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Further Characterization and Purification of the Flavin-Dependent *S*-Benzyl-L-cysteine *S*-Oxidase Activities of Rat Liver and Kidney Microsomes

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SUMMARY

Previously, we provided evidence that cysteine conjugate *S*-oxidase (*S*-oxidase) activities of rat liver and kidney microsomes may be associated with flavin-containing monooxygenases (FMOs). In this study, the biochemical properties of these activities were further investigated. When NADPH was replaced by NADH, the *S*-oxidase activities were reduced significantly. Removal of the flavin moiety from microsomes significantly reduced the *S*-oxidase activities; however, addition of exogenous FAD or FMN restored the activities of the flavin-depleted microsomes. Solubilization of hepatic or renal microsomes with Emulgen 911, Nonidet P-40, Triton X-100, or 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfate or inclusion of the sulfhydryl-reactive agents Hg^{2+}, *N*-ethylmaleimide, or iodoacetamide did not affect the *S*-oxidase activities, whereas solubilization of either hepatic or renal microsomes by cholate or heating of renal microsomes in the absence of NADPH significantly reduced the *S*-oxidase activities. In addition to male rat hepatic and renal microsomes, the *S*-oxidase activities were detected in lung microsomes of male rats and hepatic and renal microsomes of male mice and female rats and rabbits. The male rat kidney maintained the highest *S*-oxidase activity of all species and tissues examined. Whereas the aforementioned results provided further evidence for the *S*-oxidase activities being associated with FMOs, unambigious evidence for this hypothesis was provided by the purification of the activities from rat liver (580-fold) and kidney (700-fold) microsomes and by the use of the isolated proteins in polyacrylamide gel electrophoresis, flavin content determinations, amino-terminal amino acid sequence analysis, amino acid composition analysis, and substrate kinetic studies. The findings that the *S*-oxidases were immunoreactive with antibodies raised against the pig liver 1A1 isozyme but not with antibodies raised against the rabbit lung 1B1 isozyme and that the liver *S*-oxidase amino-terminal amino acid sequence was more comparable to the amino-terminal amino acid sequences of pig and rabbit liver 1A1 isozymes than to those of rabbit lung 1B1 and liver 1D1 isozymes provide evidence that the *S*-oxidases are related to the known FMO 1A1 isozymes.

The innovative studies by Ziegler and co-workers (1, 2), which demonstrated that the *N*,*N*-dimethylaniline *N*-oxidase activity of hog liver was an NADPH- and FAD-dependent amine oxidase, initiated the characterization of a new type of monooxygenase present in the microsomal fraction of the liver. FMOs are now recognized to be present in hepatic, renal, and pulmonary microsomes of a number of different mammalian species including the rat, rabbit, mouse, guinea pig, and human (3–20). The presence of multiple forms of FMO in the rabbit, guinea pig, and human has been demonstrated. These enzymes catalyze oxidation on sulfur, selenium, and nitrogen atoms of a wide range of chemicals, with overlapping substrate specificities. Distinct biochemical properties have also been recognized for the different isoforms. For example, the pig liver FMO is temperature labile and is inhibited when subjected to solubilization by cholate, whereas the rabbit lung FMO isoform is not affected by similar heating or solubilizing conditions (10, 13). Furthermore, inclusion of Hg^{2+} was found to stimulate rabbit lung *N*-oxidase activities, whereas liver FMO activities were inhibited by approximately 50% at the same Hg^{2+} concentration (6).

Recently, we identified an *S*-oxidase activity present in rat hepatic and renal microsomes that catalyzed the sulfoxidation of SBC to SBC sulfoxide (21). The *S*-oxidase activities, which were NADPH and oxygen dependent, were inhibited by the FMO alternate substrates methimazole and *N*,*N*-dimethylaniline, suggesting that an FMO may be catalyzing the reaction. Further evidence for this hypothesis was provided by the findings that inclusion of the cytochrome P-450 inhibitors metyr-

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ABBREVIATIONS: FMO, flavin-containing monooxygenase; SBC, *S*-benzyl-L-cysteine; *S*-oxidase, cysteine conjugate *S*-oxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfate; HPLC, high performance liquid chromatography.

apone and 1-benzylimidazole, antibodies to cytochrome P-450 reductase, catalase, superoxide dismutase, butylated hydroxyanisole, or the peroxidase inhibitor KCN had no effect on SBC sulfoxidation by hepatic or renal microsomes. In this report, studies were conducted to further characterize SBC sulfoxidation with respect to flavin nucleotide and NAD(P)H requirements, thermal stability in the absence of NADPH, sensitivity to sulfhydryl-reactive agents, effects of solubilization by detergents, and species, sex, and tissue distribution. In addition, the rat hepatic and renal *S*-oxidases were purified and characterized with regard to their molecular weight, flavin content, amino-terminal amino acid sequence, amino acid composition, substrate kinetics, and immunoreactivity with antibodies raised against pig liver FMO 1A1, rabbit lung FMO 1B1, or a universal FMO peptide.[1] The results provide clear evidence for the *S*-oxidases being FMO enzymes that are related to the known FMO 1A1 isozymes.

Experimental Procedures

Materials. SBC, NADPH, NADH, CHAPS, Triton X-100, sodium cholate, FAD, FMN, Cibacron Blue 3GA-agarose (type 3000-CL), Reactive Red 120-agarose (type 3000-CL), and PVDF membranes were purchased from Sigma Chemical Co. (St. Louis, MO). 2′,5′-ADP-Sepharose was obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Coomassie Blue R-250 stain was obtained from Bio-Rad (Richmond, CA). Methimazole, iodoacetamide, *N*-ethylmaleimide, mercuric chloride, 2,4-dinitrofluorobenzene, $(NH_4)_2HPO_4$, and trifluoroacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile from EM Science (Gibbstown, NJ) was used for all HPLC assays. Emulgen 911 was a generous gift from the Kao Atlas Corp. (Tokyo, Japan). Nonidet P-40 was obtained from Particle Data Laboratories, Ltd. (Elmhurst, IL). All other chemicals were of the highest quality reagent or electrophoresis grade commercially available.

Preparation of microsomes. Microsomes were prepared from male or female Sprague-Dawley rats (200–400 g; Charles River, Wilmington, MA), male $B_6C_3F_1$ mice (The Jackson Laboratory, Bar Harbor, ME), or female New Zealand white rabbits (2–3 kg; New Franken Research Rabbitry, New Franken, WI) as described previously (21). For flavin dependence studies, microsomes prepared from male Sprague-Dawley rats were resuspended in buffer containing 0.1 M KCl, 0.25 M sucrose, 5 mM EDTA, and 0.1 M KH_2PO_4, pH 7.4. The flavin was removed from the microsomes as described by Ziegler and Pettit (2). Briefly, 220 mg of KCl/ml of microsomal suspension (4–6 mg of protein/ml) were added, with stirring, on ice. When all the KCl was dissolved, microsomes were sonicated for 1 min using a Sonics (Danbury, CT) sonicator equipped with a microtip (power level 3, 45% duty level, pulse on). After sonication, the pH of the microsomal suspension was reduced to 5.0 with 1 M glacial acetic acid, and the suspension was centrifuged at 105,000 × *g* for 15 min. The pellet was resuspended in 3 M KCl, the pH was adjusted to 5.0 as described above, and the solution was recentrifuged at 105,000 × *g* for 15 min; washing of the microsomes was done twice to facilitate the removal of the flavin from the microsomes. For temperature stability studies, 1-ml portions of microsomes were removed from the stock microsomal suspension and heated for 2, 5, 10, or 20 min at 45° in the absence of NADPH. After the appropriate heating time, microsomes were placed back on ice and allowed to cool before being used for enzymatic incubations. Microsomes used for detergent effect studies were solubilized using 1% (w/v or v/v, depending on the detergent) detergent and used for enzymatic incubations. Microsomal protein concentrations were determined by the method of Lowry *et al.* (22), using bovine serum albumin as standard.

Microsomal *S*-oxidase assays. Enzymatic microsomal assays to monitor SBC sulfoxidation were carried out as described previously (21). Briefly, typical incubations contained 5 mM SBC, 2 mM NADPH, and 0.5–1.5 mg of microsomal protein or 50–200 μl of purified protein in 0.5 ml of buffer (0.1 M KH_2PO_4, 0.1 M KCl, 1 mM EDTA, pH 7.4). Enzymatic incubations were initiated by the addition of SBC after a 5-min preincubation of microsomes at 37° in the presence of NADPH; incubation times were either 15 or 20 min. Incubations were terminated by the addition of 0.5 ml of ice-cold ethanol, the protein was precipitated by centrifugation, and a 0.5-ml aliquot of the ethanol-soluble supernatant was derivatized with 2,4-dinitrofluorobenzene (0.7% in ethanol) and analyzed by HPLC as described previously (21). For studies of *S*-oxidase dependence on flavin nucleotide, either FAD (0.01–5 mM) or FMN (0.01–5 mM) was added with SBC to initiate the enzymatic incubation. Studies on the effects of sulfhydryl reagents on SBC sulfoxidation were carried out by adding mercuric chloride (0.1 mM), iodoacetamide (1 mM), or *N*-ethylmaleimide (1 mM) with SBC to initiate the incubation. In pyridine nucleotide specificity studies, NADH (2 mM) was substituted for NADPH.

Purification of SBC *S*-oxidases. *S*-Oxidase activities were purified from rat hepatic and renal microsomes by a modification of the method of Sabourin *et al.* (11). Briefly, freshly isolated microsomes from four rat livers or 100 rat kidneys (Harlan Bioproducts, Indianapolis, IN) were solubilized using 0.5% Emulgen 911 in 10 mM KH_2PO_4, 1 mM EDTA, pH 7.6 (buffer B). The solubilized microsomes were charged onto a Cibacron Blue-Sepharose column (2.5 × 5.0 cm) that had been equilibrated with buffer B. The Blue column was washed with 100 ml of buffer B and the remaining protein was eluted from the Blue column with a 0–1 M KCl gradient in buffer B and was analyzed for *S*-oxidase activity (described below). Fractions containing maximal *S*-oxidase activity were pooled (Blue pool), dialyzed against buffer A (buffer B without Emulgen 911), and charged onto a Reactive Red-agarose column (2.5 × 3.0 cm) equilibrated with buffer B. The Red column was washed with 80 ml of buffer B and the remaining protein was eluted with a 0–1 M KCl gradient in buffer B (80 ml). Fractions that contained maximal *S*-oxidase activity were pooled (Red pool), dialyzed against buffer A, and charged onto a 2′,5′-ADP-Sepharose column (1.0 × 7.0 cm). The ADP column was eluted with successive washes of 25 ml of buffer B, 25 ml of 0.1 mM NADPH in buffer B, and 1 M KCl in buffer B. Fractions from each wash were analyzed for *S*-oxidase activity and pooled with those fractions from their respective wash that contained maximal activity. The pools were concentrated with Centricon-10 concentrators (Amicon, Beverly, MA), reanalyzed for *S*-oxidase activity, and subjected to SDS-PAGE using 1.5-mm-thick 10% gels (16 × 18 cm), as described by Laemmli (23); proteins were visualized either by staining with Coomassie Blue R-250 or by silver staining as described by Giulian *et al.* (24). Molecular weight markers ranging from 14,200 to 66,000 were used to determine the molecular weight of *S*-oxidase. Protein concentrations for all steps of the purification were determined by the Pierce bicinchoninic acid microassay (Pierce Chemical Company, Rockford, IL); because we found that NADPH interfered with the assay, final protein concentrations of the samples were determined after dialysis and concentration with Centricon-10.

Determination of flavin content of purified *S*-oxidases. Flavin content was determined by reverse phase HPLC, using fluorescence detection with a Gilson 121 fluorometer equipped with a 450-nm excitation filter and an emission filter with a range of 510–650 nm. The time constant was 0.5 sec and the range was set at 0.01 relative fluorescense unit. Separation of FAD and FMN was achieved on a 5-μm, 4.6-mm × 25-cm Beckman Ultrasphere-ODS column. The mobile phase used was 89% 5 mM $(NH_4)_2HPO_4$, pH 5.5/11% acetonitrile. The flow rate was 1 ml/min and the injection volume was 100 μl. Retention times for FAD and FMN were 3.9 and 4.7 min, respectively; limits of detection for FAD and FMN were 0.5 and 0.2 pmol/injection, respec-

[1] The FMO nomenclature used here is based on structural relationships among FMO isozymes (R. Philpot, personal communication). FMOs 1A1, 1B1, and 1D1 correspond to the liver (form 1), lung, and form 2 FMOs, respectively.

tively. Samples were prepared by diluting 10 μl of purified liver (2.6 μg) or kidney (1.4 μg) *S*-oxidase with $(NH_4)_2HPO_4$, pH 5.5, to 200 μl in foil-covered microcentrifuge tubes. The samples were then boiled for 3 min to release the flavin from the protein, cooled rapidly on ice, and centrifuged for 5 min in an Eppendorf microcentrifuge. Samples were then filtered through a 0.2-μm filter before HPLC analysis. Quantitation using peak area was done by comparison with standard curves having correlation coefficients of >0.99. Control samples of FAD and FMN were also treated in the same manner, to determine the extent of their recovery after the heat treatment; the work-up procedure resulted in FAD and FMN recoveries of nearly 80% and 100%, respectively. FAD loss was not accompanied by an increase in FMN concentration, and reported values of FAD have been corrected.

Transfer of the *S*-oxidases to PVDF membranes. Purified rat hepatic and renal *S*-oxidases (12.5 μg each) were subjected to SDS-PAGE as described above, using a 1.5-mm-thick 10% gel electroblotted onto PVDF membranes, as described by Matsudaira (25). Briefly, after electrophoresis the gel was soaked in 200 ml of transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11.0] for 5 min to reduce the amount of Tris and glycine. The gel was then sandwiched between a sheet of PVDF and two sheets of Whatman 3 mm paper and placed in a Bio-Rad electroblotting apparatus. The proteins were transferred to the PVDF membrane at 300 mA for approximately 90 min, in a cold room. The PVDF membrane was washed with deionized water and stained with 0.02% Coomassie Blue in 50% methanol for 1 min. The PVDF membrane was destained (50% methanol, 10% acetic acid) for approximately 1 hr, with several changes of the destaining solution. Finally, the PVDF membrane was rinsed with deionized water, allowed to air dry, wrapped with plastic wrap, and stored at −20° until submitted for sequence analysis.

Amino acid composition and amino-terminal amino acid sequence analyses. The PVDF membrane was submitted to the University of Wisconsin Biotechnology Center for amino-terminal amino acid sequence analysis. Protein sequencing was carried out on an Applied Biosystems model 900A protein sequencer, with phenylthiohydantoin-norleucine as an internal standard (26). A portion of the PVDF membrane was cut out with a clean razor blade and sequenced using precycled polybrene-coated glass fiber filters, as recommended by the manufacturer.

The remainder of the protein on the PVDF membrane was submitted to The Medical College of Wisconsin Protein/Nucleic Acid Shared Facility of the Cancer Center for amino acid composition analysis. After hydrolysis of the protein for 20 hr at 110°, the hydrolysates were applied to a Beckman model 6300 amino acid analyzer. The amino acids were separated on an ion exchange column and subjected to postcolumn ninhydrin modification for analysis at 570 nm. Residue numbers were assigned based on 535 amino acids, because known FMOs were reported to have 532–537 amino acids (12, 14, 18–20). Standard amino acid mixtures were run at the beginning of each day. When a standard protein (bovine pancreatic chymotrypsin; Sigma) was used, the amino acid composition (excluding cysteine and tryptophan) exhibited an average 8.2% error.

Western blotting. Purified rat hepatic and renal *S*-oxidases (250 ng each), pig liver microsomes (10 μg), purified pig liver FMO 1A1 (100 ng), purified rabbit lung FMO 1B1 (100 ng), and rabbit lung microsomes (2 μg) were subjected to SDS-PAGE using 7.5% gels, immunoblotted, and immunostained as described by Tynes and Philpot (9). Briefly, after SDS-PAGE the proteins were transferred to nitrocellulose sheets and stained. The nitrocellulose sheets were treated with bovine serum albumin (3%) for 15 min at 40° to block any remaining sites on the nitrocellulose membrane. The proteins were then exposed to antibodies raised against either pig liver, rabbit lung, or a chemically synthesized FMO peptide (16), for 60 min at room temperature, followed by rabbit anti-goat IgG (1/100 dilution) and finally goat peroxidase-antiperoxidase (1/3000 dilution).

Substrate kinetics of hepatic and renal *S*-oxidases. Experiments for characterization of SBC sulfoxidation by purified hepatic and renal *S*-oxidases were conducted as described above, except that the SBC concentrations used were 0.63–5.0 mM (higher concentrations of SBC were not achievable because of its limited solubility), the protein concentrations were 5.6 (kidney) and 10.4 (liver) μg/ml, the NADPH concentation was 2 mM, the incubation time was 20 min, and the incubation buffer was 10 mM KH_2PO_4, pH 7.6, containing 1 mM EDTA. Kinetic constants (K_m and V_{max}) were determined from double-reciprocal plots ($r > 0.99$) of SBC concentration versus nmol of SBC sulfoxide formed/mg of protein/min. Experiments were also conducted to determine the ability of the *S*-oxidases to catalyze NADPH oxidation at pH 7.6 and 37° in the presence of methimazole. In these experiments, the reaction mixture (1 ml) contained 0.1 mM NADPH, 10–200 μM methimazole, 10 mM KH_2PO_4, 1 mM EDTA, and 0, 1.4 (kidney), or 2.6 (liver) μg of protein; pH 7.6 was used because it was optimal for the SBC *S*-oxidase activities of rat liver and kidney microsomes (21). NADPH oxidation was measured at 340 nm (5, 12); kinetic constants were determined from double-reciprocal plots ($r = 0.92$ for both liver and kidney *S*-oxidase) of methimazole concentration versus nmol of NADP formed/mg of protein/min.

Statistics. Data are presented as mean ± standard deviation. Statistical significance was determined by the Student's *t* test, using $p \leq 0.05$ as the criterion for significance.

Results

Dependence of SBC *S*-oxidase microsomal activity on pyridine and flavin nucleotides. To determine the pyridine nucleotide specificity of *S*-oxidase, enzymatic assays were conducted in the presence of NADPH (2 mM) or NADH (2 mM). With NADH as the cofactor, SBC sulfoxidation was reduced to approximately 25 and 10% of the activities measured with NADPH for hepatic and renal microsomes, respectively (Table 1). Removal of the flavin nucleotide from either rat hepatic or renal microsomes resulted in approximately 50 and 80% loss of SBC *S*-oxidase activity, respectively; however, addition of FAD (0.01–5 mM) to incubation mixtures completely restored both hepatic and renal SBC *S*-oxidase activities (Table 2). Interestingly, addition of FMN (0.01–5 mM) was also able to restore both hepatic and renal SBC *S*-oxidase activities (Table 2).

Effects of heat and detergent solubilization on microsomal SBC *S*-oxidase activities. When rat kidney microsomes were heated at 45° for 2–20 min in the absence of NADPH, renal SBC *S*-oxidase activity was significantly reduced; approximately 77% of the renal *S*-oxidase activity was lost at 10 min (Fig. 1). Similar heat treatments, however, had no significant effect on hepatic microsomal SBC *S*-oxidase activity. Solubilization of rat hepatic and renal microsomes with 1% solutions of the detergents Triton X-100, Nonidet P-40, Emulgen 911, or CHAPS did not significantly affect SBC sulfoxidation; however, 1% sodium cholate resulted in nearly

TABLE 1

Cofactor requirements of the *S*-oxidase activities in male rat hepatic and renal microsomes

S-Oxidase activity was determined as described in Experimental Procedures; the incubation time was 15 min. Values are expressed as mean ± standard deviation for the number of experiments in parentheses.

Cofactor	Specific activity	
	Liver	Kidney
	nmol/mg of protein/min	
NADPH	5.0 ± 1.3 (4)	6.5 ± 1.4 (3)
NADH	1.3 ± 0.5 (4)[a]	0.7 ± 0.6 (3)[a]

[a] Significantly different from NADPH ($p < 0.01$), as determined by Student's *t* test.

TABLE 2

Flavin nucleotide requirements of the S-oxidase activities in male rat hepatic and renal microsomes

S-Oxidase activities were determined as described in Experimental Procedures; the incubation time was 20 min and the FAD or FMN concentration was 5 mM. Values are expressed as the results from a typical experiment.

Fraction	Flavin nucleotide supplements to medium	Specific activity
		nmol/mg of protein/min
Liver		
Microsomes	None	2.2
	FAD	2.0
	FMN	2.3
Flavin-depleted microsomes	None	1.1
	FAD	3.3
	FMN	3.6
Kidney		
Microsomes	None	6.3
	FAD	4.3
	FMN	4.3
Flavin-depleted microsomes	None	1.4
	FAD	8.3
	FMN	11.4

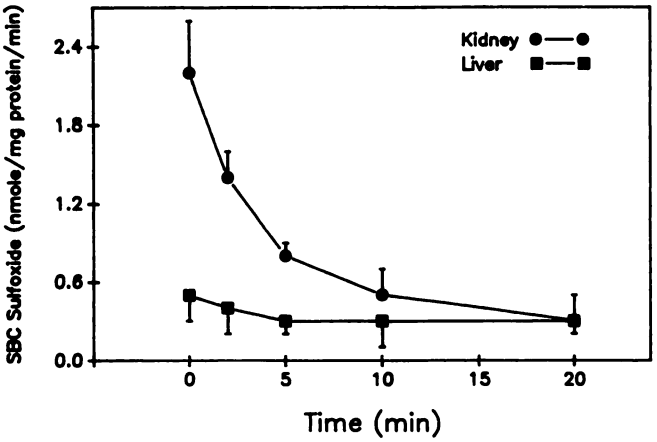

Fig. 1. Effects of heating of hepatic or renal microsomes in the absence of NADPH on SBC sulfoxide formation. S-Oxidase activity was determined as described in Experimental Procedures.

TABLE 3

Effects of detergents on S-oxidase activities in male rat hepatic and renal microsomes

S-Oxidase activity was determined as described in Experimental Procedures; the incubation time was 20 min. Values are expressed as mean ± standard deviation for the number of experiments in parentheses. The specific activity for liver and kidney controls was 1.4 ± 0.4 and 3.4 ± 1.3 nmol/mg of protein/min, respectively (five experiments).

Detergent	Activity	
	Liver	Kidney
	% of control	
Nonionic		
Triton X-100	93 (2)[a]	79 ± 11 (3)
Nonidet P-40	91 (2)[a]	82 ± 15 (3)
Emulgen 911	91 ± 10 (3)	84 ± 3 (3)
Zwitterionic		
CHAPS	84 ± 7 (3)	70 ± 15 (3)
Anionic		
Sodium cholate	32 ± 13 (4)[b]	30 ± 4 (4)[c]

[a] Values are expressed as the mean of two experiments.
[b] Significantly different from control ($p < 0.01$), as determined by Student's t test.
[c] Significantly different from control ($p < 0.05$), as determined by Student's t test.

70% loss of S-oxidase activities in both rat hepatic and renal microsomes (Table 3).

Effects of sulfhydryl-reactive agents on microsomal SBC S-oxidase activities. The effects of Hg^{2+} (0.1 mM), iodoacetamide (1 mM), or N-ethylmaleimide (1 mM) on SBC sulfoxidation by rat hepatic and renal microsomes were examined. Inclusion of mercuric chloride, iodoacetamide, or N-ethylmaleimide in incubation mixtures had no significant effect on SBC sulfoxidation by hepatic or renal microsomes (data not shown).

Species, sex, and tissue distribution of microsomal SBC S-oxidase activity. SBC S-oxidase activity was present in hepatic and renal microsomes from both male and female rats, with the male rat kidney maintaining approximately 3.3 times more activity than the female rat kidney (Table 4). In addition, SBC S-oxidase activities were also present in hepatic and renal microsomes of male mice (specific activities, mean ± standard deviation: 2.4 ± 0.1 and 2.1 ± 1.0 nmol of SBC sulfoxide/mg of protein/min, respectively) and female rabbits (specific activities, mean ± standard deviation: 3.0 ± 0.9 and 1.0 ± 0.2 nmol of SBC sulfoxide/mg of protein/min, respectively). In studies designed to determine the tissue distribution of microsomal S-oxidase activity in male rats, microsomes from the small intestine and lungs were also prepared and tested for SBC S-oxidase activity. Whereas no SBC S-oxidase activity was detected in the small intestine, the lungs exhibited a specific activity of 3.5 ± 0.8 nmol of SBC sulfoxide/mg of protein/min (mean ± standard deviation).

Purification of SBC S-oxidases. To further characterize the properties of SBC S-oxidase, the activities were purified from rat liver and kidney microsomes. The summary of a typical purification of S-oxidase from rat hepatic and renal microsomes is presented in Table 5. This table shows that nearly 580- and 700-fold purifications of S-oxidase from rat liver and kidney microsomes, respectively, were achieved. SDS-PAGE analysis of the purified enzymes showed that, whereas the kidney sample was apparently homogeneous, with a molecular weight of 56,000, the liver sample contained an additional band at 65,000 (Fig. 2). However, only the liver M_r 56,000 band was observed after subsequent purifications used to obtain the protein samples used for determinations of the flavin content, substrate kinetics, amino-terminal amino acid sequence, and amino acid composition (data not shown).

Flavin content of purified SBC S-oxidases. To determine whether the purified S-oxidases contain FAD, FMN, or both, a highly sensitive reverse phase HPLC method was developed that allowed the separation of FAD and FMN, with detection limits in the picomole range for each chemical (see Experimental Procedures). The results obtained with liver and kidney S-oxidases show that these proteins contain both FAD

TABLE 4

Sex dependence of S-oxidase in rat hepatic and renal microsomes

S-Oxidase activity was determined as described in Experimental Procedures; the incubation time was 20 min. Values are expressed as mean ± standard deviation for the number of experiments in parentheses.

Species/gender	Specific activity	
	Liver	Kidney
	nmol/mg of protein/min	
Rat, male	2.0 ± 0.1 (3)	4.9 ± 0.3 (3)
Rat, female	0.8 (2)[a]	1.5 ± 0.5 (4)

[a] Values are expressed as the mean of two experiments.

TABLE 5

Summary of purification of *S*-oxidase from rat liver and kidney microsomes

S-Oxidase activities and protein concentrations were determined as described in Experimental Procedures; the incubation time was 20 min.

Fraction	Volume	Total protein	Total activity	Specific activity	Fold purification
	ml	*mg*	*nmol/min*	*nmol/min/mg*	
Liver					
Microsomes	160	832	2579	3	1
Solubilized microsomes	155	271	3930	15	5
Blue pool	45	42	3694	89	29
Red pool	35	8.8	1138	129	42
ADP pool	0.35	0.074	132	1802	581
Kidney					
Microsomes	80	372	1786	5	1
Solubilized microsomes	79	236	1699	7	2
Blue pool	25	11.4	693	61	13
Red pool	29	1.2	497	432	90
ADP pool	0.2	0.029	97	3349	698

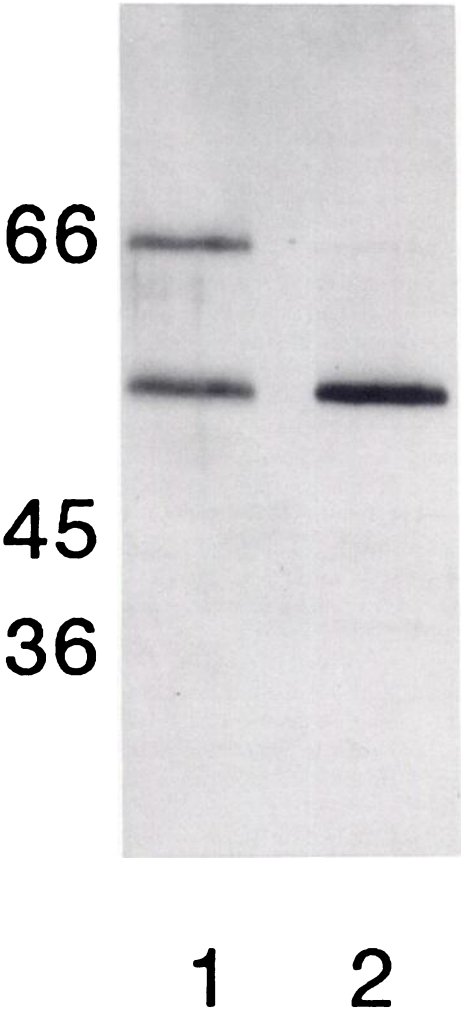

Fig. 2. SDS-PAGE analysis of *S*-oxidases purified from rat liver (*lane 1*; 430 ng) and kidney (*lane 2*; 500 ng), using a 1.5-mm-thick 10% gel. Gels were silver-stained by the method of Giulian *et al.* (24). Molecular weight standards were bovine serum albumin (66,000), ovalbumin (45,000), and glyceraldehyde-3-phosphate dehydrogenase (36,000). Liver *S*-oxidase used for determination of the flavin content and amino-terminal amino acid sequence exhibited the 56,000 band only.

TABLE 6

Flavin nucleotide content of rat liver and kidney SBC *S*-oxidases

FAD and FMN concentrations were determined by HPLC, as described in Experimental Procedures. The results obtained from two independent determinations of liver and kidney SBC *S*-oxidase are shown.

Enzyme	Flavin content		
	FAD	FMN	Total flavin
	nmol/mg of protein		
Liver *S*-oxidase	16.7	0.6	17.3
	15.9	0.6	16.5
Kidney *S*-oxidase	12.1	0.7	12.8
	11.3	0.7	12.0

and FMN (Table 6), but the FMN concentrations represented only 3.5–5.8% of the total flavin concentrations (12.0–17.3 nmol/mg of protein).

Amino-terminal amino acid sequence and amino acid composition. To provide further characterization of the purified *S*-oxidases, the amino-terminal amino acid sequences and amino acid composition of purified rat liver and kidney *S*-oxidases were determined and compared with those of selected known FMOs (Tables 7 and 8). Final analysis demonstrated that 8 and 6 pmol of a single protein with an unblocked amino-terminal end were sequenced from the PVDF membranes corresponding to liver and kidney *S*-oxidase, respectively; repetitive yields were 98 and 96%. The finding that similar yields (5.40 and 6.94 pmol) were obtained by amino acid analysis of the sequenced proteins provides further evidence for the purity of the sequenced bands. The observed amino acid sequences of liver and kidney *S*-oxidases are virtually identical and to a large extent resemble the amino-terminal amino acid sequences of known FMO isozymes (Table 7). More importantly, however, the highly conserved consensus sequence found in FAD-binding proteins (Gly-X-Gly-X-X-Gly) is present in both liver and kidney SBC *S*-oxidases beginning at residue 8. The amino-terminal amino acid sequence of liver *S*-oxidase, which is virtually identical to that derived from the cDNA sequence of rat liver 1A1 (unpublished sequence in GenBank), is more comparable to the amino-terminal amino acid sequences of pig and rabbit liver 1A1 isozymes (only one difference) than to that of rabbit lung 1B1 (eight differences) or rabbit liver 1D1 (five differences) isozymes.

The amino acid compositions of rat liver and kidney *S*-oxidases are presented in Table 8, which also contains the previously reported amino acid composition of mouse liver and kidney and pig liver FMO for reference. These results, which show that liver and kidney *S*-oxidases have large amounts of glutamate/glutamine, leucine, and valine and small amounts of histidine, arginine, and methionine, are consistent with the amino acid compositions of mouse liver and kidney and pig liver FMO isozymes. Interestingly, liver *S*-oxidase contains larger amounts of glutamate/glutamine than does kidney *S*-oxidase, whereas kidney *S*-oxidase contains a larger amount of serine, compared with liver *S*-oxidase.

Western blotting. Western blot analyses of purified liver and kidney *S*-oxidases were performed to possibly provide further evidence that the *S*-oxidases are indeed FMO isozymes and to determine immunochemical properties of these enzymes. Antibodies raised against the hog liver FMO 1A1 isozyme or the universal FMO peptide were immunoreactive with both liver and kidney *S*-oxidases, whereas no immunoreactivity was detected when rat liver or kidney *S*-oxidases were exposed to

TABLE 7

Amino-terminal amino acid sequences of purified rat liver (A) and kidney (B) SBC S-oxidases and of rat liver 1A1 (C), pig liver 1A1 (D), rabbit liver 1A1 (E), rabbit liver 1D1 (F), and rabbit lung 1B1 (G) FMO isozymes

Enzyme	Amino-terminal amino acid sequence[a]
	1[b] 5 10 15 20
A	Val-Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-X[c] - X -Leu-Glu- X -Gly-
B	Val-Lys- X -Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-
C[d]	Met-Val-Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-Cys-Cys-Leu-Glu-Glu-Gly-
D[d]	Met-Ala-Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-Cys-Cys-Leu-Glu-Glu-Gly-
E[e]	Ac -Ala-Lys-Arg-Val-Ala-Ile-Val-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ala-Ser-Ile-Lys-Ser-Cys-Leu-Glu-Glu-Gly-
F[e]	Gly-Lys-Lys-Val-Ala-Ile-Ile-Gly-Ala-Gly-Ile-Ser-Gly-Leu-Ala-Ser-Ile-Arg-Ser-Cys-Leu-Glu-Glu-Gly-
G[d]	Met-Ala-Lys-Lys-Val-Ala-Val-Ile-Gly-Ala-Gly-Val-Ser-Gly-Leu-Ile-Ser-Leu-Lys-Cys-Cys-Val-Asp-Glu-Gly-

[a] Underlining indicates differences in the amino acid sequences, compared with that of purified rat liver S-oxidase.
[b] Amino acid number.
[c] X, uncertainty in analysis of this amino acid.
[d] Deduced from cloned cDNA (14, 18) (unpublished cDNA sequence in GenBank).
[e] Obtained using purified FMO (19, 20).

TABLE 8

Amino acid composition of purified rat liver and kidney SBC S-oxidases and of mouse liver and kidney and pig liver FMO isozymes

Amino acid	Residues/molecule				
	Liver S-oxidase	Kidney S-oxidase	Mouse liver[a]	Mouse kidney[a]	Pig liver[b]
Alanine	29	30	24	28	31
Arginine	13	8	21	23	21
Aspartate/asparagine	41	38	52	46	52
Cysteine	ND[c]	ND	ND	ND	9
Glutamate/Glutamine	62	46	50	46	52
Glycine	41	40	52	63	37
Histidine	4	7	16	10	10
Isoleucine	27	25	30	25	29
Leucine	65	56	50	50	58
Lysine	16	20	26	34	39
Methionine	11	10	22	11	10
Phenylalanine	40	34	38	33	37
Proline	45	37	21	38	42
Serine	34	57	40	38	38
Threonine	37	38	24	35	33
Tryptophan	ND	ND	ND	ND	9
Tyrosine	16	18	22	17	19
Valine	53	49	49	39	44

[a] Amino acid composition data from Venkatesh *et al.* (12).
[b] Amino acid composition data from Poulsen and Ziegler (27).
[c] ND, not determined.

Fig. 3. Western blot analysis of purified rat hepatic and renal *S*-oxidases with antibodies raised to pig liver FMO 1A1 (*lanes 1-5*), rabbit lung FMO 1B1 (*lanes 6-9*), and a universal FMO peptide (*lanes 10-13*). *Lane 1*, pig liver microsomes (10 μg); *lane 2*, purified pig liver FMO 1A1 (100 ng); *lane 3*, yeast extract microsomes (200 μg); *lanes 4* and *8*, purified rat liver *S*-oxidase (250 ng); *lanes 5* and *9*, purified kidney *S*-oxidase (250 ng); *lane 6*, rabbit lung microsomes (2 μg); *lane 7*, purified rabbit lung FMO 1B1 (100 ng); *lane 10*, purified pig liver FMO 1A1 (200 ng); *lane 11*, purified rabbit lung FMO 1B1 (200 ng); *lane 12*, purified rat liver *S*-oxidase (500 ng); *lane 13*, purified rat kidney *S*-oxidase (500 ng).

TABLE 9

Kinetic constants for SBC and methimazole oxidation by purified SBC S-oxidase from rat liver and kidney

Kinetic constants were determined from double-reciprocal plots (SBC, $r > 0.99$; methimazole, $r = 0.92$), obtained as described in Experimental Procedures; values are the averages of duplicate determinations, which varied by <10%.

Enzyme	SBC			Methimazole		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
	μM	nmol/min/	mg	μM	nmol/min/	mg
Liver *S*-oxidase	4170	1887	0.5	6	253	42.2
Kidney *S*-oxidase	4160	2632	0.6	8	289	36.1

antibodies raised against the rabbit lung FMO 1B1 isozyme (Fig. 3).

Substrate kinetics. K_m and V_{max} values were determined and compared between liver and kidney *S*-oxidases for SBC and the typical FMO substrate methimazole (Table 9). In general, kinetic values were similar between liver and kidney *S*-oxidases, but SBC exhibited much lower V_{max}/K_m values than did methimazole (Table 9). As indicated in Experimental Procedures, the SBC and methimazole incubations were carried out at pH 7.6, an optimal pH for SBC sulfoxidation (21); the rate of methimazole oxidation by pig liver FMO at this pH was nearly 20% of that measured at pH 8.5 (13).

Discussion

Studies conducted by Ziegler and co-workers (1, 2) had shown that the flavin nucleotide could be stripped from pig liver microsomes by sonication and acid treatment and that exogenous FAD added to incubation mixtures could restore the FMO activity. Using this flavin depletion method, liver and kidney *S*-oxidase activities were reduced by approximately 50 and 75%, respectively, and subsequent addition of either FAD or FMN to the incubation mixture restored SBC *S*-oxidase activities of both liver and kidney microsomes (Table 2). These results, the detection of both FAD and FMN in the isolated proteins (Table 6), and the presence of the highly conserved Gly-X-Gly-X-X-Gly sequence upon amino-terminal amino acid sequence analyses of purified liver and kidney *S*-oxidases (Table 7) provide clear evidence that hepatic and renal *S*-oxidases are FMO enzymes. The FAD concentrations of purified liver and kidney *S*-oxidases are similar to those reported previously (13, 15, 27) for pig liver FMO (15.2 nmol/mg of protein), rabbit lung FMO (14 nmol/mg of protein), and guinea pig liver FMO I and II (15.3 and 19.1 nmol/mg of protein, respectively). The detection of both FAD and FMN in the *S*-oxidases and the ability of exogenous FMN to restore *S*-oxidase activities of flavin-depleted microsomes are interesting observations, because all known mammalian FMOs were reported to contain only FAD (5) and FMN was shown not to restore pig liver FMO activities (1, 2, 5). Whereas the bacterial FMO luciferase was shown to contain an FMN binding site (5, 28), the presence of different FAD and FMN binding sites on the *S*-oxidases is unlikely. The likelihood that FMN binds to the FAD binding site of liver and kidney *S*-oxidases is evidenced by the findings that FMN accounted for only a small fraction (3.5–5.8%) of the total flavin content and that FMN reconstituted the activity of flavin-depleted microsomes. FMN was reported to bind tightly to the vacant FAD site of cytochrome P-450 reductase and reconstitute activity (1, 29). Indeed, similarly to the results obtained with the *S*-oxidase activities (Table 2), FMN was reported to consistently produce greater stimulation of the cytochrome *c* reductase activity of flavin-depleted microsomes or the FAD-depleted purified cytochrome *c* reductase than did FAD (1, 29).

A characteristic property of known liver FMOs is their thermal lability in the absence of NADPH. For example, the enzyme in pig liver microsomes was totally inactivated in 1–2 min upon incubation at 50° without NADPH (5, 8, 13). The rabbit lung FMO isoform, however, was shown not to be inhibited by heat treatments, whereas the pig liver FMO isoform was inactivated when heated at 45° in the absence of NADPH (13). Rat kidney microsomes incubated in the absence of NADPH at 45° for 10 min lost approximately 80% of the *S*-oxidase activity, whereas similar heat treatments for up to 20 min did not significantly inhibit the liver *S*-oxidase activity (Fig. 1). These results suggest that hepatic and renal *S*-oxidase activities may be associated with different FMO isoforms. Solubilization of rat hepatic and renal microsomes by 1% sodium cholate caused approximately 70% loss of SBC *S*-oxidase activities, whereas Emulgen 911, Triton X-100, Nonidet P-40, and CHAPS had no significant effect on SBC sulfoxidation. These results are consistent with earlier findings that hepatic FMO isozymes are inactivated by anionic detergents, whereas the rabbit lung FMO isozyme is positively affected by 1% cholate (13, 30). Recently, solubilization of mouse liver microsomes with nonionic detergents was shown not to inhibit the NADPH oxidation of thiourea, but the NADPH-dependent oxidation of phorate, an organophosphate, was inhibited in the same detergent preparations (30).

Inclusion of Hg^{2+}, *N*-ethylmaleimide, or iodoacetamide in SBC *S*-oxidase incubations had no effect on SBC sulfoxide formation by rat hepatic or renal microsomes. These results are consistent with the previous finding that methimazole oxidation was unaffected when mouse liver homogenate was incubated with *N*-ethylmaleimide (31). On the other hand, these results are inconsistent with the findings of Devereux *et al.* (6), which showed that Hg^{2+} stimulated and inhibited FMO activities of rabbit pulmonary and liver microsomes, respectively. Although these results provide no conclusive evidence regarding the type of FMO isozyme catalyzing SBC sulfoxidation, it appears that *S*-oxidase does not contain an exposed nucleophilic site essential for SBC sulfoxidation.

S-Oxidase was present in hepatic and renal microsomes from male rats and mice and female rats and rabbits; in addition, SBC *S*-oxidase activity was also present in male rat pulmonary microsomes. These results are consistent with the findings that the FMOs are present in the liver, lungs, and kidneys of rats, rabbits, mice, guinea pigs, and humans (5, 7–20). *S*-Oxidase activities of male rat liver and kidney microsomes were nearly 2.5- and 3.3-fold higher, respectively, than those detected in the corresponding female tissues. Consistent with our results, dimethylaniline *N*-oxidation rates by male rat liver microsomes were nearly 2-fold higher than those detected with female rat liver microsomes (32). Furthermore, based on immunochemical methods, male rat liver and kidney microsomes were estimated to contain nearly 5- and 2-fold higher levels of FMO, respectively, than those present in the corresponding female tissues (7). Several reports of sex differences in FMO activities that have been attributed to sex hormone regulation have also been published (5, 7, 32, 33).

Further characterization of hepatic and renal SBC *S*-oxidase was provided by examination of the properties of the purified enzymes. Because the results described above provide strong evidence that *S*-oxidase has characteristics similar to those of the known hepatic and renal FMO isozymes, the method used by Sabourin and co-workers (11, 12) to purify the pig liver (60-fold from microsomes) and mouse liver (800-fold from solubilized microsomes) and kidney (1470-fold from solubilized microsomes) FMOs was used to purify *S*-oxidase. Using a slight modification of this method, *S*-oxidase was purified nearly 580- and 700-fold from rat hepatic and renal microsomes, respectively (Table 5). When analyzed under denaturing conditions on SDS-PAGE, both proteins exhibited apparent molecular

weights of approximately 56,000 (Fig. 2). Although the liver sample contained an additional band with a molecular weight of 65,000, this band is probably not associated with the *S*-oxidase activities, because subsequent purifications, which were used for determinations of substrate kinetics and contained essentially 1 mol of flavin/mol of protein, did not exhibit the band. These results are consistent with the molecular weights of the FMO isozymes isolated from pig liver, lung, and kidney but are slightly different from the molecular weights of 59,000 and 50,000 reported for the rat liver dimethylaniline *N*-oxidase and the thiobenzamide *S*-oxidase, respectively (5, 34, 35). As indicated above, flavin content, amino-terminal amino acid sequences, and amino acid composition of rat liver and kidney *S*-oxidase provide unequivocal evidence that these proteins are FMOs (Tables 6–8). Furthermore, whereas the Western results provide strong evidence for the rat liver and kidney *S*-oxidases being related to the pig liver FMO 1A1 isozyme (Fig. 3), further evidence for the structural similarity of the liver *S*-oxidase to the known FMO 1A1 isozymes was obtained by comparison of the amino-terminal sequences in Table 7. It should be noted, however, that these results do not exclude the possibility that other FMOs are also oxidizing SBC.

Although the FMOs are capable of catalyzing the oxidation of a wide range of substrates, physiological substrates such as cysteine, methionine, or glutathione were found not to be substrates of the hog liver FMO; furthermore, substrates bearing a second charge on the molecule, either anionic or cationic, are not substrates (4, 5, 8). The ability of *S*-oxidases to catalyze the sulfoxidation of SBC marks a new class of substrates for FMO. For both hepatic and renal *S*-oxidases, methimazole exhibited higher affinity (lower K_m value) than did SBC, but at high substrate concentration SBC would be expected to be oxidized at a higher rate (higher V_{max} value) than methimazole.

The results presented in this report provide clear evidence for the presence of *S*-oxidases in rat hepatic and renal microsomes. A recent report by Park *et al.* (36) indicated that pig liver FMO catalyzed SBC sulfoxidation, but those authors failed to detect SBC *S*-oxidase activity in either rat hepatic or renal microsomes. It appears that the discrepancy between the results published by Park *et al.* (36) and the work presented by our laboratory is most likely the result of the differences in the assays used to detect *S*-oxidase activity. The assay used by Park *et al.*, which relies on direct detection of SBC sulfoxide at 220 nm, is much less sensitive than detection of the SBC sulfoxide *N*-2,4-dinitrophenyl derivative at 365 nm, which was used in our experiments. Thus, whereas our assay allowed the detection of the *S*-oxidase activities in both rat liver and kidney microsomes, the assay used by Park *et al.* may allow the detection of only the *S*-oxidase activity with purified FMOs.

In summary, the results presented in this manuscript show that hepatic and renal *S*-oxidase activities are 1) dependent on the presence of pyridine and flavin nucleotides, 2) different in their sensitivity to heating in the absence of NADPH, 3) sensitive to solubilization by sodium cholate, 4) unaffected by the presence of sulfhydryl-reactive agents, 5) present within hepatic and renal microsomes of male and female rats and pulmonary microsomes of male rats, as well as distributed among males and/or females of a number of other different species, and 6) readily purified from rat hepatic and renal microsomes. Biochemical characterization of the isolated proteins provides strong evidence for the *S*-oxidases being FMO enzymes, which are immunologically related to the pig liver 1A1 isozyme and appear to be structurally related to FMO 1A1 isozymes. Because our studies do not exclude the possibility that other FMO isoforms could also be involved in SBC sulfoxidation, the substrate specificities of FMO isozymes and their role in cysteine *S*-conjugate metabolism warrant further investigation. Furthermore, our recent findings that the sulfoxide form of the cysteine conjugate of trichloroethylene, namely, *S*-(1,2-dichlorovinyl)-L-cysteine sulfoxide, was more reactive and nephrotoxic than the sulfide form, *S*-(1,2-dichlorovinyl)-L-cysteine (37), suggest that FMOs may play a role in the nephrotoxicity of cysteine *S*-conjugates.

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