ABSTRACT:

Flavin-containing monoxygenases (FMOs) are microsomal enzymes that catalyze the NADPH- and \( \text{O}_2 \)-dependent oxidation of many nitrogen-, sulfur-, selenium-, and phosphorus-containing compounds (Ziegler, 1993). Five isoforms of FMO have been identified thus far (FMO1-FMO5), each exhibiting its own unique species- and tissue-dependent expression (Hines et al., 1994). The predominant FMO isoform in adult human liver is FMO3. This isoform catalyzes oxidation of several important drugs and xenobiotics such as methimazole, chlorpromazine, and nicotine (Cashman et al., 1995; Overby et al., 1997), as well as the oxidation of alkyl cysteine conjugates (Ripp et al., 1997a). FMO3 has also been shown to be the major enzyme responsible for methionine \( S \)-oxidation in rat and rabbit liver microsomes (Duescher et al., 1994; Krause et al., 1996). Although methionine can also be oxidized by FMO1 and FMO2, the \( K_m \) values for these reactions are much higher (50 mM and 30 mM, respectively) than the \( K_m \) value for oxidation by FMO3 (approximately 5 mM). Recently, FMO3 has been identified as the enzyme responsible for \( N \)-oxidation of trimethylamine, and mutations in this isoform result in the human disease trimethylaminuria (Dolphin et al., 1997). Oxidation of a chemical by FMO3 can result in the formation of a metabolite that is more readily excreted than the parent chemical, as is the case for trimethylamine. FMO3-mediated oxidations can also result in increased toxicity, as is the case for S-(1,2-dichlorovinyl)-L-cysteine (DCVC) (Sausen and Elfarra, 1991; Lash et al., 1994; Ripp et al., 1997a). Thus, whether oxidation by FMO3 is a detoxication or a bioactivation reaction depends on the chemical being oxidized.

Despite the fact that FMO3 is an important enzyme for drug metabolism and is the predominant FMO isoform in human liver, little is known about its distribution in other species and tissues. FMO3 messenger RNA (mRNA) levels have been qualitatively assessed in liver from rats, rabbits, mice, hamsters, and guinea pigs (Burnett et al., 1995). However, mRNA levels have been shown to not correlate well with actual protein levels of FMO3 (Overby et al., 1997). Therefore, species differences in hepatic expression of FMO3 protein remain to be investigated. Information about the distribution of FMO3 in laboratory animals is important because these animals are used in research to model human metabolism and toxicity.

FMO3 expression has been shown to be sex-dependent in mouse liver in that females express this isoform and males do not (Falls et al., 1995). This sex-dependence appears to be due to repression of FMO3 expression by testosterone (Falls et al., 1997a). The expression of FMO3 does not appear to show sex-dependence in human liver (Cashman et al., 1993; Sadeque et al., 1993). It is not known, however, whether or not other laboratory species, such as rats, rabbits, or dogs, exhibit sex differences in FMO3 expression, or if this is a mouse-specific phenomenon.

There has been very little characterization of FMO3 levels in the kidney. FMO3 mRNA levels have been qualitatively assessed in the
Materials and Methods

**Chemicals.** DCVC, DCVC sulfoxide, and S-allyl-L-cysteine (SAC) sulfoxide were synthesized as previously described (Ripp et al., 1997a). SAC was a gift from Wakanaga Pharmaceutical of America (Mission Viejo, CA). L-Methionine, L-methionine-dl-sulfoxide, flavin adenine dinucleotide, flavin mononucleotide, and NADPH were obtained from Sigma (St. Louis, MO). 2,4-Dinitro-1-fluorobenzene and trifluoroacetic acid were obtained from Aldrich (Milwaukee, WI). High-pressure liquid chromatography (HPLC)-grade acetonitrile was purchased from EM Science (Gibbstown, NJ).

**Biological Materials.** *Escherichia coli* membrane fractions containing complementary DNA (cDNA)-expressed rabbit FMO3 or human FMO3 were obtained as previously described (Burnett et al., 1994; Itagaki et al., 1996). Flavin contents were determined by HPLC with fluorescence detection as previously described (Ripp et al., 1997a). Human liver samples were purchased from SRI International (Menlo Park, CA). All human livers were from adult Caucasians ranging in age from 22 to 45 years old. New Zealand White rabbit (8–12 weeks old) livers and kidneys were obtained from Pel-Freez Biologicals ( Rogers, AR). Sprague-Dawley rats (8–10 weeks old) were purchased from Sasco (Omaha, NE) and B6C3F1 mice (6–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Adult dog (hound-cross) livers and kidneys were obtained from control animals in experiments carried out at the School of Veterinary Medicine (Madison, WI). Microsomes were prepared by differential centrifugation as previously described (Sauses and Elfarra, 1990). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

**Enzyme Assays.** The buffer used in all experiments was 0.1 M KCl, 0.1 M KH2PO4, 5 mM EDTA, pH 7.4. Microsomes (0.2–0.7 mg liver; 0.1–0.3 mg kidney) or *E. coli* membrane fractions (0.4–0.9 ml flavin) were preincubated at 37°C for 5 min in the presence of NADPH (2 mM final) in a shaking water bath and reactions were started by the addition of substrate. Final reaction volume was 0.5 ml. Control reactions lacking NADPH or with zero incubation time were run in parallel to correct for any nonenzymatic sulfoxidation. Reactions were stopped with either 0.5 ml of ice-cold ethanol (methionine and SAC) or 0.5 ml of ice-cold 0.75% perchloric acid (DCVC). Reactions were then vortexed and centrifuged for 15 min at 3000 rpm to removed precipitated proteins. Supernatants from DCVC reactions were filtered and derivatized with 18 μl of 2,4-dinitro-1-fluorobenzene (10% v/v in ethanol). Derivation of SAC reactions was complete after heating at 60°C for 1 h. Derivatization of methionine reactions was complete after heating at 37°C for 30 min and then allowing the reaction to proceed to room temperature for at least 6 h. Derivatized reaction mixtures were stable for at least 24 h and were analyzed by HPLC with UV detection at 360 nm as previously described (Duescher et al., 1994; Ripp et al., 1997a).

**Results**

**Catalytic Activities of Human and Rabbit FMO3.** cDNA-expressed human FMO3 and rabbit FMO3 catalyzed the highly diastereoselective S-oxidation of methionine, SAC, and DCVC. Reactions were monitored by measuring formation of sulfoxide diastereoisomers using sensitive HPLC methods. Both FMO3 orthologs catalyzed formation of the d-isomer of methionine sulfoxide as 90 to 100% of the total sulfoxide produced (Table 1). Both FMO3 orthologs also exhibited 90 to 100% diastereoselectivity for SAC sulfoxide and DCVC sulfoxide formation. The predominant diastereoisomer formed was the later eluting of the two sulfoxide diastereoisomers for both SAC and DCVC, however, the absolute stereochemistry was not determined. All reactions were protein- and NADPH-dependent and were linear for at least 75 min. Kinetic constants were determined using double-reciprocal plots (Table 1). Kinetic constants for S-oxidation of the three substrates were nearly identical between the two FMO3 forms. Previous studies in this laboratory have also shown that rat liver FMO3 had a Vmax value of 3.4 mM for methionine S-oxidation (Krause et al., 1996). These results suggest that FMO3 orthologs from multiple species are catalytically very similar with respect to SAC, DCVC, and methionine S-oxidations.

**Species Differences in Expression of Hepatic and Renal FMO3.** Methionine S-oxidation was used to assess FMO3 activity. The concentration of methionine used (10 mM) was high enough to saturate FMO3, but not high enough to result in significant interference from

Enzymatically produced sulfoxides were quantified by comparing peak areas from enzymatic reactions with peak areas from standard curves generated using synthetic sulfoxide standards (r2 > 0.99 for all standard curves). Limits of detection were 140, 43, and 120 pmol/20 μl injection for SAC sulfoxide, DCVC sulfoxide, and methionine sulfoxide, respectively. All HPLC methods allowed for separation of sulfoxide diastereoisomers. Absolute stereochemistry of methionine sulfoxide diastereoisomers was previously determined by picric acid precipitation (Duescher et al., 1994); absolute stereochemistry of sulfoxides of DCVC and SAC were not determined.

**Immunoblotting.** All experiments conducted, as standards, one lane of prestained molecular weight markers (Bio-Rad, Hercules, CA) and one lane of *E. coli* membrane fractions containing cDNA-expressed rabbit FMO3. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% resolving gel), followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked with 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) in Tris-buffered saline with 0.1% Tween 20. Goat anti-rabbit FMO3 primary antibody (monospecific for FMO3, as described in Overby et al., 1997) was used at a 1:1000 dilution. Secondary antibody (donkey anti-goat IgG conjugated to alkaline phosphatase; Jackson Immunoresearch) was used at a 1:20,000 dilution. After incubation with secondary antibody, membranes were incubated with Vistra ECF alkaline phosphatase fluorescent substrate (Amersham, Arlington Heights, IL) and scanned using a Storm system FluorImager (Molecular Dynamics, Sunnyvale, CA). Immunoreactive proteins were quantified using Image Quant software (Molecular Dynamics). To control for blot to blot variability in background fluorescence, incubation time, and scan time, intensity of fluorescence of FMO3 in microsomal samples was normalized to intensity of the standard (0.7 μg of *E. coli* membrane fractions containing cDNA-expressed rabbit FMO3) for each experiment. After quantification of fluorescence, blots were rinsed and immunoreactive proteins visualized with Western Blue alkaline phosphatase substrate obtained from Promega (Madison, WI).

**Experimental Design and Statistics.** Microsomes were prepared from livers and kidneys of at least three separate animals or separate pools of animals for mouse experiments. Experiments were conducted in duplicate and results are presented as means ± S.D. of three separate experiments. Species comparisons were made using one-way analysis of variance (α = 0.05), followed by least-significant difference analysis, with p < 0.05 as the criterion for significance. Sex comparisons were made using a two-sample t test, with p < 0.05 as the criterion for significance.
FMO1 or FMO2. Liver and kidney microsomes from each of the species/sexes were initially assayed for linearity of the methionine \( S \)-oxidase reaction with time and protein concentration. All reactions were linear for at least 30 min, with the exception of male mouse liver which was linear for 10 min, and with protein concentrations from 0.4 to 1.4 mg/ml for liver and 0.2 to 0.6 mg/ml for kidney (data not shown). The \( d \)-isomer of methionine sulfoxide was formed preferentially to the \( l \)-isomer for all species and sexes. In the liver, the formation of the \( d \)-isomer ranged from 70 to 100% of the total methionine sulfoxide formed, depending on the species. In the kidney, the \( d \)-isomer ranged from 60 to 80% of the total methionine sulfoxide formed (data not shown). The microsomal methionine \( S \)-oxidase reaction with time and protein concentration. All reactions species/sexes were initially assayed for linearity of the methionine \( S \)-oxidase activity than dogs, while rats had at least 2-fold higher activity than any of the other species.

Results from immunoblotting experiments were not used to measure species differences because antibody to one FMO3 ortholog (in this case, rabbit FMO3) would not be expected to react equally well with FMO3 from other species. Therefore, immunoblotting is not a good tool for comparing expression between species.
els, there were not significant differences between males and females. Results from immunoblotting experiments agreed with those of activity assays and showed no significant differences in FMO3 expression between males and females (Fig. 1, B and C).

Hepatic methionine S-oxidase activity was also determined in male and female dog, rat, mouse, and rabbit microsomes (Fig. 2A). The levels were not significantly different between sexes of any species except the mouse, where levels were significantly lower in males than in females. Male dogs also appeared to have lower levels of activity than female dogs; however, with a p value of .09, the difference was not statistically significant.

A, methionine (10 mM) was incubated with liver microsomes and NADPH-dependent methionine sulfoxide formation was measured by HPLC. Data represent means ± S.D. from three separate animals (or three separate pooled preparations for the mouse). Males are shown by open columns and females by shaded columns. * Significantly different from female (p < .05). B, relative amounts of FMO3 in male (open columns) and female (shaded columns) liver microsomes determined by immunoblotting. Relative intensities were determined using fluorescence imaging and are presented as means ± S.D. for three separate animals.

A, methionine (10 mM) was incubated with kidney microsomes and NADPH-dependent methionine sulfoxide formation measured by HPLC. Data represent means ± S.D. from three separate animals (or three separate pools of animals for rats and mice). Males are shown by open columns and females by shaded columns. B, relative amounts of FMO3 in male (open columns) and female (shaded columns) kidney microsomes determined by immunoblotting. Relative intensities were determined using fluorescence imaging and are presented as means ± S.D. for three separate animals.

Fig. 2. FMO3 levels in liver microsomes from male and female dog, rat, mouse, and rabbit.

Fig. 3. FMO3 levels in kidney microsomes from male and female dog, rat, mouse, and rabbit.
not statistically significant. Levels of FMO3 determined with immunoblots agreed with those determined using methionine S-oxidase activity (Figs. 2B and 4A). There were no distinguishable sex differences in FMO3 levels in rats and rabbits. Male dogs had 2-fold less FMO3 than female dogs, and there was a marked difference between the male and female mouse samples, with male mouse levels being essentially undetectable.

**Assessment of Sex Differences in Renal Microsomal FMO3 Levels Using Methionine S-Oxidase Activity and Immunoblotting.** Methionine S-oxidase activity was determined for kidney microsomes from male and female dogs, rats, mice, and rabbits. No significant differences in activity were detected between males and females of any species (Fig. 3A). Microsomal samples were also analyzed by immunoblotting with antibody to recombinant rabbit FMO3, and the results agree with the activity assays in that no sex differences were distinguishable in any of the four species (Figs. 3B and 4B).

**Relationship between Methionine S-Oxidase Activity and FMO3 Levels Detected by Immunoblotting.** The levels of methionine S-oxidase activity and the levels of FMO3 determined by immunoblotting showed similar trends for all species and sexes with the exception of the mouse. The ratios of specific activity for methionine S-oxidation to relative FMO3 intensity by immunoblotting are approximately equal for dogs, rats, and humans (approximately 10:1, activity/relative intensity) (Figs. 1–3). This ratio is consistent for both males and females. Female mouse liver also exhibits an activity/intensity ratio of approximately 10:1. The ratio of activity/intensity is lower for the male and female rabbit (approximately 5:1), which is expected due to better antigenicity of rabbit FMO3 to this antibody. However, for male mouse liver and for male and female mouse kidney, there is a much higher activity/intensity ratio (approximately 100:1) than is seen in any of the other species or in the female mouse liver. In fact, immunoreactive bands were not visible in any of the three immunoblots for male mouse liver or for male or female mouse kidney (Fig. 4). Therefore, it appears that for these three tissues there is methionine S-oxidase activity that is not FMO3-mediated. The possibility that an enzyme other than FMO3 was catalyzing methionine S-oxidation was investigated in male mouse liver microsomes. Kinetic constants for methionine S-oxidation were compared between male and female mouse liver microsomes (Fig. 5). Both tissues exhibited $K_m$ values of approximately 3 mM, while the $V_{max}$ values were 2.5-fold higher for the female mouse than for the male. Stereoselectivity favoring the formation of the $d$-isomer of methionine sulfoxide (70–75% of total sulfoxide) was evident in both male and female mouse liver. Methionine S-oxidation in male mouse liver microsomes was inhibited by 50% by inclusion of methimazole (1 mM), but not inhibited by inclusion of benzyliimidazole (1 mM), potassium cyanide (1 mM), catalase (2000 U), or superoxide dismutase (500 U) (data not shown).

**Discussion**

The purpose of the studies presented here was to investigate whether the sex-dependence of FMO3 expression seen in mouse liver was apparent in other commonly used laboratory species or in humans and whether FMO3 sex-dependence was evident in the kidney. The hepatic and renal microsomal levels of FMO3 were also compared among species. The expression of FMO3 was assessed using an activity assay and by immunoblotting. There have been several reports documenting the species-, sex-, and tissue-dependence of FMO (Dannan and Guengerich, 1982; Tynes and Hodgson, 1985; Tynes and Philpot, 1987; Shehin-Johnson et al., 1995). However, all of these studies were conducted before the time when an isofrom-specific probe for FMO3 was available and most used an antibody against purified hog liver FMO (now known as FMO1) or antibody against purified rabbit lung FMO (now known as FMO2). Therefore, these studies provide valuable information regarding distribution of FMO1 and FMO2, but not FMO3. More recent studies documenting FMO3 species-, sex-, and tissue-dependence are based on mRNA analysis (Burnett et al., 1994). This study is the first to look specifically at the distribution of FMO3 at the protein level in multiple species, sexes, and tissues.

To use an enzyme activity assay to assess FMO3 levels between species, it was first necessary to establish that FMO3 orthologs from different species were catalytically comparable. This was accomplished by comparing cDNA-expressed human FMO3 with rabbit FMO3 for S-oxidation of three different FMO3 substrates, methionine, SAC, and DCVC. Kinetic constants for the two FMO3 orthologs were nearly identical. In addition, the previously determined $K_m$ value for methionine S-oxidaion by purified rat liver FMO3 (3.4 mM) was very close to that determined for human and rabbit FMO3 (3.7 and 6.4 mM, respectively). These results suggest that FMO3 orthologs are catalytically similar with respect to methionine and cysteine conjugate oxidation.
S-oxidations, and that these reactions can be used to assess FMO3 levels between species. Of the three substrates studied, methionine was chosen as a probe for microsomal FMO3 activity for several reasons. FMO3 had previously been shown to be the major methionine S-oxidase in rat and rabbit liver microsomes (Duescher et al., 1994; Krause et al., 1996). Both methionine and SAC are S-oxidized at high enough rates to easily assay by HPLC, however, SAC can also be oxidized by FMO1 with a $K_m$ value similar to FMO3 (Ripp et al., 1997a). Although methionine can also be oxidized by FMO1 and FMO2, the $K_m$ values (50 mM and 30 mM, respectively) are much higher than for FMO3 (approximately 5 mM) (Duescher et al., 1994). Therefore, methionine, at a concentration of 10 mM, is a more specific probe of FMO3 activity than SAC. DCVC is also specific for FMO3 (Ripp et al., 1997a), but its relatively high $K_m$ value, low $V_{max}$ value, and its toxicity make it less desirable for use as a probe. It is interesting to note that the $V_{max}$ value for DCVC oxidation is much lower than the $V_{max}$ values for SAC and methionine oxidations (Table 1). This suggests that the rate-limiting step for DCVC oxidation is different from that of methionine and SAC S-oxidations.

Methionine S-oxidase activity was used to compare microsomal FMO3 levels among species, within the same tissue and for the same sex. The fact that species differences in hepatic FMO3 were only noted in males suggests that the differences may be due to varying responses to testosterone, as noted for the mouse liver by Falls et al. (1997a). Species differences in kidney microsomal FMO3 were evident in both sexes. The rat had 2- to 6-fold higher levels than the other species. This could be an important consideration when conducting metabolism studies of FMO3-dependent reactions in vivo, as the rat may have considerably more kidney metabolism than other laboratory species. Although species differences were noted in liver and kidney microsomes, it should be understood that these comparisons were made based on activity normalized to microsomal protein content. Conclusions about species differences are only valid assuming similar endoplasmic reticulum protein backgrounds in different species.

Human liver microsomes exhibited some interindividual variability in FMO3 levels, but on average there were no differences between males and females. This result agrees with results from two other studies, utilizing Western blots and activity assays, that found no sex differences in FMO3 in human liver (Cushman et al., 1993; Sadeque et al., 1993). The 2-fold interindividual variability detected in this study is less than the variability detected in previous studies, however, this may be explained by the small sample size and by the relative age and race homogeneity of the human liver samples used in this study.

When FMO3 levels were assessed in the liver and kidney of rats, mice, rabbits, and dogs, only mouse and dog liver exhibited sex differences. This suggests that the testosterone repression of mouse liver FMO3 noted by Falls et al. (1997a) is species- and tissue-specific. This is an important consideration when using the mouse for metabolism studies that may involve FMO3. It is interesting to note that the results of Falls et al. showed no detectable FMO3 in male mouse liver or in male or female mouse kidney by Western or Northern blot analysis (Falls et al., 1995; Falls et al., 1997b), whereas the results presented here showed FMO3 detectable by methionine S-oxidase assay, but not by immunoblotting. The possibility that the methionine S-oxidase activity detected may have been due to an enzyme other than FMO3 was investigated in male mouse liver microsomes. The activity was inhibited by methimazole, a high-affinity FMO substrate and competitive inhibitor, but not by the P-450 inhibitor benzylidiazole, or the peroxidase inhibitor potassium cyanide, or by scavengers of reactive oxygen species. This suggests that the activity may be FMO-mediated. The $K_m$ value for methionine S-oxidation of 2.8 mM is much closer to the $K_m$ value for FMO3 than for FMO1 or FMO2, which have much higher values. FMO5, which is also present in mouse liver, has been shown to not utilize methionine as a substrate (Duescher et al., 1994). It is possible that some of the activity detected in male mouse liver microsomes may be due to the presence of FMO1 or FMO2; however, if this were the case, then contribution by FMO1 or FMO2 would also be expected in other species that are also known to express these isoforms. This would therefore increase the activity/intensity ratio for all species, which was not the case. Furthermore, the stereoselectivity of methionine oxidation in male mouse liver microsomes was similar to that observed in female mouse liver microsomes. Therefore, it appears that there is an enzyme present in male mouse liver microsomes and in male and female mouse kidney microsomes that has catalytic properties, with respect to methionine S-oxidation, similar to those of FMO3, but is not immunoreactive with FMO3 antibody. The identity of this enzyme is currently under investigation in this laboratory. Taken together, these data suggest that methionine S-oxidation, using 10 mM methionine, is a good indicator of FMO3 in liver and kidney microsomes from humans, rats, rabbits, and dogs, but, based on the immunoblott ing results, may not be a good indicator in mice.

In summary, the results presented here show that FMO3 expression is species-, tissue-, and sex-dependent and that these factors should be considered when designing experiments that may involve FMO3-dependent metabolism.

References


