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Oxidation of Cysteine *S*-Conjugates by Rabbit Liver Microsomes and cDNA-Expressed Flavin-Containing Monooxygenases: Studies with *S*-(1,2-Dichlorovinyl)-L-cysteine, *S*-(1,2,2-Trichlorovinyl)-L-cysteine, *S*-Allyl-L-cysteine, and *S*-Benzyl-L-cysteine

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SUMMARY

Rabbit liver microsomes catalyzed the highly stereoselective, NADPH- and time-dependent *S*-oxidation of *S*-benzyl-L-cysteine (SBC), *S*-allyl-L-cysteine (SAC), *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC) to their respective sulfoxides. Methimazole, a flavin-containing mono-oxygenase (FMO) substrate, inhibited *S*-oxidation of all four conjugates. The cytochrome P450 inhibitor 1-benzylimidazole did not affect SAC, SBC, or DCVC *S*-oxidation but inhibited the *S*-oxidation of TCVC. Solubilization of microsomes also inhibited TCVC activity, whereas SBC, SAC, and DCVC activities were not affected. Because these results suggested that FMOs were the major catalysts of SBC, SAC, and DCVC sulfoxidations, the four conjugates were evaluated as substrates for cDNA-expressed rabbit FMO isoforms FMO1, FMO2, FMO3, and FMO5. At equimolar concentrations (10 mM), FMO1 *S*-oxidized SBC and SAC, but no sulfoxides were detected with DCVC or TCVC. FMO3 *S*-oxidized all four conjugates. K_m values determined with FMO3 were comparable to K_m values from rabbit liver microsomes. *S*-Oxidation by FMO2 was detected only with SAC, and no sulfoxides were detected in incubations with FMO5. These results show that FMO isoforms can catalyze cysteine conjugate *S*-oxidation and that the specific isoform involved depends on the structure of the cysteine conjugate. The cysteine conjugates with more nucleophilic sulfur atoms, SAC and SBC, were much better FMO substrates than those having the less nucleophilic sulfur atoms DCVC and TCVC. The sulfoxides of TCVC and DCVC were reactive toward GSH, whereas the sulfoxides of SBC and SAC were not reactive. These results provide evidence for different chemical reactivities of these sulfoxides.

Cysteine conjugates are intermediates in the mercapturic acid pathway, which begins with conjugation of electrophilic compounds with GSH. GSH conjugates undergo cleavages by γ-glutamyl-transpeptidase and cysteinyl glycinase to yield cysteine conjugates. Cysteine conjugates can undergo *N*-acetylation to form mercapturic acids, which are excreted in urine (1, 2). An additional pathway for cysteine conjugate and mercapturic acid metabolism occurs *in vivo*, namely *S*-oxidation to form sulfoxides. For example, rats dosed with allyl chloride or SAC excreted both allylmercapturic acid and allylmercapturic acid sulfoxide in urine (3). *S*-Methyl-L-cysteine was converted to methylmercapturic acid and methylmercapturic acid sulfoxide in rats and to *S*-methyl-L-cysteine sulfoxide in humans (4, 5).

Although the studies mentioned above show that sulfoxidation is a metabolic pathway for cysteine conjugates *in vivo*, the nature of the enzyme(s) responsible for this reaction had not been investigated until this laboratory identified a novel cysteine conjugate *S*-oxidase activity in rat liver and kidney microsomes (6). This NADPH- and O_2-dependent activity catalyzed the oxidation of SBC to SBC sulfoxide. Additional studies and purification of the activity revealed that a form of FMO (E.C. 1.14.13.8) was mediating the sulfoxidation. There are currently five known isoforms of FMO (identified as FMO1, FMO2, FMO3, FMO4, and FMO5) that show tissue-specific expression and distinct substrate selectivities (7, 8).

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ABBREVIATIONS: GSH, reduced glutathione; SAC, *S*-Allyl-L-cysteine; SBC, *S*-benzyl-L-cysteine; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; TCVC, *S*-(1,2,2-trichlorovinyl)-L-cysteine; ACN, acetonitrile; β-lyase, cysteine conjugate β-lyase; AOAA, aminooxyacetic acid; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; FMO, flavin-containing monooxygenase; TFA, trifluoroacetic acid.

N-Terminal sequencing and immunoblotting revealed that the rat liver and kidney SBC *S*-oxidase activity was related to the FMO1 isoform (9). Subsequent to characterization of the SBC *S*-oxidase activity of FMOs, Park *et al.* (10) reported the *S*-oxidation of the cysteine conjugates of *cis*- and *trans*-1,2-dichloropropene, as well as SBC, and their mercapturates by purified hog liver FMO (FMO1). Park *et al.* (10) found that the cysteine conjugates were better substrates and exhibited more diastereoselective oxidation than their corresponding mercapturates for the purified hog liver FMO.

Although studies with SBC and the cysteine conjugates of *cis*- and *trans*-1,2-dichloropropene showed that FMO1 can catalyze cysteine conjugate *S*-oxidation, it is unclear whether *S*-oxidation occurs with other cysteine conjugates and what structural features are required for activity. It also is not known whether FMO isoforms other than FMO1 catalyze these reactions. The purpose of the present studies was to investigate structure-activity relationships of cysteine conjugate *S*-oxidation by rabbit liver microsomes and cDNA-expressed FMO isoforms. Rabbit liver, which is known to contain FMO1, FMO3, and FMO5 protein and FMO4 mRNA (8, 11), was used as an enzyme source so that accurate comparisons could be made between microsomal activity and the activity of recombinant FMO isoforms that were expressed from cDNA isolated from rabbit liver (with the exception of FMO2 cDNA, which was obtained from rabbit lung).

To gain insight into the structural characteristics necessary for enzymatic cysteine conjugate *S*-oxidation, four cysteine conjugates were chosen for this study. SBC is a relatively lipophilic molecule containing a nucleophilic sulfur atom and was used as a positive control because it already has been shown to be a selective substrate for FMO1 (9). SAC is a naturally occurring compound found in garlic (12) and is also a known metabolite of allyl halides/esters (3). SAC, which also contains a nucleophilic sulfur atom, was used to examine whether a more water-soluble compound would be a substrate for any of the FMO isoforms. DCVC and TCVC, the cysteine conjugates of trichloroethylene and tetrachloroethylene, respectively, are less water soluble than SAC and contain vinylic sulfur atoms as well as electron-withdrawing chlorine atoms, properties that make the sulfur atom of these compounds much less nucleophilic than that of SAC or SBC. By studying these four substrates, the importance of nucleophilicity and lipophilicity to cysteine conjugate *S*-oxidation by different FMO isoforms could be examined. Preliminary results have been presented previously (13).

Experimental Procedures

Materials. NADPH, SBC, tetrachloroethylene, L-cysteine, AOAA, GSH, H_2O_2, catalase, superoxide dismutase, FAD, and FMN were purchased from Sigma Chemical (St. Louis, MO). Trichloroethylene, methimazole, 1-benzylimidazole, 2,4-dinitrofluorobenzene, and TFA were purchased from Aldrich Chemical Co. (Milwaukee, WI). SAC was kindly provided by Wakunaga of America (Mission Viejo, CA). HPLC-grade ACN was purchased from EM Science (Gibstown, NJ). All other chemicals were of the highest grade commercially available.

Proton NMR spectra were conducted by the Nuclear Magnetic Resonance Facility at Madison (Madison, WI) on a Bruker spectrometer at 500 MHz using D_2O/DCl as solvent. Chemical shifts are reported in ppm from 3-(trimethylsilyl)-tetradeutero sodium propionate.

Positive-ion FAB-MS analyses were performed with a Kratos MS-50 ultra high-resolution mass spectrometer (Manchester, England) fitted with a Kratos DS-55 data system. Xenon was used as the FAB gas with a direct insertion probe and 3-nitrobenzyl alcohol matrix.

Synthesis and characterization of cysteine *S*-conjugates and sulfoxides. DCVC, DCVC sulfoxide, and SBC sulfoxide were synthesized as described in earlier reports from this laboratory (6, 14).

SAC sulfoxide was synthesized essentially as described by Stoll and Seebeck (12). Briefly, SAC (0.1 g) was dissolved in 3 ml of H_2O and 200 μl of 30% H_2O_2 was added. The solution was stirred at room temperature for 5 hr, and product was recovered by rotary evaporation followed by precipitation with ice-cold ethanol. Reaction yield was 48% and was 96% pure as determined by HPLC with detection at 220 nm. ^{1}H NMR [(1:1 mixture of two diastereomers): 5.92 ppm (m, 2H; vinyl protons), 5.55 ppm (d, 2 H, J = 10.5; vinyl protons), 5.51 ppm (d, 1 H, J = 17; vinyl proton), 5.49 ppm (d, 1 H, J = 17; vinyl proton), 4.26 ppm (dd, 1 H, J = 8, 4; α proton), 4.22 ppm (t, 1 H, J = 7; α proton), 3.85 ppm (m, 2 H; allyl protons) 3.67 ppm (m, 2 H; allyl protons), 3.44 ppm (dd, 1 H, J = 14, 6.5; β proton), 3.41 ppm (m, 2 H; β protons), 3.23 ppm (dd, 1 H, J = 14, 7.5; β proton)]. FAB-MS, *m/z* range 80–200 (relative intensity): 88 (100) [M + 1]-CH_2CHCH_2SO; 178 (50) [M + 1].

TCVC was synthesized as described by McKinney *et al.* (15). Briefly, tetrachloroethylene (0.075 mol) dissolved in acetone was added slowly to a solution of disodium cysteinate (0.075 mol) in liquid ammonia and stirred for approximately 30 hr. The ammonia was allowed to evaporate and TCVC was recovered by dissolving the residue in water and precipitating with ethanol/water adjusted to pH 5 with acetic acid. The crude TCVC precipitate (≈80% pure as determined by HPLC) was purified on a silica gel column, eluted with 80% ACN/water, and recrystallized from water for a final purity > 95% and a yield of 19%. ^{1}H NMR [(ppm): 3.50 (t, 1 H, J = 5; α proton), 3.36 (dd, 1 H, J = 11, 4; β proton), 3.22 (dd, 1 H, J = 11, 6; β proton)]. FAB-MS, *m/z* range 100–400 (relative intensity): 250 (100)[M + 1], 252 (100)[M + 3], 254 (30)[M + 5], 256 (5)[M + 7], isotope cluster consistent with three chlorines.

TCVC sulfoxide was synthesized by adding 30% H_2O_2 (150 μl) to a solution of TCVC (0.030 g) in formic acid (0.5 ml) then stirred at 4° for 40 min. Formic acid was evaporated under a stream of N_2 and TCVC sulfoxide was precipitated by the addition of 20 ml of ice-cold ether. Product was collected by vacuum filtration and was >95% pure as determined by HPLC with a yield of 75%. ^{1}H NMR [(1:1 mixture of two diastereomers, A and B): 4.62 ppm (dd, 1 H, J = 8, 4; α proton A), 3.68 ppm (dd, 1 H, J = 14, 8; β proton A), 3.80 ppm (dd, 1 H, J = 14, 4; β proton A), 4.65 ppm (t, 1 H, J = 6; α proton B), 3.68 ppm (d, 1 H, J = 6; β proton B), 3.68 ppm (d, 1 H, J = 6; β proton B)]. FAB-MS, *m/z* range 80–400 (relative intensity): 88 (100) [M + 1]-CCl_2CClSO; 266 (60)[M + 1], 268 (60)[M + 3], 270 (20)[M + 5], 272 (4)[M + 7], isotope cluster consistent with three chlorines.

Microsomal preparations and incubations. The buffer used in all experiments was 0.1 M KCl, 0.1 M KH_2PO_4, 5 mM EDTA, pH 7.4, unless otherwise stated. Livers from male New Zealand White rabbits (8–12 weeks of age) were obtained from Pelfreeze (Brown Deer, WI). "Washed" microsomes were prepared as described previously (6). Solubilized microsomes were prepared by dilution of washed microsomes 1:1 with buffer containing 1% Emulgen 911 (a gift from Kao Corporation, Tokyo, Japan) and stirring on ice for 1 hr. The solubilized microsomes were then centrifuged at 100,000 × g for 1 hr to pellet the lipid fraction and the supernatant was used in *S*-oxidase assays. Protein concentrations for rabbit microsomes were determined by the method of Lowry *et al.* (16) using bovine serum albumin as a standard.

Typical microsomal incubations were carried out as follows: microsomal suspensions (0.5–1.5 mg of protein) were preincubated with NADPH (or buffer for -NADPH controls) for 5 min at 37° in a shaking water bath and reactions were started by addition of substrate.

(Note: due to limited solubility, DCVC, TCVC, and SBC stock solutions were heated at 60° and several drops of NaOH were added to aid dissolution. Stocks were reequilibrated to 37° and final pH was 7.4–7.8, bringing pH of the assay to 7.4–7.6. Final reaction volume was 0.5 ml with 2 mM NADPH and 10 mM substrate. SBC and SAC incubations were stopped after 20 min by addition of 0.5 ml of ice-cold ethanol. DCVC incubations were stopped after 75 min with 0.5 ml of 0.75% perchloric acid. Ethanol could not be used in DCVC reactions because DCVC sulfoxide was found to be not stable in ethanol. TCVC incubations were stopped after 75 min by addition of 150 μl 2% perchloric acid. Reaction mixtures were then centrifuged for 20 min at 4° in a Beckman model TJ-6R benchtop centrifuge (Beckman Instruments, Palo Alto, CA) to remove precipitated proteins. Supernatants from SBC, DCVC, and TCVC reactions were then filtered using 0.2 μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI) and analyzed directly by HPLC (described below). SAC reactions were derivatized before HPLC analysis as follows: 18 μl of 10% 2,4-dinitrofluorobenzene in ethanol was added to 0.7 ml of reaction supernatant followed by 12 μl 1 M $NaHCO_3$ to bring the pH to approximately 8.5; this mixture was then heated at 60° for 1 hr, filtered as described above, and analyzed by HPLC. This procedure allowed complete derivatization of up to 50 mM SAC and metabolites and the products were stable for the duration of HPLC analysis.

For enzyme incubations in the presence of inhibitors, superoxide dismutase, or catalase, the inhibitor/reactive oxygen species scavenger was added with NADPH and preincubated for 5 min followed by addition of substrate for final assay concentrations of 1 mM 1-benzylimidazole, 1 mM potassium cyanide, 537 units superoxide dismutase, or 2800 units catalase. Methimazole was added simultaneously with substrate for a final assay concentration of 1 mM.

cDNA-expressed FMOs. *Escherichia coli* membrane fractions containing cDNA-expressed rabbit FMO1, FMO2, FMO3, FMO5, or expression vector control were obtained as described previously (17). Flavin content of the membrane fractions was determined by HPLC with fluorescence detection as described previously (9), except a Shimadzu RF-551PC fluorescence detector with excitation $\lambda = 447$ nm and emission $\lambda = 530$ nm was used. Limits of detection were 0.5 and 0.2 pmol/100 μl injection for FAD and FMN, respectively.

Enzymatic incubations with cDNA-expressed FMOs were carried out as follows: *E. coli* membrane fractions (20–100 μl, containing 0.2–1.0 nmol flavin) were preincubated with NADPH in a 37° shaking water bath for 5 min followed by addition of substrate for a final reaction volume of 0.5 ml with 10 mM substrate and 2 mM NADPH. Controls, lacking protein, substrate, or NADPH, were run in parallel. AOAA (1 mM) was included in some incubations to stabilize reactants and products (see Results). Reactions were stopped and analyzed as described for rabbit liver microsomal reactions.

HPLC analyses. HPLC analyses were performed using a Beckman 125 Solvent Module (dual pumps, gradient controlled) and Beckman 166 Detector on a Beckman ODS 5 μm reverse-phase C-18 column (4.6 mm × 25 cm; Beckman Instruments) and a Brownlee Newguard guard column (Rainin Instruments, Woburn, MA). Flow rate was 1 ml/min and injections (20 μl) were made with a Gilson 234 Autoinjector equipped with a Rheodyne 7010 injector (Beckman Instruments, Irvine, CA). UV scans were obtained using a Beckman diode array detector (model 168; Beckman Instruments). Beckman System Gold software (Beckman Instruments) was used for system control and data analysis. Mobile phase for pump A was 1% ACN in water, pH adjusted to 2.5 with TFA for SBC, DCVC, and TCVC analysis methods. Pump B mobile phases were 50% ACN, pH 2.5 for SBC and TCVC and 25% ACN for DCVC. Mobile phases for SAC analyses were 1% ACN (pump A) and 75% ACN (pump B) in water, 0.1% TFA, pH adjusted to 4.5 with 1 N NaOH. Gradients were as follows: for SBC, initial 10% B, at 3 min increased to 35% B over 4 min, at 13 min returned to 10% B; for SAC, initial 15% B, at 6 min increased to 60% B over 5 min, at 14 min returned to 15% B over 5 min; for DCVC, initial 25% B, at 4 min increased to 60% B over 2.5 min, at 11.5 min returned to 5% B over 3 min; for TCVC, initial 25% B, at 5 min increased to 60% B over 3 min, at 13 min returned to 25% B over 2.5 min. Detection limits and retention times are summarized in Table 1.

Enzymatically produced sulfoxides were quantified by comparing peak areas of enzymatic reactions with peak areas of standard curves generated using synthetic sulfoxide standards ($r > 0.99$ for all curves). Limits of detection shown in Table 1 were the lowest detectable concentrations in the linear range of the standard curve. Sulfoxide recoveries were determined by comparing HPLC peak areas of known sulfoxide concentrations under standard curve conditions (buffer only) with peak areas under incubation conditions (in presence of rabbit liver microsomes, NADPH, 37°). Recoveries for SAC sulfoxide and SBC sulfoxide were 100% and 94%, respectively, for 20-min incubations. Recoveries for DCVC sulfoxide and TCVC sulfoxide were 71% and 73%, respectively, for 75-min incubations; TCVC and DCVC data were corrected for recovery. Stereoselectivity (expressed as percentage of later-eluting of two diastereomers) was determined by dividing the peak area of the later-eluting of two sulfoxide diastereomers by the sum of the peak areas of both diastereomers, and multiplying by 100.

Sulfoxide stability studies. Solutions (3 ml) of each sulfoxide (3 mM) were incubated in buffer at 37° in a shaking water bath in the absence or presence of GSH (10 mM). Aliquots (100 μl) of each solution were taken at various times and diluted with 200 μl ice-cold buffer and kept on ice until HPLC analysis. To better separate TCVC sulfoxide diastereomers from GSH, the gradient for the previously described HPLC method was modified as follows: initial 10% B, at 4.5 min increased to 50% B over 3 min, at 11.5 min returned to 10% B over 3 min, end at 15 min. The retention times for TCVC sulfoxide diastereomers using this method were 9.5 and 10 min. The HPLC method for SAC sulfoxide was modified as no derivatization was needed due to the higher concentrations used in these studies. The method was 100% A isocratic for 15 min, using direct detection at 220 nm. Retention times for SAC sulfoxide diastereomers were 3.6 and 3.7 min. DCVC sulfoxide and SBC sulfoxide HPLC methods were essentially as described previously.

Aliquots (100 μl) of incubation mixtures were taken immediately after mixing the sulfoxides with GSH or buffer (time zero) and then at indicated times thereafter. Sulfoxide peak area in the presence of GSH was compared with sulfoxide peak area in buffer only at each time point (expressed as percent control). Half-lives were calculated by plotting log[% control] versus time.

Statistics. Data are expressed as mean ± standard deviation of the indicated number of individual rabbit livers. Statistical significance was determined by two-sample *t* test, or paired *t* test in the case of total versus solubilized microsomes, using $p < 0.05$ as the criterion for significance.

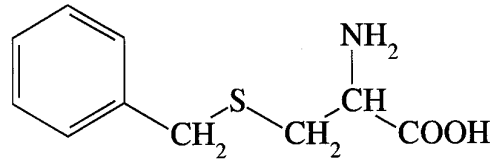
Results

Highly sensitive HPLC methods were developed for the separation and detection of four cysteine conjugates and their respective sulfoxide diastereomers (Fig. 1, Table 1). SBC, DCVC, and TCVC methods employed direct detection,

TABLE 1

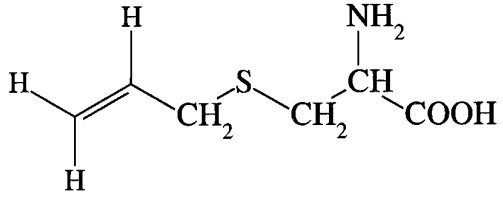
HPLC methods for detection of cysteine conjugates and sulfoxides

	Detection λ	Retention time		Sulfoxide detection limit
		Parent compound	Sulfoxide diastereomers	
	nm	*min*	*min*	*pmol/20 μl*
SBC	220	14.2	9.1, 9.4	110
SAC	360	14.5	13.1, 13.3	140
DCVC	220	11.6	4.6, 5.0	43
TCVC	225	11.4	4.5, 5.0	37



S-Benzyl-L-Cysteine

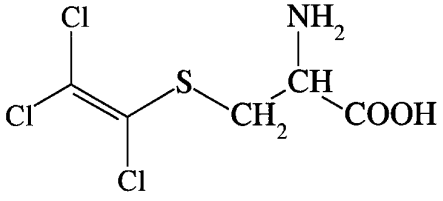
(SBC)



S-Allyl-L-Cysteine

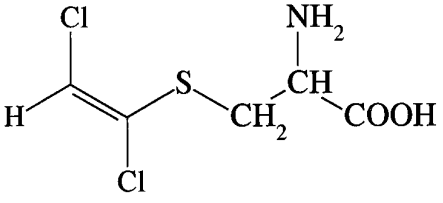
(SAC)

Fig. 1. Chemical structures of the four cysteine *S*-conjugates.



S-(1,2,2-Trichlorovinyl)-L-Cysteine

(TCVC)



S-(trans-1,2-Dichlorovinyl)-L-Cysteine

(DCVC)

whereas SAC, due to its high water solubility and low molar absorptivity, was derivatized with 2,4-dinitrofluorobenzene before analysis. Because the chiral identity of the sulfoxide diastereomers was not determined, the earlier eluting of the two diastereomers will be referred to as "diastereomer 1" and the later-eluting diastereomers will be referred to as "diastereomer 2."

Incubation of male rabbit liver microsomes with 10 mM SBC, SAC, DCVC, or TCVC at 37°, pH 7.4, produced time- and NADPH-dependent product peaks that coeluted with synthetic standard sulfoxides (Fig. 2); reactions were linear for at least 1 hr for SAC and SBC incubations and for at least 75 min for DCVC and TCVC incubations. Control reactions (lacking substrate or enzyme) were carried out in parallel and no peaks coeluting with sulfoxide standards were detected. Sulfoxidation in the absence of NADPH was negligible, except with SAC, in which a small amount of sulfoxide was formed during the derivatization process; SAC data were corrected for this nonenzymatic oxidation. UV scans of the products peaks of SBC, SAC, and DCVC reactions matched the UV scans of synthetic sulfoxides (data not shown). Attempts at obtaining a UV scan of the TCVC reaction product peak were unsuccessful due to the small amount of product formed.

The kinetics of *S*-oxidation of each of the four cysteine conjugates by rabbit liver microsomes were studied using double-reciprocal plots (Table 2). The apparent K_m values (mM) were 3.6, 4.8, 18, and 86 for SAC, TCVC, SBC, and DCVC, respectively. The fact that lower K_m enzymes might exist was considered; however, experiments carried out at lower substrate concentrations showed no evidence of lower K_m forms (data not shown). The V_{max}/K_m ratios for oxidation of SBC and SAC were an order of magnitude higher than the V_{max}/K_m ratios for DCVC and TCVC. All reactions were highly stereoselective; diastereomer 2 accounted for >95% of sulfoxide peak area in incubations with SBC, SAC, and DCVC. TCVC reactions exhibited more variability; diastereomer 2 comprises 65–100% of sulfoxide peak area.

To characterize the nature of the sulfoxidation reaction, effects of adding various scavengers of reactive oxygen species to microsomal incubations were investigated. Addition of superoxide dismutase, catalase, or the peroxidase inhibitor

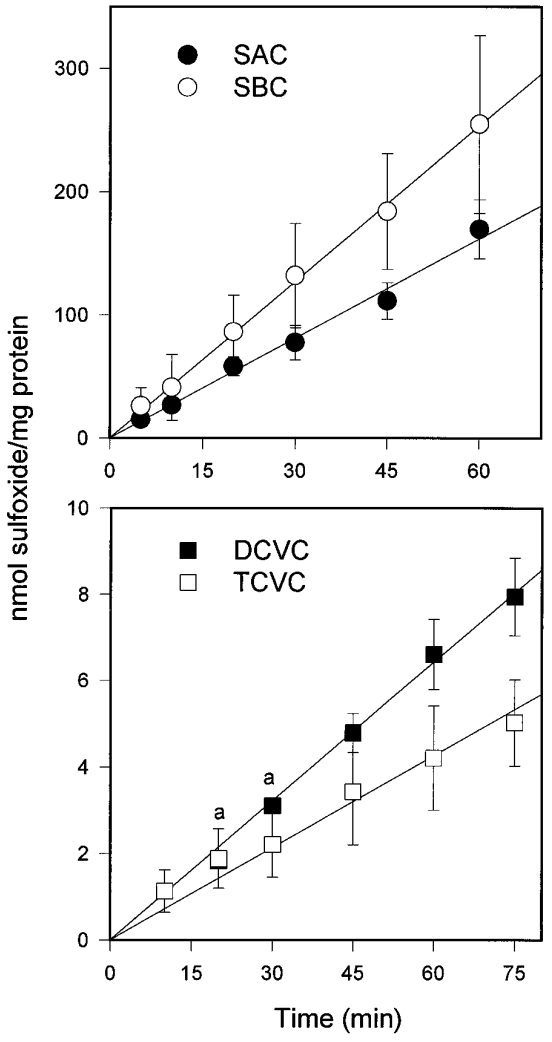


Fig. 2. Time courses for *S*-oxidation by rabbit liver microsomes. Incubations were carried out as described in Experimental Procedures, with 10 mM of each cysteine conjugate and 2 mM NADPH. Data are mean ± standard deviation of three separate experiments using three different rabbit livers. Only one data point was used because the other two replicates were below the limit of detection.

TABLE 2

Apparent kinetic constants for cysteine conjugate *S*-oxidation by rabbit liver microsomes

Kinetic constants were determined as described in Experimental Procedures using double-reciprocal plots. All r values > 0.98. Incubations contained 2 mM NADPH, 0.5–1.0 mg of rabbit liver microsomal protein, and the following concentrations of substrate: 1–10 mM for TCVC and SBC, 1–30 mM for SAC, and 10–30 mM for DCVC.

Data represent the mean of three experiments, except SAC, which is the mean of four experiments.

Substrate	V_{max}	K_m	V_{max}/K_m
	nmol/mg protein·min	*mM*	
SAC	3.0	3.6	0.84
SBC	6.6	18.0	0.37
TCVC	0.1	4.8	0.02
DCVC	0.7	86.0	0.01

potassium cyanide resulted in only small changes in levels of *S*-oxidation (within ±15% of control levels) for any of the four cysteine conjugates tested (data not shown).

The cysteine conjugate *S*-oxidations could potentially be mediated by cytochrome P450 enzymes or FMOs because both of these enzyme systems are microsomal, require NADPH, and are capable of oxidation of compounds containing a sulfur atom. To distinguish between P450s and FMOs, the cytochrome P450 inhibitor 1-benzylimidazole or the FMO alternate substrate methimazole was added to microsomal incubations (Table 3). SBC, SAC, and DCVC *S*-oxidations were not inhibited by 1-benzylimidazole, but methimazole inhibited *S*-oxidation by nearly 100% for SBC and DCVC and 80% for SAC. TCVC *S*-oxidation was inhibited by approximately 90% by both compounds. These data suggest that FMOs may be involved in SBC, SAC, and DCVC oxidations and that TCVC oxidation may be catalyzed by both FMOs and P450s.

To further investigate the nature of the sulfoxidation reactions, microsomes were solubilized with 0.5% Emulgen 911 detergent and assayed for activity of each of the four cysteine conjugates (Fig. 3). Solubilization removes the proteins from the microsomal membrane, a procedure that inactivates cytochrome P450s but does not inactivate FMOs (18, 19). The specific activity of DCVC *S*-oxidation remained approximately equal in total versus solubilized microsomes. SBC and SAC activity increased somewhat upon solubilization, although this increase was not statistically significant. Solubilization decreased TCVC activity to approximately 17% the activity of total microsomes. These results again suggest that FMOs may play the predominant role in *S*-oxidation of SBC, SAC, and DCVC and are partially involved in *S*-oxidation of TCVC.

Because results with inhibitors and detergent solubilization suggested that FMOs were involved in the *S*-oxidation of the four cysteine conjugates, the ability of recombinant FMOs to catalyze this reaction was examined. Before *S*-oxidase activities of the FMO isoforms were assessed, recovery experiments were performed to determine the stability of the four cysteine conjugates and their sulfoxides in the presence of *E. coli* membrane fractions. In contrast to the results with rabbit liver microsomes, in which recoveries of the four cysteine conjugates and their sulfoxides were nearly 100% (with the exception of DCVC sulfoxide and TCVC sulfoxide, which had recoveries of approximately 70%), recoveries in *E. coli* membrane fractions were lower, ranging from 50 to 80%. This lack of stability also was observed in incubations with vector controls and was not NADPH dependent. The most striking finding with the recovery experiments was that with

TABLE 3

Effects of the cytochrome P450 inhibitor, 1-benzylimidazole, and the FMO inhibitor, methimazole, on *S*-oxidation of SBC, SAC, DCVC, and TCVC by rabbit liver microsomes

Specific activity (nmol sulfoxide/mg protein·min) was determined by measuring NADPH (2 mM)-dependent conversion of the indicated substrates (10 mM) to the corresponding sulfoxides in the presence of male rabbit liver microsomes. Control activities were 2.78 ± 0.74, 2.99 ± 0.94, 0.10 ± 0.01, and 0.070 ± 0.03 for SBC, SAC, DCVC, and TCVC, respectively. Results are presented as mean ± standard deviation for three experiments using three different rabbits, except TCVC, which is the mean ± standard deviation of four experiments.

Treatment	Specific Activity			
	SBC	SAC	DCVC	TCVC
		%		
+1 mM 1-Benzylimidazole	122 ± 11	135 ± 49	88 ± 2.5	19 ± 25[a]
+1 mM Methimazole	0[a,b]	18 ± 5[a]	0[a,b]	6.8 ± 13[a]

[a] Significantly different from control; $p < 0.05$ using a two-sample t test.
[b] Sulfoxide levels were below the limit of detection for all three determinations.

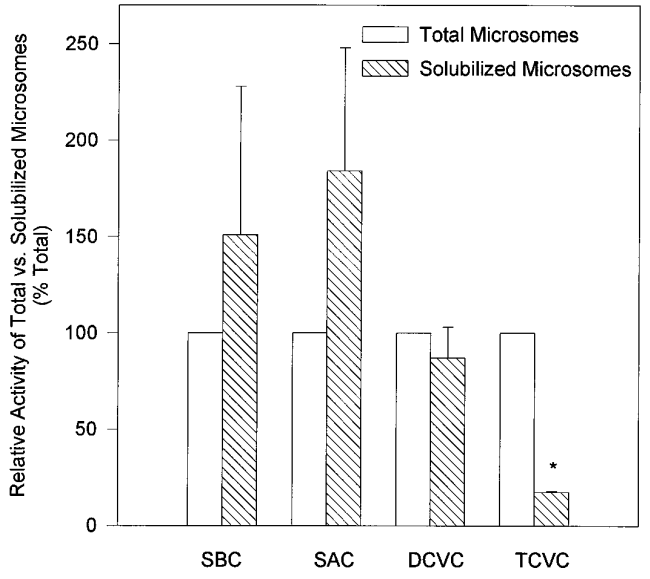


Fig. 3. *S*-Oxidation of cysteine conjugates by total rabbit liver microsomes versus microsomes solubilized with 0.5% Emulgen 911. Data are mean ± standard deviation relative to activity in total microsomes of four rabbit livers. Activity in total microsomes was 3.61 ± 2.0, 3.06 ± 0.28, 0.15 ± 0.03, and 0.07 ± 0.01 for SBC, SAC, DCVC, and TCVC, respectively. *, Significantly different from total microsomes, $p < 0.05$.

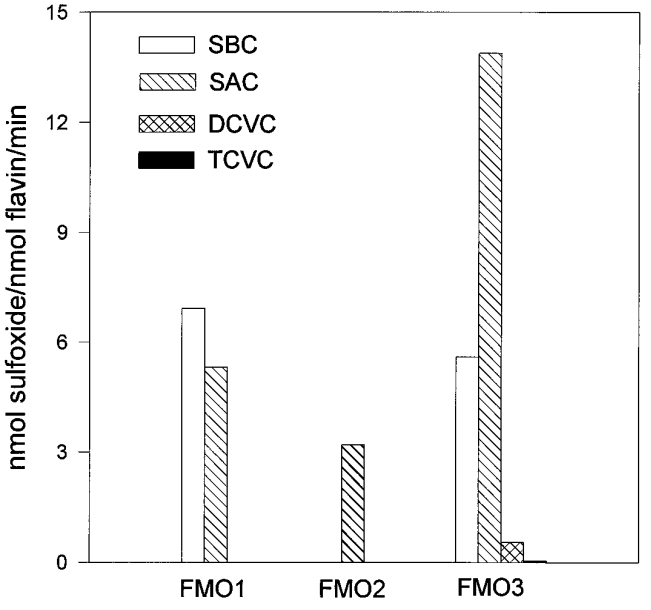


Fig. 4. Cysteine conjugate *S*-oxidase activities of *E. coli* membrane fractions containing cDNA-expressed rabbit FMOs. Incubations contained 10 mM substrate, 2 mM NADPH, 1 mM AOAA, and membrane fractions (0.2–1.0 nmol flavin). Reactions times were 20 min for SBC and SAC and 75 min for TCVC and DCVC. Results are the average of two separate experiments that varied by less than 20%. Stereoselectivity, the percentage of sulfoxide peak area comprising diastereomer 2, was >92% for all reactions.

all four cysteine conjugate sulfoxides, stability was markedly diastereospecific, that is, in all cases, there was nearly complete loss of diastereomer 1. Another observation of the recovery experiments was the strong thiol odor detected upon incubation of the parent cysteine conjugates. This suggested that the stability problems were caused by a β-lyase-type enzyme present in *E. coli* membrane fractions that was cleaving the cysteine conjugates to thiols. Indeed, addition of 1 mM AOAA, a general β-lyase inhibitor, to incubations restored recoveries to nearly 100% for all four cysteine conjugates and their sulfoxides. It also restored sulfoxide diasteromeric ratios to that of standards. Therefore, 1 mM AOAA was included in incubations with cDNA-expressed FMOs to ensure stability of reactants and products.

E. coli membrane fractions containing cDNA-expressed rabbit FMO isoforms 1, 2, 3, and 5 were assayed with 10 mM of each of the four cysteine conjugates (Fig. 4). cDNA-Expressed FMO1 catalyzed *S*-oxidation of SBC and SAC with specific activities (nmol sulfoxide/nmol flavin per min) of 6.9 and 5.3, respectively. With FMO2, *S*-oxidation was observed only with SAC, with specific activity of 3.2. Membrane fractions containing FMO3 catalyzed the *S*-oxidation of all four substrates. Specific activities for SAC and SBC with FMO3 were 13.9 and 5.9, respectively. Much lower specific activities were seen with DCVC and TCVC with specific activities of 0.55 and 0.04, respectively. No *S*-oxidation was observed with any of the cysteine conjugates with FMO5. All reactions were linear for at least 75 min (data not shown). Attempts at detecting DCVC and TCVC *S*-oxidation by FMOs 1,2, or 5 by increasing enzyme concentration in incubations still resulted in no detectable sulfoxidation. All reactions were highly stereoselective, and diastereomer 2 was detected almost exclusively. To determine whether AOAA had any effect on reactions other than improving stability of reactants and products, all reactions were also performed in the absence of AOAA. The results were nearly identical to those shown in Fig. 4, except that specific activities were slightly lower, probably due to the instability of the sulfoxides in *E. coli* membrane fractions.

Apparent kinetic constants for *S*-oxidation of SAC, DCVC, and TCVC were determined with recombinant FMOs; SBC kinetics had been determined previously with both purified rat liver FMO1 and FMO3 and cDNA-expressed rabbit FMOs (20, 21). These previous studies had shown that both FMO1 and FMO3 catalyzed *S*-oxidation of SBC and that FMO1 was the more efficient catalyst with a lower K_m value and a higher V_{max}/K_m ratio than FMO3 with both purified rat liver enzymes and recombinant FMOs. A summary of the kinetic constants for SAC, DCVC, and TCVC is shown in Table 4. SAC had a K_m value of 4.1 mM with FMO1, 45 mM with FMO2, and 5.5 mM with FMO3. However, FMO3 catalyzed the *S*-oxidation of SAC at a higher rate than FMO1 or FMO2, with a V_{max}/K_m ratio 2-fold higher than that of FMO1 and 16-fold higher than that of FMO2. The K_m value for DCVC and FMO3 was 54 mM and for TCVC was 7.7 mM, and the overall rates for these reactions were very low. The apparent kinetic constants and stereoselectivities determined with the cDNA-expressed FMOs were very similar to those determined in rabbit liver microsomes and support the role of FMOs in microsomal reactions.

Sulfoxidation of DCVC results in formation of an α,β-unsaturated compound, which is known to be a highly reactive Michael acceptor and nephrotoxin (14, 22). To explore whether sulfoxidation increases the reactivity of the other three conjugates, TCVC, SAC, SBC, and their respective sulfoxides were incubated with the biological nucleophile GSH and reactivities compared with those of DCVC and DCVC sulfoxide. Solutions of each compound (3 mM) were incubated at 37° in the presence and absence of 10 mM GSH

TABLE 4

Apparent kinetic constants for cysteine conjugate *S*-oxidation by cDNA-expressed rabbit FMOs

Kinetic constants were determined as described in Experimental Procedures using double-reciprocal plots, all r values > 0.98. Units for K_m are mM, units for V_{max} are nmol sulfoxide/nmol flavin·min. Incubations contained 2 mM NADPH, 1 mM AOAA, and the following concentrations of substrate: 5–30 mM SAC with FMO1, 1–20 mM SAC with FMO3, 10–50 mM DCVC, and 2.5–15 mM TCVC. N.D., TCVC and DCVC kinetics were not determined with FMO1 due to no detectable activity at 10 mM substrate. SAC-apparent kinetic constants with FMO2 were: K_m = 45.0, V_{max} = 9.5, and V_{max}/K_m = 0.21.

Substrate	FMO1			FMO3		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
SAC	4.1	6.1	1.5	5.5	18.2	3.3
DCVC	N.D.	N.D.	N.D.	53.6	3.0	0.06
TCVC	N.D.	N.D.	N.D.	7.7	0.06	0.01

(Fig. 5). Aliquots of the incubation mixtures were taken at various time points and analyzed by HPLC to monitor disappearance of cysteine conjugate, sulfoxide, and GSH peaks as well as formation of any potential adduct peaks. The sulfoxide peak areas in the presence of GSH were compared with sulfoxide peak areas in buffer alone at each indicated time point. DCVC sulfoxide was the most unstable of the compounds tested with a half-life in the presence of GSH of approximately 1 min. In fact, the reaction was so fast that there was significant loss of DCVC sulfoxide even at time zero, which was immediately after mixing the sulfoxide with GSH. Time-dependent formation of three peaks with retention times later than either DCVC sulfoxide or GSH were observed (data not shown), consistent with an earlier report of DCVC sulfoxide-GSH adduct peaks (14). No loss of DCVC sulfoxide was detected in the absence of GSH for the 5 min duration of the time course. In contrast to DCVC sulfoxide, the parent compound DCVC was stable for at least 24 hr at 37° in presence or absence of GSH.

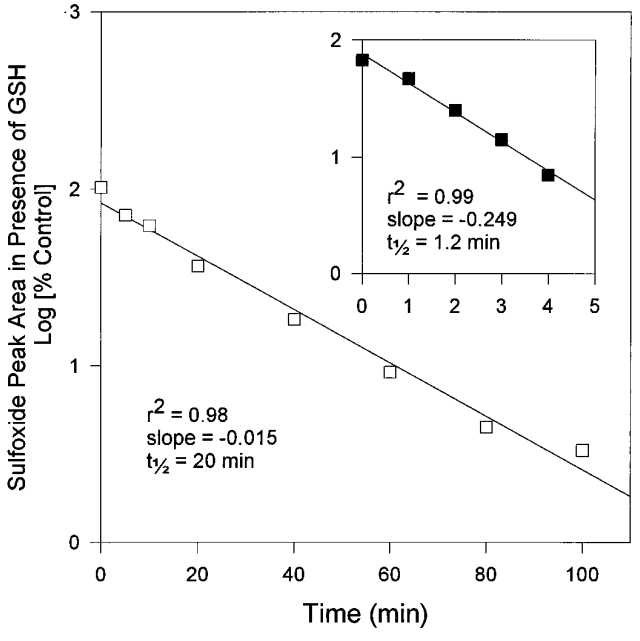


Fig. 5. Stability of sulfoxides in the presence of GSH. Solutions of each sulfoxide (3 mM) were incubated with GSH (10 mM) in buffer at 37°. Disappearance was monitored by HPLC and expressed as the log of percentage of sulfoxide in the absence of GSH. Experiments were conducted twice with nearly identical results. The half-lives of DCVC sulfoxide (■) and TCVC sulfoxide (□) in the presence of GSH were 1.2 min and 20 min, respectively. In the absence of GSH, TCVC sulfoxide decreased by 12% over the 100-min time course, and DCVC sulfoxide did not decrease over the 5-min time course. SBC sulfoxide and SAC sulfoxide were completely stable in the presence or absence of GSH for at least 24 hr (not shown).

TCVC sulfoxide was somewhat more stable than DCVC sulfoxide in the presence of GSH, with a half-life of 20 min. Time-dependent decreases in TCVC sulfoxide and GSH peaks were accompanied by time-dependent increases in two peaks with retention times intermediate to those of TCVC sulfoxide and GSH (data not shown). In the absence of GSH, TCVC sulfoxide was much more stable, with loss of 12% over the 100-min duration of the time course. The parent compound TCVC was stable for at least 24 hr in the presence or absence of GSH. These results indicate that the sulfoxides of DCVC and TCVC are capable of reacting with GSH, suggesting that sulfoxide formation of haloalkenyl *S*-conjugates could lead to formation of electrophilic metabolites. In contrast to DCVC sulfoxide and TCVC sulfoxide, SBC sulfoxide and SAC sulfoxide were very stable in the presence of GSH. No change in peak area of SBC, SAC, or their sulfoxides was seen for 24 hr in either the presence or absence of GSH.

Discussion

The results presented in this manuscript show that rabbit liver microsomes and cDNA-expressed FMOs catalyze the *S*-oxidation of SBC, SAC, DCVC, and TCVC. Although the formation of DCVC sulfoxide had been hypothesized previously based on the ability of DCVC to inhibit SBC *S*-oxidation in rat liver and kidney microsomes (6), this is the first report of detection of enzymatically produced DCVC sulfoxide, as well as TCVC sulfoxide and SAC sulfoxide.

Results with cDNA-expressed rabbit FMO isoforms showed that FMO3 catalyzed *S*-oxidation of all four cysteine conjugates, whereas FMO1 catalyzed only SAC and SBC *S*-oxidation, FMO2 catalyzed only SAC *S*-oxidation, and FMO5 was not active with any of the cysteine conjugates tested. No FMO4 was available for these studies. These results are somewhat surprising, as it is believed that the substrate-binding channel of FMO3 is far more restrictive than that of FMO1 (23, 24). However, the results presented here, considered with the fact that methionine is a better substrate for FMO3 than FMO1 (20, 21), suggest that FMO3 may be more selective than FMO1 for sulfoxidation of *S*-alkyl or *S*-alkenyl cysteine and homocysteine conjugates, whereas FMO1 may be more selective for *S*-benzyl conjugates and related compounds. The apparent K_m values for oxidation of SAC, DCVC, and TCVC determined with cDNA-expressed FMOs were similar to those determined with rabbit liver microsomes. Also, both microsomes and recombinant FMOs formed diastereomer 2 in great excess of diastereomer 1.

These data strongly support a role for FMOs in microsomal cysteine conjugate oxidation and, in particular, a role for FMO3 in alkenyl cysteine conjugate oxidation.

SAC and SBC were much better substrates for rabbit liver microsomes and for cDNA-expressed FMO1 and FMO3 than DCVC or TCVC. This is likely due to the fact that the sulfur of the allyl and benzyl compounds is more nucleophilic than the sulfur of the vinyl compounds, in agreement with the tendency for FMOs to oxidize only "soft" nucleophiles (25). DCVC, having a sulfur atom more nucleophilic than that of TCVC, would be expected to be oxidized more efficiently than TCVC for the same reason. With FMO3, SAC seemed to be a better substrate than SBC, and DCVC was better than TCVC. Although the results obtained with FMO3 suggest that nucleophilicity of the sulfur atom is a more important determinant of cysteine conjugate *S*-oxidation than lipophilicity, steric factors also may have affected the outcome of these experiments.

Although evidence presented here shows that SAC, SBC, and DCVC *S*-oxidations are mediated primarily by FMOs, *S*-oxidation of TCVC seems also to involve other microsomal enzymes. Recently, Werner *et al.* (26) have shown that the *N*-acetyl forms of TCVC and DCVC could be *S*-oxidized by rat liver microsomes and these authors provided evidence for cytochrome P450 form 3A in these oxidations. Although cytochrome P450 3A forms may be involved in *S*-oxidation of *N*-acetyl TCVC, further investigation is needed to determine the potential activity with TCVC.

The importance of *S*-oxidation to cysteine conjugate metabolism and disposition is unknown at this time. The finding that SAC is metabolized fairly efficiently to its sulfoxide by FMO3, and to a lesser extent by FMO1 and FMO2, is of interest because SAC, a component of garlic, has antiproliferative effects on some tumor cells (27, 28) and anticancer effects in animals (29, 30). The mechanism of SAC's anticancer effects is unknown and may involve SAC itself or a metabolite. DCVC and TCVC are potent nephrotoxins and mutagens (31, 32). Previously, DCVC sulfoxide had been shown to undergo rapid Michael addition with GSH *in vitro* and *in vivo* and DCVC sulfoxide administered to rats resulted in hepatic and renal GSH depletion (14). This indicates that *S*-oxidation is a bioactivation reaction rather than a detoxication reaction for DCVC. In the present studies, TCVC sulfoxide was found to react rapidly with GSH (Fig. 5), although not as rapidly as DCVC sulfoxide, whereas the parent compound TCVC was much more stable. This suggests that *S*-oxidation of TCVC also may represent a bioactivation reaction. The high reactivity of these sulfoxides with GSH suggest that *S*-oxidation may contribute to toxicity of these conjugates; however, the low *S*-oxidation rates seen with TCVC and DCVC and the high K_m value seen with DCVC suggest that *S*-oxidation is probably only a minor metabolic pathway for these compounds *in vivo*. Results with SBC and SAC sulfoxides suggest that these compounds do not react readily with nucleophiles. This indicates that reactivity of sulfoxides is dependent on chemical structure.

In conclusion, the data presented here show that enzymes in rabbit liver microsomes catalyze the *S*-oxidation of SAC, SBC, DCVC, and TCVC. Results with inhibitors, solubilization, and comparison of apparent kinetic constants and stereoselectivity of microsomal reactions with cDNA-expressed FMOs suggests that FMO3 and FMO1 are the main enzymes involved, with FMO3 being more selective for the alkenyl cysteine conjugates. The cysteine conjugates with more nucleophilic sulfur atoms were better substrates for FMOs, whereas lipophilicity did not seem to be a major factor in cysteine conjugate substrate selectivity. The α,β-unsaturated products, DCVC sulfoxide and TCVC sulfoxide, were much more reactive with GSH than the sulfoxides of SAC or SBC showing that reactivity of cysteine conjugate sulfoxides depends on chemical structure.

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