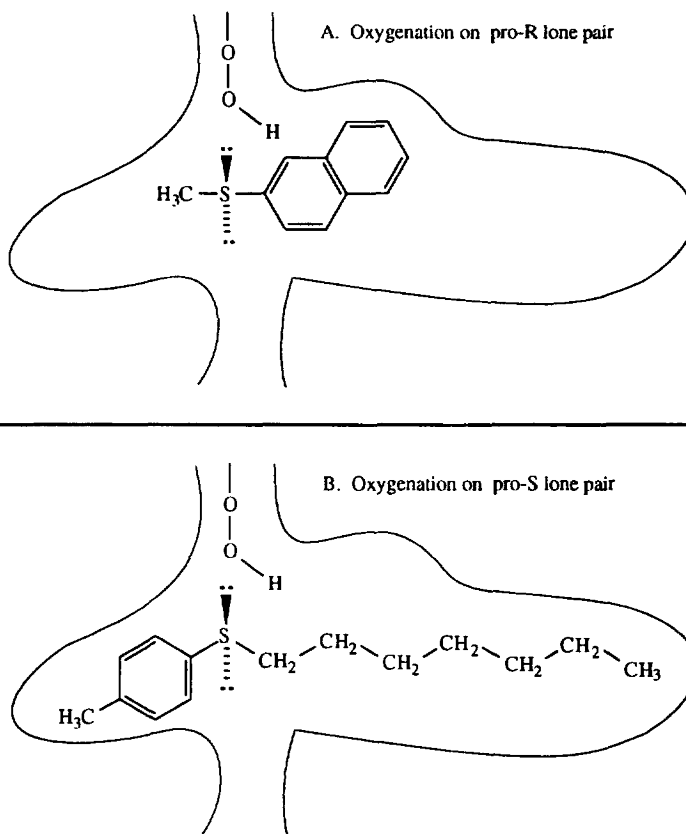

Figure 2. Proposed orientation of substrates in FMO2 active site.

were purchased from Sigma. HPLC grade hexane and 2-propanol were obtained from Optima. The analytical Chiralcel OB column was purchased from Baker and the analytical and preparatory (*R,R*) Whelk-O1 columns were obtained from Regis. Rabbit lung FMO2 and minipig liver FMO1 were purified as previously described.[4a]

Syntheses

Methyl, ethyl, *n*-propyl, *n*-butyl p-tolyl sulfides were synthesized and characterized as described previously.[4a,d] The *n*-pentyl, *n*-hexyl and *n*-heptyl derivatives were prepared in a similar manner by the reaction of p-thiocresol with the appropriate alkyl iodide in methanol under nitrogen. Reactions were monitored by thin-layer chromatography (TLC). Purification by column chromatography (SiO_2/hexanes) gave >95% yield for each analog. The *n*-alkyl 2-naphthyl sulfides were prepared by the same method, utilizing 2-naphthalene thiol and the corresponding alkyl iodide. The identity of all products was confirmed by proton NMR and all substrates were >95% pure as assessed by GC/FID.

Racemic sulfoxides were generated essentially as reported previously[4a,d] by reacting 1 mmol of sulfide with 1.25 mmol of sodium m-periodate (Na_2IO_4) in methanol at 4°C. Reactions were monitored by TLC. Reactions were quenched by the addition of water, extracted into methylene chloride, and purified by column chromatography over silica (hexanes/2-propanol). All reactions gave >95% yield. Products were characterized by proton NMR and were >95% pure as assessed by normal-phase HPLC.

Enzyme incubations

Typically, enzyme reactions contained 25 pmol of purified FMO, 100 mM glycine, 25 mM pyrophosphate (pH 8.5) and 1 µmol NADPH in a volume of either 0.99 or 2.97 mL. After a 3 minute preincubation period at 37°C, substrate was added in methanol to give a final incubation volume of 1 mL or 3 mL and incubated for 10 additional minutes. Control incubations were conducted in the absence of NADPH. Reactions were terminated by the addition of 10 mL of ice cold dichloromethane. 0.5 mg NaCl and 5 µg of an appropriate sulfoxide internal standard were added and reactions were extracted by vortexing for 1 minute, the aqueous layer was removed, and the organic layer was evaporated under a stream of nitrogen. The residue was reconstituted in a small volume of 2-propanol and analyzed by normal-phase HPLC. Sulfoxide metabolite peaks were collected in silanized glass concentration tubes, concentrated under a stream of nitrogen, and reanalyzed by chiral-phase HPLC.

HPLC analyses

Normal-phase HPLC separations were performed on an Alltech silica 10 µm column (250 mm × 4.6 mm) using a mobile phase of hexane/2-propanol (80/20–95/5) isocratically at 2 mL/min, with UV detection at 254 nm. Metabolites were quantitated by comparison of the metabolite peak area/internal standard peak area ratio to a standard curve constructed by extracting known amounts of each compound. Chiral-phase separation was performed either on 250 mm × 4.6 mm Chiralcel OB (Baker) or (*R*,*R*) Whelk-O1 (Regis) columns with a mobile phase of hexane/2-propanol, 60/40–90/10 and flow rates of 1–1.5 mL/min.

Determination of absolute configuration

Enantiomerically pure methyl, ethyl, *n*-propyl and *n*-butyl p-tolyl sulfoxides [>98% e.e.] were obtained by reaction of the appropriate alkyl Grignard reagent with either the (+)- or (−)-menthyl p-toluenesulfinate as described previously.[4a,d] This reaction is known to proceed with inversion of stereochemistry[7] and after purification by column chromatography (SiO_2 with hexanes/2-propanol), the (*R*)- or (*S*)-sulfoxide was obtained from (*S*)- or (*R*)-menthyl p-toluenesulfinate, respectively. On Chiralcel OB, the (*S*)-methyl, ethyl, *n*-propyl and *n*-butyl sulfoxides eluted first, and on the (*R*,*R*) Whelk-O1 column the order of elution was reversed. We confirmed that the order of elution did not change for the longer *n*-alkyl chain p-tolyl derivatives by stereospecific synthesis of (*R*)-(+)-p-tolyl hexyl sulfoxide from reaction of (−)-menthyl p-tolyl sulfinate with hexyl magnesium bromide, $[\alpha]^{D}{}_{25}=+112\pm3$ (c=1 mg/mL, acetone).

A convenient chiral synthesis of the *n*-alkyl 2-naphthyl sulfoxides is not available. However, it has been reported that all dextrorotatory aryl alkyl sulfoxides possess the (*R*)-configuration.[8] Therefore, we isolated sufficient quantities (6–7 mg) of the two enanatiomers of methyl 2-naphthyl sulfoxide by repetitive chromatography on a preparatory scale (*R*,*R*) Whelk-O1 column (1 cm×25 cm) to assign stereochemistry by rotation. Both enantiomers eluting from (*R*,*R*) Whelk-O1 were collected, the pooled samples evaporated to dryness and the products redissolved in acetone to 0.64 mg/mL for polarimetry. The more stereochemically pure fraction was found to be dextrorotatory and so was assigned the (*R*)-configuration, $[\alpha]^{D}{}_{25}=+108\pm5$ (c=0.64 mg/mL, acetone). In accordance with the behavior of the alkyl p-tolyl sulfoxides this order of elution was reversed on Chiralcel OB. These assignments are consistent with a previous report from our laboratory which assigned the stereochemistry of methyl 2-naphthyl sulfoxide based on our observation that FMO1 and FMO5 isoforms behave, respectively, as (*R*)- and (*S*)-sulfoxide synthases for methyl aryl sulfides.[9]

Acknowledgements

This work was supported in part by NIH grant GM43511 to AER. MBF was supported by NIH Training Grant GM07750. The authors would like to thank Dr Richard M. Philpot (NIEHS) for the generous gift of expressed FMO2, Dr Dieter Lang for critically reading the manuscript, and Cliff Fisher for many helpful discussions.

References

1. (a) Ziegler, D. M. *Annu. Rev. Pharmacol. Toxicol.* **1993**, *33*, 179–199. (b) Ziegler, D. M. *TiPS* **1990**, *11*, 321–324. (c) Ziegler, D. M. Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds, in *Enzymatic Basis of Detoxication*, Jakoby, W. B., Ed., Vol.1, Academic Press, New York, **1980**, 201–227.
2. (a) Villaverde, C.; Alvarez, A. I.; Redondo, P.; Voces, J.; Del Estal, J. L.; Prieto, J. G. *Xenobiotica* **1995**, *25*, 433–441. (b) Cashman, J. R.; Park, S. B.; Yang, Z.; Washington, C. B.; Gomez, D. Y.; Giacomini, K. M.; Brett, C. M. *Drug Metab. Dispos.* **1993**, *21*, 587–597. (c) Cashman, J. R. *Chem. Res. Toxicol.* **1995**, *8*, 165–181. (d) Cashman, J. R. *Mol. Pharmacol.* **1989**, *36*, 497–503.
3. (a) Lomri, N.; Yang, Z.; Cashman, J. R. *Chem. Res. Toxicol.* **1993**, *6*, 800–807. (b) Nagata, T.; Williams, D. E.; Ziegler, D. M. *Chem. Res. Toxicol.* **1990**, *3*, 372–376. (c) Poulsen, L. L.; Taylor, K.; Williams, D. E.; Masters, B. S. S.; Ziegler, D. M. *Mol. Pharmacol.* **1986**, *30*, 680–685.
4. (a) Rettie, A. E.; Bogucki, B. D.; Lim, I.; Meier, G. P. *Mol. Pharmacol.* **1990**, *37*, 643–651. (b) Sadeque, A. J. M.; Eddy, A. C.; Meier, G. P.; Rettie, A. E. *Drug Metab. Dispos.* **1992**, *20*, 832–839. (c) Rettie, A. E.; Meier, G. P.; Sadeque, A. J. M. *Chem.–Biol. Interact.* **1995**, *96*, 3–15. (d) Rettie, A. E.; Lawton, M. P.; Sadeque, A. J. M.; Meier, G. P.; Philpot, R. M. *Arch. Biochem. Biophys.* **1994**, *311*, 369–377.
5. (a) Light, D. R.; Waxman, D. J.; Walsh, C. *Biochemistry* **1982**, *21*, 2490–2498. (b) Allenmark, S. G.; Andersson, M. A. *Tetrahedron: Asymmetry* **1996**, *7*, 1089–1094. (c) Ozaki, S.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 7056–7064. (d) Carrea, G.; Redigolo, B.; Riva, S.; Colonna, S.; Gaggero, N.; Battistel, E.; Bianchi, D. *Tetrahedron: Asymmetry* **1992**, *3*, 1063–1068. (e) Ottolina, G.; Pasta, P.; Carrea, G.; Colonna, S.; Dallavalle, S.; Holland, H. L. *Tetrahedron: Asymmetry* **1995**, *6*, 1375–1386. (f) Ryerson, C. C.; Ballou, D. P.; Walsh, C. *Biochemistry* **1982**, *21*, 2644–2655. (g) Fujimori, K.; Matsuura, T.; Mikami, A.; Watanabe, Y.; Oae, S.; Iyanagi, T. *J. Chem. Soc. Perkin Trans. 1* **1990**, 1435–1440. (h) Auret, B. J.; Boyd, D. R.; Henbest, H. B.; Ross, S. *J. Chem. Soc. C* **1968**, 2371–2374.
6. (a) Bharme, S.; Bhagwat, S. V.; Shankar, S. K.; Williams, D. E.; Ravindranath, V. *J. Pharm. Exp. Ther.* **1993**, *267*, 555–559. (b) Bharme, S.; Bhagwat, S. V.; Shankar, S. K.; Boyd, M. R.; Ravindranath, V. *Brain Res.* **1995**, *672*, 276–280.
7. (a) Solladié, G.; Hutt, J.; Girardin, A. *Synthesis* **1987**, 173. (b) Mislow, K.; Green, M. M.; Laur, P.; Melillo, J. T.; Simmons, T.; Ternay, A. L., Jr. *J. Am. Chem. Soc.* **1965**, *87*, 1958–1976.
8. Boyd, D. R.; Walsh, C. T.; Chen; Y.-C. J., S-Oxygenases, II: Chirality of Sulfoxidation Reactions, in: *Sulfur-Containing Drugs and Related Compounds*, Damani, L. S. Ed., Vol. 2, Part A, Ellis Horwood, Chichester, England, **1989**, 67–100.
9. Fisher, M. B.; Lawton, M. P.; Atta-Asafo-Adjei, E.; Philpot, R. M.; Rettie, A. E. *Drug Metab. Dispos.* **1995**, *23*, 1431–1433.
10. Lam, L. K. P.; Hui, R. A. H. F.; Jones, J. B. *J. Org. Chem.* **1986**, *51*, 2047–2050.

(Received in USA 12 December 1996)