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Sulphoxidation of ethyl methyl sulphide, 4-chlorophenyl methyl sulphide and diphenyl sulphide by purified pig liver flavin-containing monooxygenase

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1. The biotransformation of ethyl methyl sulphide (EMS), 4-chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) to their corresponding sulphoxides by purified flavin-containing monooxygenase (FMO) is described.

2. Purified pig liver flavin-containing monooxygenase catalysed the sulphoxidation of EMS, CPMS and DPS to their corresponding sulphoxides and the reactions followed single enzyme Michelis–Menten kinetics.

3. The apparent K_m and V_{max} for the sulphoxidation of EMS were 1.38 ± 0.05 mM and 78.74 ± 3.9 nmoles mg^{-1} protein min^{-1}, respectively. The apparent K_m and V_{max} for the sulphoxidation of CPMS were 0.185 ± 0.03 mM and 103 ± 5.0 nmoles mg^{-1} protein min^{-1}, respectively. The apparent K_m and V_{max} for the sulphoxidation of DPS were 0.068 ± 0.002 mM and 49.26 ± 2.05 nmoles mg^{-1} protein min^{-1}, respectively.

4. A significant reduction of the sulphoxidation of these simple sulphides was observed with addition of 1-naphthylthiourea in the incubation medium. On the other hand, incorporation of catalase and superoxide dismutase into the incubation media produced no appreciable inhibition of the observed sulphoxidation of the sulphides.

5. These results suggest that FMO is responsible, at least in part, for the sulphoxidation of nucleophilic sulphides as well as for the oxidation of sulphur atoms that reside within or adjacent to aromatic systems.

Introduction

Sulphide and sulphoxide functionalities are frequently encountered in agricultural chemicals and medicinal agents and may determine or influence the biological fate of the parent molecule (Ziegler 1980). Sulphides are readily converted to sulphoxides *in vitro* and *in vivo* due to the readily accessible lone pair of electrons on the divalent sulphur atom (Ziegler 1990). The psychoactive phenothiazine drugs, for example, are metabolized to their corresponding sulphoxides in various animal species and in man (Traficante *et al*. 1979). Sulphoxides, on the other hand, may be oxidized to the corresponding sulphones or reduced to sulphides. The sulphoxide drug, sulphinpyrazone, undergoes both reduction to the corresponding sulphide and oxidation to the sulphone after parenteral administration in rats (Renwick 1982).

The mammalian flavin-containing monooxygenase (FMO) is a family of enzymes mainly distributed in the liver, lung and kidney of mammals (Ziegler

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1988). FMOs exhibit broad substrate specificity for catalysing sulphur and nitrogen functional groups, which are common in therapeutic drugs (Ziegler 1990). Additionally, FMO is a major contributor to the metabolism of agricultural chemicals and may be responsible for producing biologically active metabolites (Ziegler 1988). To date, five distinct members of this protein family, designated FMO1–5, have been identified in mammals including humans. FMO1, the dominant form of FMO in pig liver, has been reported to S-oxygenate a number of nucleophilic sulfur-containing substrates (Lawton *et al.* 1994). There is a considerable amount of information available on the pig liver FMO1 compared with the human form. However, the substrate specificity of human FMO1 appears to be considerably more restricted than that of the pig FMO1. Therefore, differences in the types of nucleophiles accepted must be considered in attempting to extrapolate the extensive structure–activity studies available for the pig FMO1 to the human FMO1 (Kim and Ziegler 2000). Interestingly, the specific role of the FMOs in oxidative metabolism of xenobiotics in mammals is not well defined due to lack of specific and selective inhibitors for the FMO forms.

Ethyl methyl sulphide (EMS), 4-chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) (figure 1) are simple sulphides that are encountered in petroleum distillates and in the environment. These compounds are also intermediates in the manufacture of other xenobiotics. EMS, CPMS and DPS are biotransformed to their corresponding sulphoxides *in vitro* and *in vivo* (Nnane and Damani 1999, 2002). The cytochrome P450 or the FMO enzyme systems are

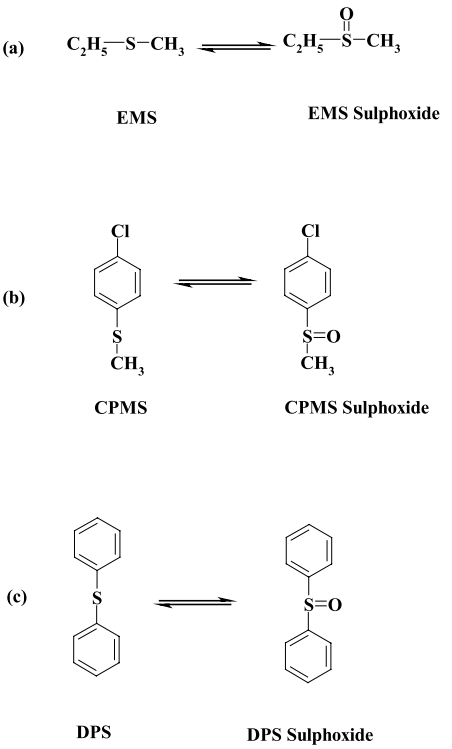

Figure 1. Sulphoxidation of (a) ethyl methyl sulphide (EMS), (b) 4-chlorophenyl methyl sulphide (CPMS) and (c) diphenyl sulphide (DPS) to their corresponding sulphoxides.

thought to mediate these S-oxidation reactions depending on the nucleophilicity of the substrates (Poulsen and Ziegler 1995). It has been reported that FMO is responsible for the sulphoxidation of the more nucleophilic aliphatic sulphides to their corresponding sulphoxides while both cytochrome P450 and the FMO are involved in the oxidation of sulphur atoms that reside within or adjacent to aromatic or heterocyclic ring systems (Hoodi and Damani 1984, Nnane and Damani 2002). Previous studies have also reported that the metabolism of a simple dialkyl sulphide such as EMS to its sulphoxide is predominantly mediated by FMO whereas sulphoxidation of an alkylaryl sulphide such as CPMS is mediated by both cytochrome P450 and the FMO in rat liver microsomes (Nnane and Damani 1999, 2002). These studies also indicate that the sulphoxidation of DPS to its sulphoxide is mediated almost exclusively by cytochrome P450 in rat liver microsomes. The aim of the present study was to determine the specific role of purified pig liver FMO in the sulphoxidation of these simple sulphides to their corresponding sulphoxide metabolites. The information obtained on the sulphoxidation of these simple sulphides by the pig liver FMO may be extrapolated to S-oxidation reactions involving more complex xenobiotics bearing the thioether functionality.

Materials and methods

Chemicals

Ethyl methyl sulphide (98%), 4-chlorophenyl methyl sulphide (98%), diphenyl sulphide (98%), *n*-propyl sulphone, 1-naphthylthiourea, 4-chlorophenyl phenyl sulphone, nicotinamide adenine dinucleotide ($NADP^+$), glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD) superoxide dismutase and catalase (11 000 units mg^{-1} protein) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The sulphoxides of EMS, CPMS and DPS were synthesized in our laboratory by methods described by Madesclaire (1986). The purity of each sulphoxide was >98%. All other reagents and organic solvents of HPLC or analytical grades were purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified pig liver FMO, which gave an electrophoretically single protein band, was kindly supplied by Dr D. M. Ziegler of the University of Texas, Austin.

Incubation procedures

Purified pig liver FMO was reconstituted in phosphate buffer (2 mM, pH 7.4) at a protein concentration of 4 mg ml^{-1}. Incubations were carried out in duplicate with the reconstituted enzyme in 25 ml Erlenmeyer flasks at 37°C using a shaking water bath in three separate occasions ($n = 3$). A typical incubation mixture consisted of a cofactor solution (0.5 ml), reconstituted enzyme (1 ml, 0.1 mg ml^{-1} protein) and substrate (20 µl) in acetone plus 0.480 µl distilled water (0–5 mM) in a total volume of 2 ml. The cofactor solution consisted of $NADP^+$ (1 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 unit) and magnesium chloride (10 mM, 50% w/w aqueous solution) in sodium phosphate buffer (0.2 M, pH 7.4). Metabolism was initiated by the addition of the reconstituted enzyme and terminated by adding NaOH (0.5 ml, 0.1 M) to the incubation mixture. The purified enzyme was omitted in control incubations. In another series of incubation flasks, EMS, CPMS and DPS were incubated with the purified enzyme and a modified cofactor solution containing superoxide dismutase (1000 units/flask) and catalase (11 000 units mg^{-1} protein). The metabolic inhibition reactions were carried out at 37°C in a shaking water bath using the optimum incubation time of 15 min for EMS, CPMS and DPS. A saturating substrate concentration of 2.5, 0.5 and 0.25 mM for EMS, CPMS and DPS, respectively, was used routinely for the metabolic inhibition studies. The biologically formed sulphoxides were extracted and measured by chromatographic assay methods.

Sample preparation and construction of calibration curves

The levels of the sulphoxide of EMS in the incubation mixture were monitored by a gas chromatographic assay method. To the incubation samples, sodium hydroxide (50 µl, 0.1 M), the internal standard, *n*-propyl sulphone (10 µl, 1 mg ml^{-1}) and sodium chloride (0.1 g) were added. The samples were then extracted with dichloromethane (2 × 5 ml). The pooled extracts were evaporated to dryness, reconstituted in 20 µl methanol and aliquots (5 µl) of the reconstituted extracts injected onto the

GLC column. The calibration curves for quantitation of EMSO were constructed by spiking varying amounts (0–50 $\mu g\,ml^{-1}$) into extraction tubes containing inactivated enzyme preparation, EMS (2.5 mM, cofactors and the internal standard, *n*-propyl sulphone (10 µl, 1 $mg\,ml^{-1}$), in a total volume of 2 ml of incubation mixture.

The calibration curves for quantitation of CPMSO were constructed by spiking varying amounts (0–50 $\mu g\,ml^{-1}$) into extraction tubes containing inactivated enzyme preparation (1 ml), CPMS (0.5 mM), cofactors and the internal standard, $CPPSO_2$ (20 µl, 1 $mg\,ml^{-1}$), in a total volume of 2 ml incubation mixture. The analytes were extracted with diethyl ether (2 × 5 ml) using a mechanical bench test tube shaker for 10 min followed by centrifugation at 400*g* for 10 min. The organic layers were evaporated to dryness in a water bath at 40°C. The extracts were reconstituted in a small volume (50 µl) of methanol and an aliquot (10 µl) of the concentrate was injected onto the HPLC column. The ratios of the peak area of the sulphoxide divided by the peak area of the internal standard were plotted against actual concentrations of the sulphoxide in the mixture. Similarly, the calibration curves for DPSO were constructed by spiking varying amounts (0–50 $\mu g\,ml^{-1}$) into extraction tubes containing inactivated enzyme preparation (1 ml), DPS (0.5 mM), cofactors and the internal standard, $CPPSO_2$ (20 µl, 1 $mg\,ml^{-1}$), in a total volume of 2 ml incubate. The mixtures were extracted with diethyl ether (2 × 5 ml) using a mechanical bench test tube shaker for 10 min followed by centrifugation at 400*g* for 10 min. After HPLC analysis, the peak areas for DPSO and the internal standard were measured for the construction of calibration curves as described for CPMSO above.

GC method

Gas chromatography was performed on an HP 5840A gas chromatograph (Hewlett-Packard Co., Avondale, PA, USA), equipped with a flame ionization detector and a glass column (200 × 0.4 cm). Separation of EMSO and *n*-propyl sulphone was achieved on a glass column packed with 10% Carbowax 20 M on Chromosorb W (Hewlett-Packard). The optimum working conditions for analysis of EMSO and *n*-propyl sulphone were: nitrogen flow rate, 30 $ml\,min^{-1}$, injection port temperature, 200°C, column temperature, 170°C; and detector temperature, 250°C.

HPLC method

The high-performance liquid chromatographic (HPLC) system used consisted of an hp® HPLC system (series 1050) equipped with a multichannel solvent delivery system coupled to an autosampler and a photodiode array detection system operated at 260 nm (Hewlett Packard). Chromatographic separation and quantitation of CPMS, DPS and their corresponding sulphoxides was achieved using a reversed-phase Spherisorb® 5ODS column (15 × 0.46 cm) obtained from HPLC technology (Macclesfield, UK). The analytical column was protected by a guard column packed with pellicular ODS packing material. The mobile phase was composed of water and tetrahydrofuran (THF) (45:55 v/v) at a flow rate of 0.5 $ml\,min^{-1}$ for the study of CPMS sulphoxidation. For DPS sulphoxidation studies, the mobile phase composition was changed to THF/water (50:50 v/v) at a flow rate of 0.5 $ml\,min^{-1}$.

Data analysis

Least-squares regression analysis on SigmaPlot® for Windows® (SPSS Science Chicago, IL, USA) was used to evaluate calibration curves and the enzyme kinetics. The Lineweaver–Burk transformation of the Michaelis–Menten rate equation was used to evaluate the enzyme kinetics in this study:

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}},$$

where $[S]$ is the concentration of the substrate, V is the rate of sulphoxide formation, V_{max} is the maximum rate of sulphoxide formation and K_m is the substrate concentration when one-half of the maximum rate of sulphoxide formation is attained. K_m, V_{max} and K_i were calculated by least-squares fit to the kinetic equations. One-way analysis of variance (ANOVA) on SigmaStat® for Windows was used to obtain probabilities (p) and compare different treatment groups. The Bonferroni *post-hoc* test was used for the determination of the level of significance. $p < 0.05$ was considered as statistically significant.

Result and discussion

EMSO ($R_t = 3.9$ min) and the internal standard, *n*-propyl sulphone ($R_t = 7.5$ min) were separated satisfactorily by using the 10% Carbowax 20 M on Chromosorb W column and the calibration curve for EMSO was linear and reproducible over the range examined ($r \geq 0.997$). The inter- and intraday vari-

ation at the lower concentrations was $< 10\%$, and $< 5\%$ at the higher concentrations.

Satisfactory separation of CPMSO ($R_t = 4.8$ min) and the internal standard, $CPPSO_2$ ($R_t = 9.6$ min), was achieved by using the reversed-phase HPLC conditions described. Similarly, satisfactory separation of DPSO ($R_t = 5.6$ min) and the internal standard, $CPPSO_2$ ($R_t = 10.2$ min), was achieved by using the reversed-phase HPLC conditions described. The calibration curves for measuring the sulphoxide of CPMS or DPS were linear ($r \geq 0.995$) in the range 0–50 $\mu g\,ml^{-1}$ and the assay methods were reproducible with low inter- and intra-assay variation of $< 13.5\%$. The assay methods were simple, fairly rapid and allowed accurate estimation of enzymically formed sulphoxides of CPMS and DPS *in vitro*.

The purified pig liver FMO catalysed the sulphoxidation of the model sulphides, EMS, CPMS and DPS to their corresponding sulphoxides under the incubation conditions used. The sulphoxidation of EMS, CPMS and DPS increased linearly with respect to time (0–30 min) and protein concentration (0.02–0.2 $mg\,ml^{-1}$) and followed a typical Michaelis–Menten profile with respect to substrate concentration (0–5 mM). A Lineweaver–Burk plot of EMS sulphoxidation was linear (figure 2). The apparent K_m and V_{max} for the sulphoxidation of EMS were 1.38 ± 0.05 mM and 78.74 ± 3.9 nmoles mg^{-1} protein min^{-1}, respectively. Similarly, Lineweaver–Burk plots of CPMS and DPS sulphoxidation were linear (figures 3 and 4). The estimated apparent K_m and V_{max} for the sulphoxidation of CPMS were 0.185 ± 0.03 mM and 103 ± 5.0 nmoles mg^{-1} protein min^{-1}, respectively. The estimated apparent K_m and V_{max} for the sulphoxidation of DPS were 0.068 ± 0.002 mM and 49.26 ± 2.05 nmoles mg^{-1} protein min^{-1}, respectively.

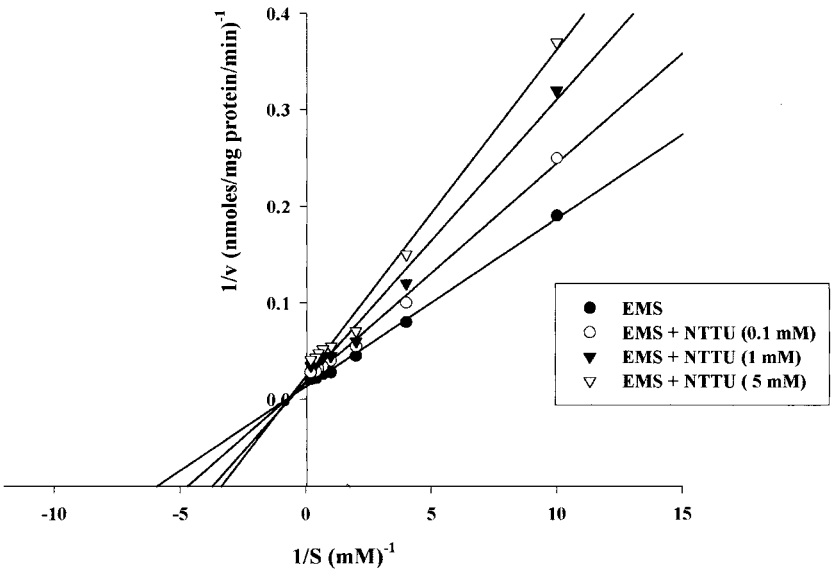

Figure 2. Lineweaver–Burk plots of ethyl methyl sulphide (EMS) sulphoxidation and the inhibitory effects of 1-naphthylthiourea (NTTU) on EMS sulphoxidation by purified pig liver flavin-containing monooxygenase. The incubation conditions were protein content = 0.1 $mg\,ml^{-1}$; incubation time = 15 min and EMS concentrations = 0.1–5 mM. Inhibitor concentrations were 0.1, 1 and 5 mM. Values are the means of three separate experiments ($n = 3$). The spread was 5–10% of the means.

A significant ($p < 0.05$) and concentration-dependent reduction of the sulphoxidation of these simple sulphides was observed with the addition of 1-naphthylthiourea, a known inhibitor of FMO, in the incubation medium (figures 2–4). The results indicate that 1-naphthylthiourea competitively inhibited the sulphoxidation of EMS by purified pig liver FMO with a $K_i = 8.49 \pm 0.51$ mM (figure 2). Analysis of the sulphoxidation of CPMS and DPS by purified pig liver FMO in the presence of 1-naphthylthiourea suggest that the inhibitor non-competitively inhibited the sulphoxidation of CPMS and DPS with K_i of 10.95 ± 0.78 and 6.8 ± 0.34 mM, respectively (figures 3 and 4). On the other hand, incorporation of catalase and superoxide dismutase into the incubation media produced no appreciable inhibition ($p > 0.05$) of the observed sulphoxidation of these simple sulphides (table 1). Non-enzymatic oxidation of the sulphides was negligible since the incorporation of catalase and superoxide dismutase into the incubation media produced no statistically significant inhibition of the observed sulphoxidation of the compounds. If reactive oxygen species played a significant role in the oxidation of these substrates then the inclusion of catalase and superoxide dismutase into the incubation medium would have markedly inhibited the metabolic reactions.

Previous studies have reported that the sulphoxidation of EMS by rat liver microsomes is mediated predominantly by the flavin-containing monooxygenase (Nnane and Damani 1999). Our present findings with purified pig liver FMO support the contention that FMO is responsible for the sulphoxidation of the more nucleophilic aliphatic sulphide, such as EMS where the sulphur atom resides adjacent to alkyl groups, to its corresponding sulphoxide. In a separate report, it

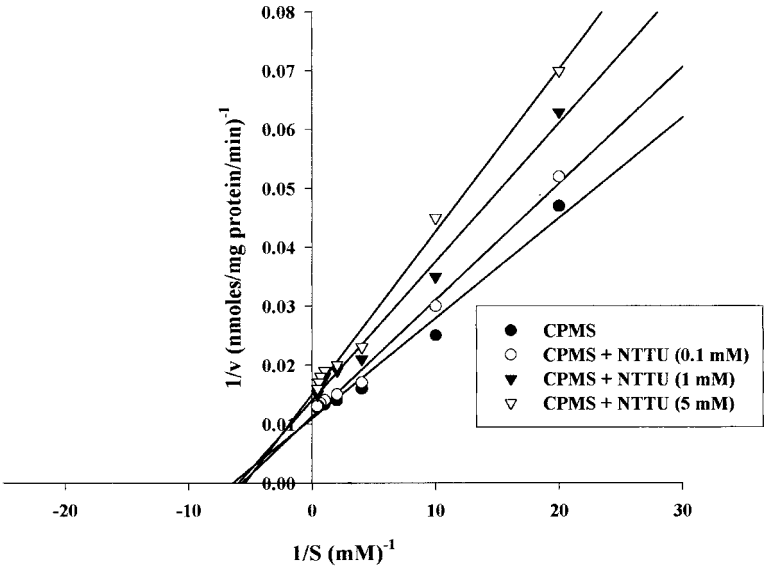

Figure 3. Lineweaver–Burk plots of 4-chlorophenyl methyl sulphide (CPMS) sulphoxidation and the inhibitory effects of 1-naphthylthiourea (NTTU) on the sulphoxidation of CPMS by purified pig liver flavin-containing monooxygenase. The incubation conditions were protein content = 0.1 mg ml^{-1}; incubation time = 15 min and CPMS concentrations = 0.05–2.5 mM. Inhibitor concentrations were 0.1, 1 and 5 mM. Values are the means of three separate experiments ($n = 3$). The spread was 5–10% of the means.

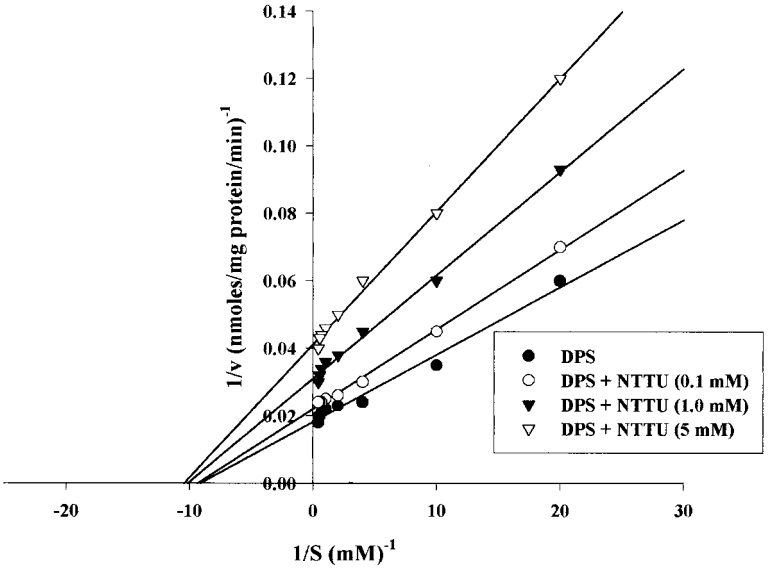

Figure 4. Lineweaver–Burk plots of the sulphoxidation of diphenyl sulphide (DPS) and the inhibitory effects of 1-naphthylthiourea (NTTU) on the sulphoxidation of DPS by purified pig liver flavin-containing monooxygenase. The incubation conditions were protein content = 0.1 mg ml^{-1}; incubation time = 15 min and DPS concentrations = 0.05–0.25 mM. Inhibitor concentrations were 0.1, 1 and 5 mM. Values are the means of three separate experiments ($n = 3$). The spread was 5–10% of the means

Table 1. Effects of catalase and superoxide dismutase on the sulphoxidation of ethyl methyl sulphide (EMS), 4-chlorophenyl methyl sulphide (CPMS) and diphenyl sulphide (DPS) by purified pig liver flavin-containing monooxygenase.

	Enzyme activity (nmoles mg^{-1} protein min^{-1})	
Substrate	Purified FMO	Purified FMO + catalase/superoxide dismutase
EMS	44.5 ± 4.08	40.95 ± 3.28*
CPMS	73.88 ± 5.20	67.97 ± 5.44*
DPS	41.77 ± 2.10	40.12 ± 2.40*

Incubation conditions were protein content = 0.1 mg ml^{-1}; incubation time = 15 min. Substrate concentrations were 2.5 mM for EMS, 0.5 and 0.25 mM for DPS metabolism studies.

* $p > 0.05$. Data are the means (± SD) from three separate experiments ($n = 3$).

was observed that the sulphoxidation of CPMS is mediated by both cytochrome P450 and FMO in rat liver microsomes (Nnane and Damani 2001). This finding was in agreement with another study where it was reported that the sulphoxidation of a simple alkylaryl sulphide was mediated by both cytochrome P450 and the flavin-containing monooxygenase (Waxman *et al.* 1982). Thus, the findings in the present study with purified pig liver FMO demonstrate that FMO has a role in the sulphoxidation of sulphides of intermediate nucleophilicity such as CPMS, where the sulphur atom resides adjacent to both alkyl and aryl groups.

The formation of the sulphoxide of DPS was also dependent on the presence of the purified pig liver FMO. The effects of catalase and superoxide dismutase on the S-oxidation of DPS by pig liver FMO was evaluated to rule out the participation of reactive oxygen species in this metabolic reaction. The sulphoxidation of DPS by pig liver FMO was not significantly ($p > 0.05$) affected by the addition of catalase or superoxide dismutase in the incubation medium (table 1). These results indicate that reactive oxygen species were not involved in the oxidation of DPS. On the other hand, addition of with 1-naphthylthiourea, an inhibitor of FMO, in the incubation mixture caused a significant decrease ($p < 0.05$) in DPS sulphoxidation in a concentration-dependent manner (figure 4). These results suggest that DPS is catalysed to its corresponding sulphoxide by purified pig liver FMO. Previous studies have indicated that the S-oxidation of simple diaryl sulphides was mediated mainly by cytochrome P450 monooxygenase (Hoodi and Damani 1984, Nnane and Damani 2002). In contrast, the present study clearly demonstrates a significant role for the FMO in the sulphoxidation of a diaryl sulphide such as DPS. Therefore, it would appear that the FMO contributes to the sulphoxidation of less nucleophilic thioethers such as DPS, where the sulphur atom resides adjacent to aromatic groups, in addition to its established role in the S-oxidation of nucleophilic compounds bearing the sulphur atom.

In conclusion, our studies demonstrate the capacity of purified pig liver FMO to catalyse the oxidation of both nucleophilic substrates such as EMS and substrates of intermediate nucleophilicity such as CPMS and also contributes to the S-oxidation of electrophilic substrates such as DPS. These simple sulphides may be used as probes for assessing the relative contribution of the microsomal monooxygenases to sulphoxidation reactions and the information generated on the sulphoxidation of these simple sulphides by the pig liver FMO may be used to predict the S-oxidation of more complex xenobiotics bearing the thioether functional groups in humans.

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