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THE FLAVIN-CONTAINING MONOOXYGENASE OF MOUSE KIDNEY

A COMPARISON WITH THE LIVER ENZYME

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Abstract—Flavin-containing monooxygenase (FMO; EC 1.14.13.8) was purified from mouse kidney microsomes and compared to that isolated from mouse liver microsomes. The purified enzymes from kidney and liver appeared as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with an apparent molecular weight of 58,000 daltons. On wide range (pH 3.5 to 9.0) isoelectric focusing, FMOs from kidney and liver resolved as a single band with an isoelectric point of 8.2. The enzymes from both kidney and liver have a pH optimum of 9.2. Thiobenzamide-S-oxidation catalyzed by both enzymes was sensitive to inhibition by the competitive inhibitors thiourea and methimazole. At an *n*-octylamine concentration of 3 mM, thiobenzamide-S-oxidation by the kidney FMO was increased by 122% and that by the liver FMO by 148%. K_m and V_{max} values were determined and compared between the two tissue enzymes for xenobiotic substrates containing nucleophilic nitrogen, sulfur or phosphorus atoms. In general, for most FMO substrates, K_m and V_{max} values were similar between kidney and liver FMO with only a few exceptions. The K_m and V_{max} values for fenthion for kidney were only half of those observed for liver FMO. Fonofos was unusual in having a low K_m as well as a low V_{max} for both tissue enzymes. Anti-sera developed to the FMO purified from kidney and liver showed cross-reactivity with each purified enzyme as well as with a protein with the same molecular weight as the purified FMO present in both kidney and liver microsomes. These bands showed equal intensity based on an equivalent amount of protein. Analysis of kidney and liver FMO by proteolytic digestion followed by visualization of peptides by silver staining or immunoblotting showed only minor differences between the enzymes of the two tissues. The amino acid composition of both mouse kidney and liver FMO was low in methionine and histidine and rich in aspartate/asparagine, glutamate/glutamine, leucine, valine and glycine. Edman degradation of the purified mouse kidney and liver FMO provided a single amino acid sequence of the NH_2-terminus. This sequence matched exactly with the cDNA-deduced sequence reported for the pig and rabbit liver beginning with the fifth amino acid and contained the highly conserved FAD-binding domain Gly-X-Gly-X-X-Gly, commonly found in a number of other FAD-binding proteins. These studies indicate that the renal and hepatic forms of FMO from mouse are similar enzymes that are immunologically related and show only a few minor differences.

The flavin-containing monooxygenase (FMO; EC 1.14.13.8) has been studied extensively and characterized from the liver of pigs, mice, rats and rabbits and from the lungs of rabbits. This enzyme, located in the endoplasmic reticulum, has been shown to oxygenate a variety of nucleophilic nitrogen-, sulfur- and phosphorus-containing drugs, pesticides and other xenobiotics. The ability of this enzyme to oxygenate a variety of xenobiotics is important in both activation and detoxification processes [1–6]. The broad specificity of FMO towards various substrates appears to be the result of catalytic diversity of a single protein since FMO from pig liver [7] and rabbit liver and lungs [8] has been shown to be the product of single genes. Physiologically, this enzyme may play a role in the maintenance of disulfide bonds for protein synthesis through oxygenation of cysteamine to the disulfide, cystamine [9]. Although no inducers of FMO have been reported, the enzyme activity has been shown to increase with age [10], gestation [11], and by the administration of glucocorticoids [11] and may be under the control of sex steroids [12].

The FMO occurs widely in animal tissues and its activity and/or its presence have been detected by immunochemical methods in the liver, kidney, lungs, skin, brain and other tissues, with the highest concentration occurring in liver, kidney and lungs [13, 14]. Considerable variation appears to exist among tissues of the same animal in the occurrence of multiple forms of FMO. FMOs, isolated from the liver of pigs [15], rats [16], and rabbits [17] appear to be single proteins with molecular weights between 56,000 and 59,000 daltons based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Recently, Ozols [18] isolated two structurally related forms of FMO from rabbit liver differing in molecular weight by only 500, indicating the presence of two distinct proteins representing different gene products. FMO purified from mouse liver was reported to be present as two equally staining bands with molecular weights of 56,000 and 58,000 [19] although Western blotting with anti-pig liver FMO indicated the presence of only a single immunoreactive band in the mouse liver microsomes [15]. However, it is not known whether the low molecular weight band was a product of degeneration, contaminant or artifact of purification and/or the electrophoretic procedures used.

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Although FMO has been purified and characterized from the mouse liver, less extensive information is available on the properties of FMO purified from other tissues. Tynes and Hodgson [20] have reported activities towards various FMO substrates by kidney microsomes from rat, mouse and rabbit. So far few attempts have been made to purify or characterize the kidney FMO in mouse or any other species. It is clear from recent studies [21, 22], however, that the FMO plays a significant role in the metabolism of xenobiotics in the kidney with activity per milligram of microsomal protein comparable to or higher than that in the liver. The presence of an FMO in kidney microsomes of the rat has been implicated recently as a possible enzyme in the bioactivation of nephrotoxic and mutagenic cysteine *S*-conjugates to their corresponding sulfoxides [23]. The present studies were undertaken, therefore, to purify and characterize kidney FMO and compare it to that of liver FMO to more fully understand and evaluate the differences in FMO isolated from different tissues of the same species.

MATERIALS AND METHODS

Materials. $NADP^+$, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, methimazole, imipramine, chlorpromazine, thioridazine, trypsin, *Staphylococcus aureus* V8 protease and goat anti-rat IgG were obtained from the Sigma Chemical Co., St. Louis, MO. Thiobenzamide and *n*-octylamine were obtained from the Aldrich Chemical Co., Milwaukee, WI (*n*-octylamine was used as the hydrochloride, prepared by the equimolar addition of HCl). Phorate, disulfoton, fenthion, nicotine, fonofos and aldicarb were purchased from Chem Service, Westchester, PA. Thiourea and *N,N*-dimethylaniline were obtained from the Fisher Scientific Co., Fairlawn, NJ. All other chemicals and materials were of the highest quality commercially available. Emulgen 911 was a gift from the Kao Atlas Corp., Japan.

Preparation of microsomes. Microsomes were prepared from the kidney and liver of 25–30 g male CD-1 mice by differential centrifugation as previously described [19]. The microsomal pellets obtained were resuspended in 0.05 M potassium phosphate, pH 7.6, containing 0.25 M sucrose, 0.1 mM EDTA, and stored under nitrogen at −80°.

Purification of flavin-containing monooxygenase. The FMO from kidney and liver was purified according to the method of Sabourin *et al.* [19] with slight modifications. The microsomal preparations (from 200 livers or 600 kidneys) were suspended in 0.01 M potassium phosphate, pH 7.6, 20% glycerol, 0.1 mM EDTA (Buffer A) to obtain a concentration of 10 mg of protein/mL. Microsomes were then solubilized by the addition of an equal amount of Buffer A containing 2% Emulgen 911. The resulting solution was passed over a column containing 100 mL of DEAE-Sepharose CL 6B equilibrated with Buffer A containing 1% Emulgen 911 (Buffer B). Under these conditions FMO does not bind to the DEAE column. This step generally gives a 1.5- to 2-fold initial purification. The unbound material eluting from the column was applied to a column of Blue Sepharose 4B pre-equilibrated with Buffer B. Following extensive washing of the column, FMO was eluted with a linear gradient of 0–1 M KCl in Buffer B. Fractions containing peak FMO activity were pooled (Blue Pool), dialyzed for 24 hr against Buffer A, and then applied to a column containing Red Sepharose pre-equilibrated with Buffer B. The FMO was again eluted with a linear gradient of 0–1 M KCl and fractions containing peak FMO activity were pooled (Red Pool) and dialyzed against Buffer A. The dialyzed material was applied to a column of 2′5′-ADP Sepharose which had been pre-equilibrated with Buffer A containing 0.5% Emulgen 911 (Buffer C). The FMO was eluted from the column with Buffer C containing 0.1 mM NADPH. The purified FMO (ADP Pool) was stored in small aliquots at −80°.

Enzyme assays. FMO activity was measured by monitoring NADPH oxidation at 340 nm [15] or by monitoring thiobenzamide-S-oxidation at 370 nm [24] at 37° using an Aminco DW-2 double beam spectrophotometer.

NADPH oxidation was measured using 0.1 M Tricine/KOH, pH 8.5, 0.1 mM EDTA, 0.1 mM NADPH. The final volume was 1 mL. Enzyme (0–100 μL) was added to both sample and reference cuvette, and the endogenous rate of NADPH oxidation was measured. The substrate in 5–10 μL of water or acetonitrile was then added to the sample cuvette and the substrate-dependent rate of NADPH oxidation was measured. Reduced glutathione at a final concentration of 1 mM was added in assays using methimazole to prevent an initial lag phase in NADPH oxidation [25].

Thiobenzamide-S-oxidation was measured in a 1-mL reaction mixture containing 0.1 M Tricine/KOH, pH 8.5, 0.1 mM EDTA, 0.1 nM thiobenzamide (added in 5 μL of acetonitrile), an NADPH-regenerating system (0.25 nM $NADP^+$, 2.0 mM glucose-6-phosphate and 0.5 units/mL glucose-6-phosphate dehydrogenase) and 0–20 μL of enzyme. Kinetic data were obtained using at least five to seven different substrate concentrations ranging from approximately 0.5 to 10.0 times the K_m value. Enzyme activity was determined twice at each substrate concentration and kinetic constants were calculated from double-reciprocal plots.

Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin (BSA Fraction V, Sigma) as a standard. Protein concentration in samples containing Emulgen 911 was determined by the fluorescamine technique of Bohlen *et al.* [26] using BSA as a standard.

Competitive inhibition studies were conducted under standard assay conditions described above, with the competitive inhibitor added simultaneously with the substrate. Reaction rates were linear for 2–3 min and initial rates were measured. For pH activity profiles, 0.1 M Tricine/KOH, 0.1 mM EDTA, 0.1 mM thiobenzamide were used. All assays were conducted in duplicate or triplicate, and the standard deviation was less than 5% of the mean.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions was performed using 7.5 or

12.5% gels in a Tris–glycine buffer system [27]. The proteins were visualized by staining with Coomassie brilliant blue (Bio-Rad). Isoelectric focusing was performed on an LKB 2117 Multiphor II electrophoresis system using LKB Ampholine PagPlates. The broad range precast, 11.0 cm gel (pH 3.5 to 9.9) was 5% polyacrylamide with 2.4% Ampholine. Gels were prefocused for 30 min at 10° and 25 W without samples. Ten microliters of each diluted sample was applied as droplets at six locations in separate lanes excluding 1 cm from the cathode and anode ends. Electrofocusing was performed for 2.5 hr at a constant power of 25 W. The proteins were visualized by staining with Coomassie brilliant blue. A 1-cm lane was sliced into twenty 0.5-cm pieces and eluted for 24 hr at 4° in 0.5 mL of distilled water for measurement of pH.

Immunology. Polyclonal antibodies to FMO purified from kidney and liver were elicited in male CD-1 rats. A primary dose consisting of 50 μg of purified FMO emulsified in Freund's complete adjuvant was injected intraperitonially. At 2- and 4-week intervals a booster dose of 25 μg of purified protein was administered in Freund's incomplete adjuvant. The rats were killed 2 weeks later and the blood was collected from a heart puncture. Serum was prepared by centrifuging the clotted blood at 10,000 *g* for 15 min at 4°. The immune serum thus collected was stored at −85° and used for enzyme characterization studies without further purification.

Immunoblotting was performed by transferring proteins from an SDS–PAGE gel onto nitrocellulose according to Towbin *et al.* [28]. The excess binding sites were blocked with 2% BSA in 0.01 M Tris–HCl, pH 8.0, 0.15 M NaCl (Tris-buffered saline, TBS). The nitrocellulose filters were incubated with a 1:1000 dilution of the immune serum in TBS containing 1% BSA for 2 hr at room temperature with constant agitation. The blots were washed three times for 10 min each in TBS containing 0.5% BSA and incubated with a 1:1000 dilution of goat anti-rat IgG conjugated to alkaline phosphatase in TBS containing 1% BSA for 2 hr at room temperature. The blots were then washed four times in TBS containing 0.5% BSA and transferred to a tray containing 0.07 M bromo-4-chloro-3-indolyl phosphate and 0.06 M nitroblue tetrazolium in 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 0.04 M $MgCl_2$ and then incubated at room temperature in the dark for 5 min for visualization of immunoblotted proteins.

Proteolysis and peptide analysis. Purified FMO samples from kidney and liver were dialyzed against 0.01 M potassium phosphate, pH 7.6, 20% glycerol, 0.1 mM EDTA. Digestion was carried out by treating FMO with protease (2 μg/4 μg purified FMO) or trypsin (1 μg/4 μg purified FMO) in 0.01 M potassium phosphate, pH 7.6, 0.1 mM EDTA at 37°. Digestions were carried out for the indicated times and were terminated by the addition of SDS–PAGE sample buffer followed by heating at 100° for 2 min. Peptides formed were separated using 12.5% SDS–PAGE, and were analyzed by silver staining (Bio-Rad) and immunoblotting.

Amino acid composition and sequence determination. The amino acid composition and sequence analysis of purified mouse liver FMO was done by the Sequencing Facility of North Carolina State University. Mouse liver FMO (ADP Pool), purified as described above, was applied to an Aquapore octyl 300 Å reverse phase (Brownlee, 2.1 nm × 3 cm) high performance liquid chromatograph (HPLC) in 0.1% trifluoroacetic acid (TFA) graded to 0.1% TFA in 80% acetonitrile/15% isopropyl alcohol in water. Absorbance of the protein at 280 and 214 nm was determined. The desalted and lyophilized sample of FMO eluted from the HPLC column was hydrolyzed under vacuum in Corning glass tubes by gas-phase HCl hydrolysis at 150° for 1 hr. After hydrolysis, the phenylthiocarbamyl derivatives formed were analyzed in a Pico-Tag amino acid analysis column (Waters) according to the procedure of Bidlingmyer *et al.* [29] which is described in detail in Manual No. 88140 of Waters Associates. Amino acid analysis of mouse kidney FMO was performed by the ion exchange-ninhydrin method [30]. Automated sequence analysis of the FMO was performed with a gas-phase microsequencer (model 470A, Applied Biosystems) and the aqueous TFA conversion method of Hewick *et al.* [31].

Table 1. Purification of flavin-containing monooxygenase from the mouse kidney and liver

Material	Protein (mg)	Total activity (nmol/TBSO/min)	Specific activity (nmol/TBSO/min/mg)	Recovery (%)	Purification factor
		Mouse kidney FMO			
Sol. microsomes	14,600	15,600	1.1	—	1.0
DEAE Pool	11,200	16,700	1.5	107	1.4
Blue Pool	360	10,000	30.0	64	25.0
Red Pool	20	6300	310.0	40	270.0
ADP Pool	1.6	2600	1600.0	17	1470.0
		Mouse liver FMO			
Sol. microsomes	6400	8800	1.4	—	1.0
DEAE Pool	4600	11700	2.5	130	1.8
Blue Pool	170	6800	40.0	78	29.0
Red Pool	20	3900	190.0	44	140.0
ADP Pool	2.2	2500	1140.0	28	800.0

TBSO, thiobenzamide-*S*-oxide.

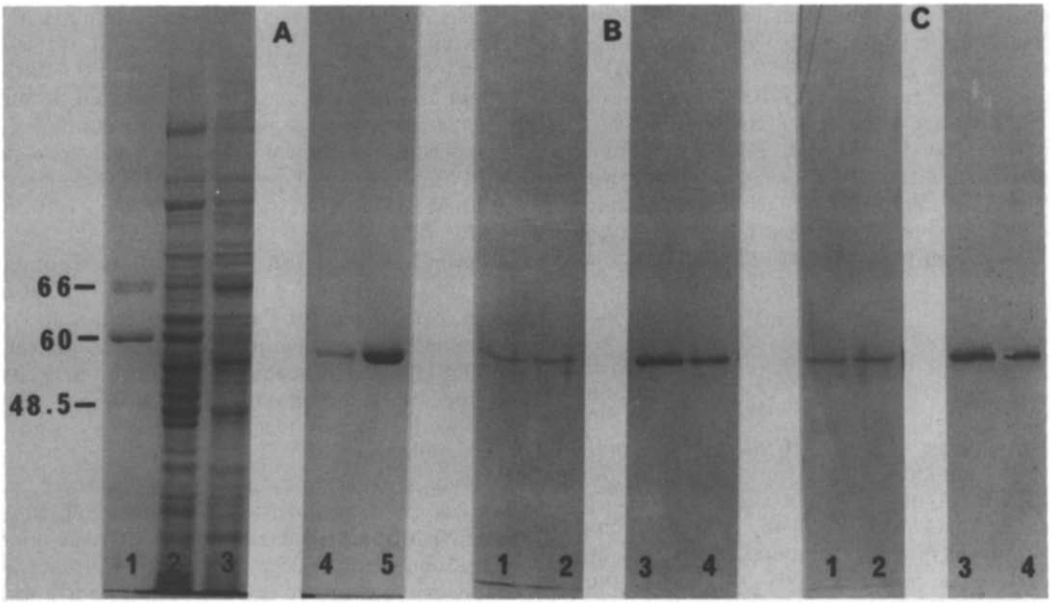

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel (7.5%) electrophoretic and Western blot analysis of mouse kidney and liver microsomes and purified FMO. (A) Electrophoretic analysis; 1, molecular weight markers (Bio-Rad): bovine serum albumin (66,000), catalase (60,000), and fumarase (48,500); 2, liver microsomes (40 μg); 3, kidney microsomes (40 μg); 4, liver FMO (1 μg) and 5, kidney FMO (4 μg). Immunoreactivity of anti-mouse liver (B) and anti-mouse kidney (C) FMO sera with microsomes and purified FMO from mouse liver and kidney; 1, liver microsomes (40 μg); 2, kidney microsomes (40 μg); 3, liver FMO (1 μg); 4, kidney FMO (1 μg).

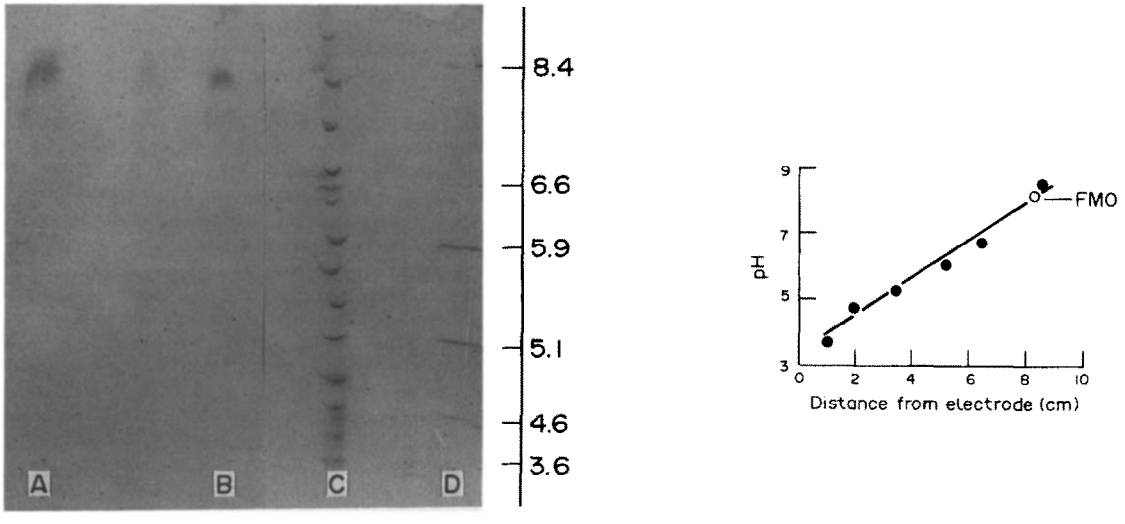

Fig. 2. Wide range (pH 3.5 to 9.0) isoelectric focusing of mouse kidney and liver FMO. Lane A, liver FMO (2 μg); lane B, kidney FMO (2 μg); lane C, liver microsomes (20 μg); and lane D, isoelectric focusing markers. Inset shows the calibration curve for various isoelectric focusing markers and FMO.

RESULTS

Table 1 summarizes the purification of FMO achieved from mouse kidney and liver microsomes using identical procedures. FMO purified from the kidney had higher specific activity and purification factor compared to liver. SDS–PAGE analysis of microsomes and FMO purified from kidney and liver is shown in Fig. 1. FMO purified from kidney and livers separated as a single band with an apparent molecular weight of 58,000. Kidney and liver FMOs were subjected to wide range (pH 3.5 to 9.0) isoelectric focusing (Fig. 2). The kidney and liver FMO electrofocused as a single band with an isoelectric point of 8.2 (Fig. 2).

The pH dependency of the purified kidney and

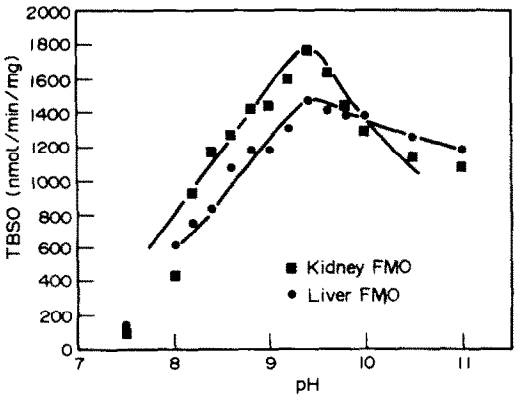

Fig. 3. Effect of pH on thiobenzamide-S-oxidation activity by the purified mouse liver and kidney FMO. Enzyme activity was measured in duplicate using 0.1 M Tricine/KOH, 0.1 mM EDTA. Enzyme activity was measured using 1.2 μg of purified FMO. TBSO, thiobenzamide-*S*-oxide.

Table 2. Effects of methimazole, thiourea and *n*-octylamine on thiobenzamide-S-oxidation activity of FMO purified from mouse kidney and liver

Substrate	Concentration (mM)	Activity (%of control) Kidney FMO	Liver FMO
Methimazole	0.1	32	34
	0.5	59	68
Thiourea	0.1	37	36
	0.5	78	68
n-Octylamine	1.0	117	128
	3.0	122	148

Thiobenzamide-S-oxidation was determined in the presence of various concentrations of indicated substrates (N = 2–3). Control enzyme activities of purified kidney and liver FMO were 1060 and 1260 nmol TBSO produced/min/mg protein, respectively. Enzyme activity was measured using 1.2 μg of purified FMO.

liver FMO towards thiobenzamide-S-oxidation is shown in Fig. 3. The pH optima for both kidney and liver FMO were seen at pH 9.2. The results using various concentrations of *n*-octylamine on thiobenzamide-S-oxidation are shown in Table 2. Stimulation of thiobenzamide-S-oxidation activity by *n*-octylamine occurred with both kidney and liver FMO. At a 3 mM concentration of *n*-octylamine (highest concentration tested), kidney FMO activity was increased to 122% of controls compared to 148% by liver FMO (Table 2). Thiobenzamide-S-oxidation by FMO purified from kidney and liver was inhibited to an equal extent in the presence of the competing substrates, methimazole and thiourea (Table 2).

Various compounds were tested as substrates, and their apparent K_m and V_{max} values were determined at pH 8.5 for the purified mouse kidney FMO and compared with those of liver FMO (Table 3). In these studies, the endogenous rates of NADPH oxidation were negligible compared to substrate-dependent rates and, therefore, were ignored in all calculations. In general, for most substrates tested, the K_m and V_{max} values were generally similar for both kidney and liver FMO with few exceptions. The K_m values for nicotine and thiobenzamide for liver FMO were twice those of kidney FMO. Diethylphenylphosphine had very low K_m and very high V_{max} values for both kidney and liver FMO. Among organophosphorous pesticides, fenthion showed marked differences between the two tissue enzymes. The V_{max} and K_m values for kidney FMO with fenthion as a substrate were half those observed for liver FMO (Table 3).

Antibodies developed to FMO purified from kidney and liver were used to test the cross-reactivity of FMOs from both tissues by immunoblotting. Microsomes, purified FMOs and the various anti-sera were used at identical concentrations for comparative studies. As shown in Fig. 1, anti-sera developed to FMO purified from either kidney or liver cross-reacted with equal intensity with both purified enzymes as well as with the enzymes present in the microsomes of the two tissues. The antibodies were not inhibitory, however, as indicated by the lack of thiobenzamide-S-oxidation by purified mouse liver FMO even after incubation of the enzyme with anti-serum for 1 hr at 37° (data not shown).

Peptides formed by limited proteolytic digestion of kidney and liver FMO were analyzed by SDS–PAGE followed by silver staining or immunoblotting. As shown in Fig. 4, silver staining of peptides obtained from protease or trypsin digestion revealed only minor differences between kidney and liver FMO (Fig. 4). Some differences observed were due to minor impurities present in the purified samples used for digestion. Immunoblots of protease digests of kidney and liver FMO reacted with anti-kidney FMO produced identical patterns, whereas the tryptic digests revealed some minor differences between the enzymes of the two tissues (Fig. 5). Some very low molecular weight peptides which were not detected by silver staining were clearly resolved in tryptic digests of kidney FMO compared to liver FMO by the immunochemical method (Fig. 5).

The amino acid composition of mouse kidney and liver FMO compared to that of pig liver FMO [32] is shown inTable 4. There was a predominance of aspartate/asparagine, glutamate/glutamine, leucine, valine and glycine, whereas methionine and histidine were found to be in the lowest amounts. The purified mouse kidney and liver FMO was subjected to Edman degradation. This analysis resulted in a sequence of 19 and 15 residues from the NH_2-terminus for kidney and liver FMO, respectively (Fig. 6). The primary sequence obtained in the present study is compared with that reported for pig liver and rabbit liver and lung FMO (Fig. 6).

DISCUSSION

The FMO was purified from mouse kidney and

Table 3. Kinetic constants for selected substrates oxidized by purified flavin-containing monooxygenase from mouse kidney and liver*

Substrate	K_m (μM)		V_{max} (nmol NADPH/min/mg)	
	Kidney FMO	Liver FMO	Kidney FMO	Liver FMO
Thiocarbamide				
Thiorea	8.5	8.8	1270	1430
Methimazole	14.3	10.9	1080	1050
Thioamide				
Thiobenzamide†	12.5	28.6	1490	1320
Tertiary amines				
N,*N*-Dimethylaniline	114.0	116.0	1320	1250
Imipramine	31.7	47.6	1140	1290
Chlorpromazine	3.2	2.3	1820	2330
Nicotine	128.0	250.0	300	410
Arylalkyl sulfide				
Thioridazine	53.6	50.0	1250	950
Phosphine				
Diethylphenylphosphine	1.7	2.0	1670	1860
Phosphonate				
Fonofos	11.1	6.8	270	270
Organothiophosphates				
Disulfoton	1.8	1.4	980	950
Fenthion	11.1	23.2	490	1000
Phorate	18.5	18.9	930	1030
Carbamates				
Aldicarb	385.0	196.0	830	710

* Activity was determined by measuring NADPH oxidation using 1.2 μg of purified FMO. Values are the averages of duplicate determinations which varied by less than 10%.

† Thiobenzamide-*S*-oxide formation was measured in the presence of thiobenzamide.

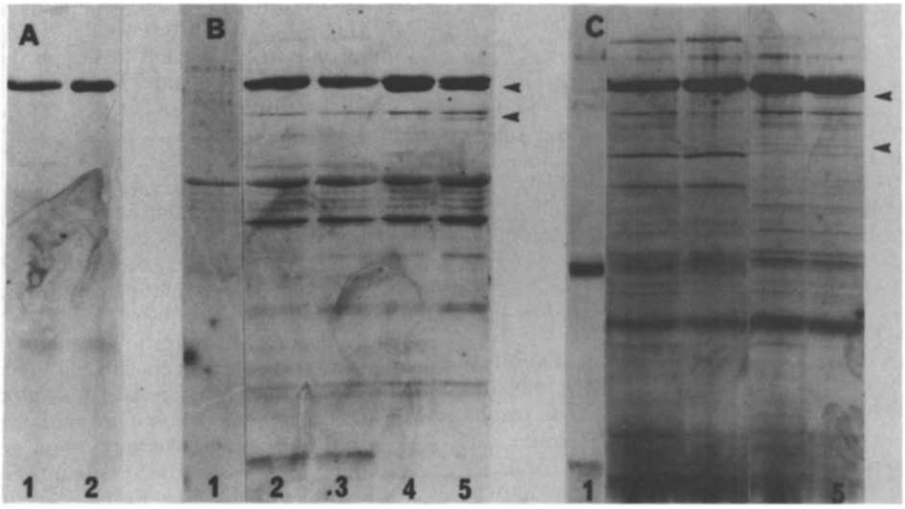

Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel (12.5%) electrophoresis and silver staining of mouse kidney and liver FMO digested with protease and trypsin. (A) 2 μg of undigested liver (1) and kidney (2) FMO. (B) 1 μg of V8 protease (1), 4 μg of liver (2,3) and kidney (4,5) FMO digested with protease for 30 min (2,4) or 60 min (3,5). (C) 1 μg of trypsin (1), 6 μg of liver (2,3) and kidney (4,5) FMO digested with trypsin for 30 min (2,4) or 60 min (3,5). Arrows indicate apparent differences between the two tissue enzymes. Details of proteolysis and silver staining procedures are described in Materials and Methods.

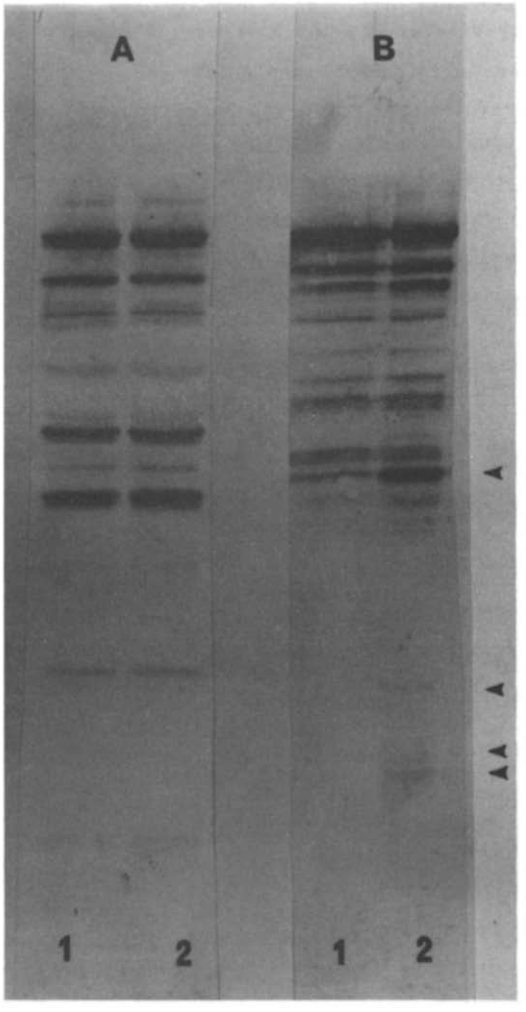

Fig. 5. Immunoblots of peptides formed by the digestion of 4 μg of kidney and liver FMO. Liver (1) and kidney (2) FMOs were digested with protease (A) or trypsin (B) for 30 min at 37°. Following digestion, proteins were subjected to electrophoresis, transferred to nitrocellulose, and analyzed for reactivity with anti-serum to mouse kidney FMO. Arrows indicate apparent differences between the enzymes of the two tissues.

Table 4. Amino acid composition of the purified flavin-containing monooxygenase of mouse kidney, mouse liver and pig liver

Amino acid	Residues per molecule		
	Mouse kidney	Mouse liver	Pig liver*
Alanine	28	24	31
Arginine	23	21	21
Aspartate/Asparagine	46	52	52
Cysteine	ND†	ND	9
Glutamate/Glutamine	46	50	52
Glycine	63	52	37
Histidine	10	16	10
Isoleucine	25	30	29
Leucine	50	50	58
Lysine	34	26	39
Methionine	11	22	10
Phenylalanine	33	38	37
Proline	38	21	42
Serine	38	40	38
Threonine	35	24	33
Tryptophan	ND	ND	9
Tyrosine	17	22	19
Valine	39	49	44
Total	536	537	570

* Amino acid composition data of pig liver are from Poulsen and Ziegler [32].
† ND = not determined.

liver using slight modification of the procedure of Sabourin *et al.* [19]. On SDS–PAGE, FMO from kidney and liver separated as a single band with an apparent molecular weight of 58,000. In a previous study, Sabourin *et al.* [19] reported that the purified mouse liver FMO contained two equally stained bands with molecular weights of 56,000 and 58,000. However, in the present study, mouse liver FMO purified several times from different batches of microsomes revealed the presence of only a single band on SDS–PAGE analysis. Previous immunoblotting studies with mouse liver microsomes using antibodies developed to mouse or pig liver FMO also revealed the presence of only a single immunoreactive band [13, 14, 33]. The appearance of two bands in purified mouse liver FMO in the earlier study, therefore, might be an artifact of purification and/or the electrophoretic procedures used [33]. The kidney and liver FMO purified by identical procedures did not reveal the presence of multiple bands. The occurrence of multiple forms (two or three) of FMO has been reported previously from lungs of rabbits [14, 33] and guinea pigs [14] and recently from the liver of rabbits [18] and guinea pigs [34].

Anti-sera developed to FMO purified from mouse kidney and liver were also used to study the immunologic relationship between the purified FMOs as well as the enzymes present in intact microsomes. The results showed that FMOs present in both tissues of the mouse cross-react with each other. The intensity of staining of different FMOs with different immune sera using the same amount of protein was not different, further supporting an immunological relationship between the kidney and liver enzymes. Antibodies developed to pig liver FMO have been shown to cross-react with FMO present in liver microsomes of hamster, pig and guinea pig [14], and from pig, mouse, rat and rabbit [13, 14] and microsomes from kidney and lungs of pig, mouse and rat [14]. Similarly, a cDNA probe to pig liver FMO has been shown to hybridize with mRNA isolated from pig lungs and kidneys [7], but not rabbit lungs [8]. The anti-rabbit lung antibodies did not cross-react with either microsomes or purified FMO from mouse kidney or liver indicating that neither mouse kidney nor liver contains the lung form of FMO (data not shown). This is in contrast to the rabbit kidney which has been shown to express

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      1                5                10                15                20
A     Val-Lys-(cys)-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-(Cys)-
B           Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-
C     Met-Ala-Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-Cys-
D     Met-Ala-Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-Cys-
E     Met-Ala-Lys-Lys-Val-Ala-Val-Ile-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ile-Ser-Leu-Lys-Cys-
F           Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-Ser-
G     Gly-Lys-----Lys-Val-Ala-Ile-Ile-Gly-Ala-Gly-Ile-Ser-Gly-Leu-Ala-Ser-Ile-Arg-Ser-
```

Fig. 6. Comparison of the NH_2-terminal amino acid sequences of FMOs from (A) mouse kidney; (B) mouse liver; (C) pig liver deduced from cloned cDNA [7]; (D and E) rabbit liver and lung, respectively, deduced from cloned cDNA [8] and (F and G) Form 1, R-1 and Form 2 peptide of rabbit liver, respectively, obtained using Edman degradation [18]. Positions in the sequences not found in the pig liver FMO are underlined.

the lung form of FMO by Western blotting [14] and recently by Northern blotting [8] techniques. Analysis of proteolytic fragments of kidney and liver FMO followed by silver staining or immunoblotting provided almost identical patterns with some minor differences, suggesting that the mouse kidney and liver FMOs are similar but not identical enzymes. The possibility that the differences seen are due to the presence of more than one isozyme must be entertained. However, if this is the case it is unlikely to be the form of the enzyme expressed in the lung (see above) and must be a new and yet uncharacterized form of the enzyme. This latter possibility would, in itself, be of interest and importance.

Purified FMOs from mouse kidney and liver had pH optima of 9.2 and behaved similarly with respect to inhibition of thiobenzamide-S-oxidation by the competing substrates, methimazole and thiourea, indicating that both tissue enzymes are catalytically similar. The high alkaline pH optima of FMOs appear to result from the high isoelectric points of the native proteins (Fig. 2). Thiobenzamide-S-oxidation was activated to a greater extent by *n*-octylamine in liver compared to kidney FMO indicating some variation in FMO isolated from different tissues of the same animal. This activation of FMO by *n*-octylamine, an inhibitor of P450, presumably occurs by an interaction of *n*-octylamine at an allosteric (regulatory) site distinct from the catalytic site causing an increased rate of breakdown of enzyme bound hydroperoxyflavin [1].

Compounds which were shown to be substrates for the purified mouse liver FMO were found to be also substrates for the purified kidney FMO. The V_{max} and K_m values for various substrates tested were similar between kidney and liver FMO with only a few exceptions. Fonofos, which undergoes oxidative desulfuration by FMO to form an oxon [6, 35], was unusual in that, although the K_m was low with both tissue enzymes, the V_{max} value was also very low with both enzymes. A similar low K_m associated with a low V_{max} has been reported with mouse liver microsomes for fonofos and sulprofos [36] and with purified mouse and lig liver FMO for sulprofos [37]. Fenthion was also unusual in having K_m and V_{max} values for kidney only one-half of those observed for liver FMO. Aldicarb was not a good substrate for either tissue enzyme. Thioether containing carbamates have been reported to be poor substrates of mouse and pig liver FMO [37, 38]. The structural changes on the thioether moiety that affect the nucleophilicity and/or the steric hindrance of the sulfur atom appear to decrease the rate of sulfoxidation by FMO [38]. The variation observed in V_{max} values among certain substrates has been attributed to the stimulation of FMO by lipophilic substrates, but not by other substrates, in a manner similar to *n*-octylamine [25]. Since the rate-limiting step is reactivation of the enzyme (i.e. regeneration of the hydroperoxyflavin), this would supposedly be the process which is stimulated. Depletion of oxygen in the assay mixture may also lead to apparent low V_{max} values [25]. The inability of certain lipophilic substrates to fully saturate the FMO due to solubility limits may also result in lowered V_{max} values [36]. Diethylphenylphosphine had a very low K_m for both kidney and liver FMO and the K_m for purified mouse liver FMO (2 μM) was identical to that obtained for mouse liver microsomes [36]. However, at substrate concentrations above 5–10 μM, rates were decreased for both enzymes compared to rates obtained at lower concentrations. This inhibition of FMO activity at higher concentrations of diethylphenylphosphine was reported to occur in kinetic measurements using mouse liver microsomes [36]. The K_m and V_{max} values determined for the purified mouse liver FMO for various substrates are comparable to that reported earlier by Sabourin and Hodgson [25] and Smyser *et al.* [37] for the same enzyme. The minor differences in K_m and V_{max} values can be attributed to variations in enzyme assay conditions such as lower pH (8.1) buffer and/or the use of 3 mM *n*-octylamine in previous studies.

The amino acid composition of mouse kidney and liver FMO closely resembles that reported for the pig liver FMO, wherein aspartate/asparagine, glutamate/glutamine, leucine, valine and glycine were shown to be present in the highest amounts and methionine and histidine in the lowest amounts [7, 32]. Previous attempts to sequence pig liver FMO from this laboratory as well as other laboratories have been unsuccessful and this has been attributed to the blocked amino terminus [7]. However, when

purified FMO from mouse kidney and liver was subjected to Edman degradation, a sequence of 19 and 15 residues from the NH_2-terminus was obtained for kidney and liver FMO, respectively. This indicates that the purified FMO from mouse kidney and liver, unlike that of pig [39] or rabbit liver FMO, may not contain a blocked NH_2-terminus or that the unblocked terminus is produced during the purification procedures used in this study. Although FMO has been purified from several mammalian species, protein sequence information has been available only recently for pig liver [7], rabbit liver [8, 18], rabbit lung [8] and guinea pig liver FMO [34]. The primary structure of mouse kidney and liver FMO is in exact agreement with the predicted NH_2-terminal amino acid sequence of pig and rabbit liver FMO beginning with the fifth amino acid [7, 8]. It can also be seen that the FAD binding site (Gly-X-Gly-X-X-Gly) of mouse kidney and liver FMO is highly conserved and is common with other FMO enzymes (Fig. 6). It is apparent from the present studies that although mouse kidney and liver are immunologically related, they exhibit some minor differences with respect to substrate specificity, activation by *n*-octylamine, amino acid composition, peptide analysis and NH_2-terminal sequence data. The extent and final explanation of the minor differences between the kidney and liver FMO remain to be resolved by the determination of the primary structures of the enzymes from the two tissues.

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REFERENCES

1. Ziegler DM, Microsomal flavin-containing monooxygenase: Oxygenation of nucleophilic nitrogen and sulfur compounds. In: *Enzymatic Basis of Detoxification* (Ed. Jacoby WB), Vol. 1, pp. 201–227. Academic Press, New York, 1980.
2. Ziegler DM, Flavin-containing monooxygenases: Catalytic mechanism and substrate specificities. *Drug Metab Rev* **6**: 1–32, 1988.
3. Ziegler DM, Flavin-containing monooxygenases—Enzymes adapted for multisubstrate specificity. *Trends Pharmacol Sci* **11**: 321–324, 1990.
4. Hodgson E and Levi PE, The flavin-containing monooxygenase as a sulfur oxidase. In: *Metabolism of Xenobiotics* (Eds. Gorrod JW, Oelschlager H and Caldwell J), pp. 81–88. Taylor & Francis, London, 1988.
5. Hodgson E and Levi PE, Species, organ and cellular variation in the flavin-containing monooxygenase. *Drug Metabol Drug Interact* **6**: 219–233, 1989.
6. Hodgson E and Levi PE, Metabolism of organophosphorous compounds by the flavin-containing monooxygenase. In: *Organophosphates: Chemistry, Fate and Effects* (Eds. Chambers JE and Levi PE), Academic Press, New York, in press.
7. Gasser R, Tynes RE, Lawton MP, Korsmeyer KK, Ziegler DM and Philpot RM, The flavin-containing monooxygenase expressed in pig liver: Primary sequence, distribution, and evidence for a single gene. *Biochemistry* **29**: 119–124, 1990.
8. Lawton MP, Gasser R, Tynes RE, Hodgson E and Philpot RM, The flavin-containing monooxygenase enzymes expressed in rabbit liver and lung are products of related but distinctly different genes. *J Biol Chem* **265**: 5855–5861, 1990.
9. Ziegler DM and Poulsen LL, Protein disulfide bond synthesis: A possible intracellular mechanism. *Trends Biochem Sci* **2**: 79–83, 1977.
10. Das ML and Ziegler DM, Rat liver oxidative *N*-dealkalase and *N*-oxidase activities as a function of animal age. *Arch Biochem Biophys* **140**: 300–306, 1970.
11. Devereaux TR and Fouts JR, Effect of pregnancy or treatment with certain steroids on N,N-dimethylaniline demethylation and N-oxidation by rabbit liver or lung microsomes. *Drug Metab Dispos* **3**: 254–258, 1975.
12. Duffel MW, Graham JM and Ziegler DM, Changes in dimethylaniline *N*-oxidase activity of mouse liver and kidney induced by steroid sex hormones. *Mol Pharmacol* **19**: 134–139, 1981.
13. Dannan GA and Guengerich FP, Immunochemical comparison and quantitation of microsomal flavin-containing monooxygenases in various hog, mouse, rat, rabbit, dog, and human tissues. *Mol Pharmacol* **22**: 787–794, 1982.
14. Tynes RE and Philpot RM, Tissue- and species-dependent expression of multiple forms of mammalian microsomal flavin-containing monooxygenase. *Mol Pharmacol* **31**: 569–574, 1987.
15. Ziegler DM and Poulsen LL, Hepatic microsomal mixed-function amine oxidase. *Methods Enzymol* **52**: 142–151, 1978.
16. Kimura T, Kodayama M and Nagata C, Purification of mixed-function amine oxidase from rat liver microsomes. *Biochem Biophys Res Commun* **110**: 640–645, 1983.
17. Tynes RE, Sabourin PJ, Hodgson E and Philpot RM, Formation of hydrogen peroxide and *N*-hydroxylated amines catalyzed by pulmonary flavin-containing monooxygenases in the presence of primary alkylamines. *Arch Biochem Biophys* **251**: 654–664, 1986.
18. Ozols J, Liver microsomes contain two distinct NADPH-monooxygenases with NH_2-terminal segments homologous to the flavin-containing NADPH monooxygenase of *Pseudomonas fluorescens*. *Biochem Biophys Res Commun* **163**: 49–55, 1989.
19. Sabourin PJ, Smyser BP and Hodgson E, Purification of the flavin-containing monooxygenase from mouse and pig liver microsomes. *Int J Biochem* **16**: 713–720, 1984.
20. Tynes RE and Hodgson E, Catalytic and substrate specificity of the flavin-containing monooxygenase in microsomal systems: Characterization of the hepatic, pulmonary and renal enzymes of the mouse, rabbit, and rat. *Arch Biochem Biophys* **240**: 77–93, 1985.
21. Kinsler S, Levi PE and Hodgson E, Hepatic and extrahepatic microsomal oxidation of phorate by the cytochrome P-450 and FAD-containing monooxygenase systems in the mouse. *Pest Biochem Physiol* **31**: 54–60, 1988.
22. Kinsler S, Levi PE and Hodgson E, Relative contributions of the cytochrome P450 and flavin-containing monooxygenase to microsomal oxidation of phorate following treatment of mice with phenobarbital, hydrocortisone, acetone, and piperonyl butoxide. *Pest Biochem Physiol* **37**: 174–181, 1990.
23. Sausen PJ and Elfarra AA, Cysteine conjugate *S*-oxidase. Characterization of a novel enzymatic activity

in rat hepatic and renal microsomes. *J Biol Chem* **265**: 6139–6145, 1990.

24. Cashman JR and Hanzlik RP, Microsomal oxidation of thiobenzamide. A photometric assay for the flavin-containing monooxygenase. *Biochem Biophys Res Commun* **98**: 147–153, 1981.
25. Sabourin PJ and Hodgson E, Characterization of the purified microsomal FAD-containing monooxygenase from mouse and pig liver. *Chem Biol Interact* **51**: 125–139, 1984.
26. Bohlen P, Stein S, Dairman W and Undenfriend S, Fluorometric assay of proteins in the nanogram range. *Arch Biochem Biophys* **155**: 213–220, 1973.
27. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
28. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
29. Bidlingmyer BA, Cohen SDA and Tarvin TL, Rapid analysis of amino acids using pre-column derivatization. *J Chromatogr* **336**: 93–104, 1984.
30. Ozols J, Amino acid analysis. *Methods Enzymol* **182**: 587–601, 1990.
31. Hewick RM, Hunkapillar MW, Wood LE and Dreyer WJ, A gas-liquid solid phase peptide and protein sequencer. *J Biol Chem* **256**: 7990–7997, 1981.
32. Poulsen LL and Ziegler DM, The liver microsomal FAD-containing monooxygenase. Spectral characterization and kinetic studies. *J Biol Chem* **254**: 6449–6455, 1979.
33. Sabourin PJ, Tynes RE, Smyser BP and Hodgson E, The FAD-containing monooxygenase of lung and liver tissue from rabbit, mouse and pig: Species and tissue differences. In: *Biological Reactive Intermediates III* (Eds. Kocsis JJ, Jollow DJ, Witmer CM, Nelson JO and Snyder R), pp. 222–263. Plenum Publishing, New York, 1986.
34. Yamada H, Yuno K, Oguri K and Yoshimura H, Multiplicity of liver microsomal flavin-containing monooxygenase in guinea pig: Its purification and characterization. *Arch Biochem Biophys* **280**: 305–312, 1990.
35. Hajjar NP and Hodgson E, The microsomal FAD-dependent monooxygenase as an activating enzyme: Fonofos metabolism. In: *Biological Reactive Intermediates II* (Eds. Snyder R, Parke DV, Kocsis JJ, Jallow DJ, Gibson CG and Witmer CM), pp. 1245–1253. Plenum Publishing, New York, 1982.
36. Tynes RE and Hodgson E, Magnitude of involvement of the mammalian flavin-containing monooxygenase in the microsomal oxidation of pesticides. *J Agric Food Chem* **33**: 471–479, 1985.
37. Smyser BP, Sabourin PJ and Hodgson E, Oxidation of pesticides by purified microsomal FAD-containing monooxygenase from mouse and pig liver. *Pest Biochem Physiol* **24**: 368–374, 1985.
38. Hajjar NP and Hodgson E, Sulfoxidation of thioether-containing pesticides by the flavin-adenine dinucleotide-dependent monooxygenase of pig liver microsomes. *Biochem Pharmacol* **31**: 745–752, 1982.
39. Guan S, Falick AM and Cashman JR, N-Terminus determination: FAD and NADP binding domain mapping of hog liver flavin-containing monooxygenase by tandem mass spectrometry. *Biochem Biophys Res Commun* **172**: 937–943, 1990.