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Stereochemistry of the Microsomal Glutathione S-Transferase Catalyzed Addition of Glutathione to Chlorotrifluoroethene†

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ABSTRACT: The stereochemistry of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione formation was studied in rat liver cytosol, microsomes, *N*-ethylmaleimide-treated microsomes, 9000*g* supernatant fractions, purified rat liver microsomal glutathione S-transferase, and isolated rat hepatocytes. The absolute configuration of the chiral center generated by the addition of glutathione to chlorotrifluoroethene was determined by degradation of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione to chlorofluoroacetic acid, followed by derivatization to form the diastereomeric amides *N*-(*S*)-α-methylbenzyl-(*S*)-chlorofluoroacetamide and *N*-(*S*)-α-methylbenzyl-(*R*)-chlorofluoroacetamide, which were separated by gas chromatography. Native and *N*-ethylmaleimide-treated rat liver microsomes, purified rat liver microsomal glutathione S-transferase, rat liver 9000*g* supernatant, and isolated rat hepatocytes catalyzed the formation of 75-81% (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione; rat liver cytosol catalyzed the formation of equal amounts of (2*R*)- and (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione. In rat hepatocytes, microsomal glutathione S-transferase catalyzed the formation of 83% of the total *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione formed. These observations show that the microsomal glutathione S-transferase catalyzes the first step in the intracellular, glutathione-dependent bioactivation of the nephrotoxin chlorotrifluoroethene.

Glutathione S-transferases (EC 2.5.1.18) catalyze the addition of glutathione to electrophilic substrates. Glutathione S-conjugate formation in the liver, followed by conversion to mercapturates, serves to detoxify potentially harmful xenobiotics. Alternatively, glutathione S-conjugate formation is an important bioactivation mechanism for several classes of compounds (Anders, 1990; Vamvakas & Anders, 1990). The nephrotoxicity of several haloalkenes is attributable to hepatic glutathione S-conjugate formation, metabolism of the gluta-

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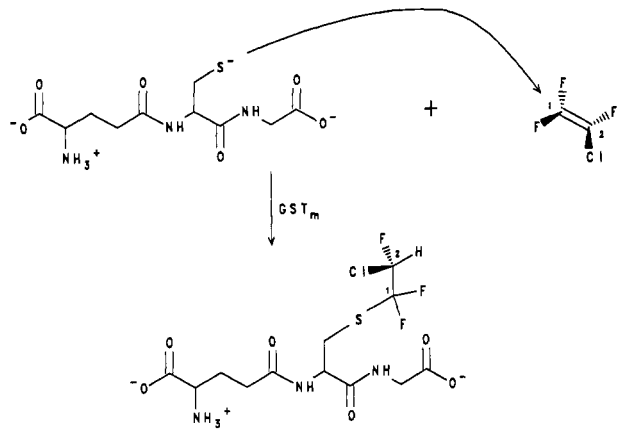

FIGURE 1: Metabolism of chlorotrifluoroethene to (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione. GST_m, microsomal glutathione S-transferase.

thione S-conjugates to the corresponding cysteine S-conjugates, translocation to the kidney, and bioactivation of the cysteine S-conjugates by cysteine conjugate β-lyase (Anders et al., 1988; Dekant et al., 1989). Chlorotrifluoroethene is a potent nephrotoxin that undergoes bioactivation by the β-lyase pathway. The glutathione S-transferase catalyzed regiospecific addition of glutathione to chlorotrifluoroethene yields *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione (CTFG)[1] (Dohn et al., 1985a). Both CTFG and the corresponding cysteine S-conjugate *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine are nephrotoxic in vivo and cytotoxic in vitro (Dohn et al., 1985b). The cysteine conjugate β-lyase dependent bioactivation of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine yields 2-chloro-1,1,2-trifluoroethanethiol, which loses HF to afford the acylating agent chlorofluorothionoacetyl fluoride (Dekant et al., 1987). The nephrotoxicity of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine is associated with the thionoacylation of cellular macromolecules by chlorofluorothionoacetyl fluoride (Dohn et al. 1985b).

The glutathione S-transferase catalyzed addition of glutathione to chlorotrifluoroethene results in the formation of a new chiral center in CTFG, and the reaction is under stereochemical control (Dohn et al., 1985a) (Figure 1). The GST_m-catalyzed formation of CTFG generates one diastereomer of CTFG preferentially, whereas the GST_c-catalyzed reaction yields a 1:1 mixture of diastereomers (Dohn et al., 1985a).

The objectives of the present study were to determine the absolute configuration of the new chiral center in CTFG, to develop a method for quantification of CTFG diastereomer formation in subcellular fractions, in hepatocytes, and by purified GST_m, and to determine the relative contributions of GST_c and GST_m to CTFG formation in isolated rat hepatocytes. To achieve the last objective, we have exploited the observation that CTFG of different diastereomeric composition is produced by GST_m and GST_c to enable the quantification of the relative contributions of GST_c and GST_m to intracellular S-conjugate formation.

[1] Abbreviations: CTFG, *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione; GST, glutathione S-transferase; GST_c, cytosolic glutathione S-transferases; GST_m, microsomal glutathione S-transferase; (2*S*)-CTFG, (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione, and (2*R*)-CTFG, (2*R*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione, where 2*S* and 2*R* refer to the absolute configuration of the chiral center at carbon 2 of the *S*-(2-chloro-1,1,2-trifluoroethyl) group in *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione.

Materials and Methods

Male Long-Evans rats were obtained from Charles River (Charleston, SC). Ethyl chlorofluoroacetate was obtained from PCR Inc. (Gainesville, FL), and chlorotrifluoroethene was purchased from Mathison Gas Products (Buffalo, NY). All other chemical reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). γ-Glutamyltranspeptidase (type I) was obtained from Sigma (St. Louis, MO). *N*-Dodecylpyridoxal bromide was prepared by the method of Kondo et al. (1985). GST_m was purified by the method of Morgenstern et al. (1982). Microsomes were activated with *N*-ethylmaleimide as previously described (Morgenstern et al., 1982). A Bruker 270-MHz NMR spectrometer operating at 270.13 MHz was used to record ^1H NMR spectra; ^1H chemical shifts are expressed in parts per million downfield from tetramethylsilane. Gas chromatography/mass spectrometry (GC/MS) analyses were performed with a Hewlett-Packard 5970 mass selective detector coupled to a Hewlett-Packard 5880A gas chromatograph fitted with a 25 m × 0.2 mm × 0.5 μm film thickness HP-1 cross-linked methylsilicone gum column; an injection splitter was used, and the injector, interface, and column temperatures were 200, 250, and 120 °C, respectively.

Resolution of Chlorofluoroacetic Acid. (*R,S*)-Chlorofluoroacetic acid was resolved according to the method of Bellucci et al. (1969). Melting points and optical rotations were in agreement with literature values. The optical purity of the resolved acids was determined by derivatization with (*S*)-(−)-α-methylbenzylamine and resolution of the diastereomeric amides by gas chromatography.

Synthesis of N-(S)-α-Methylbenzyl-(R,S)-chlorofluoroacetamide. *N*-(*S*)-α-Methylbenzyl-(*R,S*)-chlorofluoroacetamide was prepared by reacting 1 equiv of (*S*)-(−)-α-methylbenzylamine, 0.5 equiv of (*R,S*)-chlorofluoroacetic acid, 1 equiv of 1,3-dicyclohexylcarbodiimide, and 1.3 equiv of 1-hydroxybenzotriazole hydrate in 5 mL of dry tetrahydrofuran at 0 °C for 12 h. The solvent was removed in vacuo, and the residue was suspended in water. The suspension was washed twice with diethyl ether; the organic layer was washed successively with 1 N HCl, saturated $NaHCO_3$, and saturated NaCl and then dried over anhydrous Na_2SO_4 and filtered. The white, crystalline product melted at 55–56 °C; ^1H NMR ($CDCl_3$) δ 7.4 (m, 5 H), 6.5 (s, 1 H), 6.3 (d, J_{H-F} = 50 Hz, 1 H), diastereomer is shifted upfield 0.03 ppm, 5.2 (m, 1 H), 1.5 (m, 3 H). GC/MS analysis of the diastereomeric amide showed two peaks with retention times of 21.6 and 22.3 min, which showed identical mass spectra: MS *m/z* (relative abundance) 215 (28%, M^+), 180 (100%, M − Cl), 105 (79%, $M-CHCH_3C_6H_5$).

Degradation of CTFG to Chlorofluoroacetic Acid. CTFG was suspended in 2.5 mL of phosphate buffer (100 mM, pH 8.5). γ-Glutamyltranspeptidase (10 units, type I) and 30 mg of glycylglycine were added, and the mixture was allowed to react for 12 h at room temperature; the mixture was then lyophilized. The degradation product *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine was purified by preparative HPLC on a Whatman ODS column with 10% acetonitrile/90% water containing 0.1% trifluoroacetic acid as the mobile phase.

Degradation of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine to chlorofluoroacetic acid was accomplished by suspending all of the *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine isolated by HPLC in 5 mL of phosphate buffer (100 mM, pH 8.5); 20 μL of cetyltrimethylammonium chloride and 1 mg of *N*-dodecylpyridoxal bromide were added, and the mixture was allowed to react for 2 h at 37 °C. *N*-Dodecylpyridoxal

bromide catalyzes β-elimination reactions with β-substituted amino acids (Kondo et al., 1985). Treatment of *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine with *N*-dodecylpyridoxal bromide gives 2-chloro-1,1,2-trifluoroethanethiol, which loses HF to afford chlorofluorothionoacetyl fluoride; hydrolysis of the thionoacyl fluoride gives chlorofluoroacetic acid (Dekant et al., 1987).

Chlorofluoroacetic acid was derivatized by a modification of the method of Ozawa and Tsukioka (1987). The entire reaction mixture from the previous step was acidified to pH 2 with concentrated HCl, and solid KCl was added to make a 2% (w/v) solution. A solution of 0.1 mmol of (*S*)-(−)-α-methylbenzylamine and 0.25 mmol of 1,3-dicyclohexylcarbodiimide in 1 mL of ethyl acetate was mixed with the chlorofluoroacetic acid containing solution, and 0.75 mmol of 1-hydroxybenzotriazole and an additional 3 mL of ethyl acetate were added. The mixture was allowed to react for 12 h at room temperature. Solid KCl was added to a final concentration of 10% (w/v), and the solution was extracted twice with ethyl acetate. The organic layer was washed successively with 1 N HCl, saturated $NaHCO_3$, and saturated NaCl and then dried over anhydrous Na_2SO_4. The resulting brown liquid was analyzed by GC/MS.

Biosynthesis of CTFG. Biosynthetic CTFG from liver subcellular fractions was prepared as previously described (Dohn et al., 1985a). CTFG was prepared with purified GST_m as the catalyst by incubation of 460 mg of glutathione and 5 mL of enzyme solution (180 units with 1-chloro-2,4-dinitrobenzene as the substrate) in 100 mL of 100 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA in a 1-L filter flask fitted with a septum and a large balloon. Chlorotrifluoroethene gas (200 mL, room temperature) was admitted via the septum, and the mixture was incubated for 2 h at 37 °C. After incubation, the pH of the mixture was adjusted to 2 with trifluoroacetic acid, and precipitated protein was removed by centrifugation. The supernatant was lyophilized, and CTFG was purified as described above. Hepatocytes were isolated by the method of Moldéus et al. (1978) and incubated (10^6 cells/mL) at 37 °C in a 1-L filter flask fitted with a septum and a large balloon. After the flask was flushed with 95% O_2/5% CO_2, approximately 200 mL of chlorotrifluoroethene (at room temperature) was added, and the mixture was incubated for 75 min. Ethanol (50% final concentration) was added to precipitate proteins. Samples were lyophilized and then purified as previously described (Dohn et al., 1985a).

Statistical Analyses. Statistical significance was assessed by Student's *t*-test or analysis of variance. A level of $p < 0.05$ was chosen for acceptance or rejection of the null hypothesis.

Results

The first experimental objective was to determine the absolute configuration of the predominant diastereomer of the new chiral center in the 2-chloro-1,1,2-trifluoroethyl group of CTFG produced by GST_m catalysis and to develop a method for quantification of the diastereomeric composition of CTFG. This method was then employed to study the stereochemistry of CTFG formation in isolated hepatocytes and in subcellular fractions. Attempts to resolve the methyl esters of diastereomeric (*R,S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine by GC/MS failed. Furthermore, ^{19}F NMR analysis of CTFG did not provide a sufficiently accurate determination of the relative quantities of diastereomers.

The objective was accomplished by degrading CTFG to chlorofluoroacetic acid, whose absolute configuration is known (Bellucci et al., 1969) (Figure 2). (*R,S*)-Chlorofluoroacetic acid was resolved with (+)-dehydroabeitylamine (Bellucci et al., 1969), and the enantiomers were derivatized with (*S*)-(−)-α-methylbenzylamine to form the diastereomeric amides *N*-(*S*)-α-methylbenzyl-(*R*)-chlorofluroacetamide and *N*-(*S*)-α-methylbenzyl-(*S*)-chlorofluoroacetamide (Figure 2). Baseline resolution of the diastereomers was obtained by GC/MS; peaks on the chromatogram were identified as *N*-(*S*)-α-methylbenzyl-(*R*)-chlorofluoroacetamide (21.6 min) and *N*-(*S*)-α-methylbenzyl-(*S*)-chlorofluoroacetamide (22.3 min) (Figure 3).

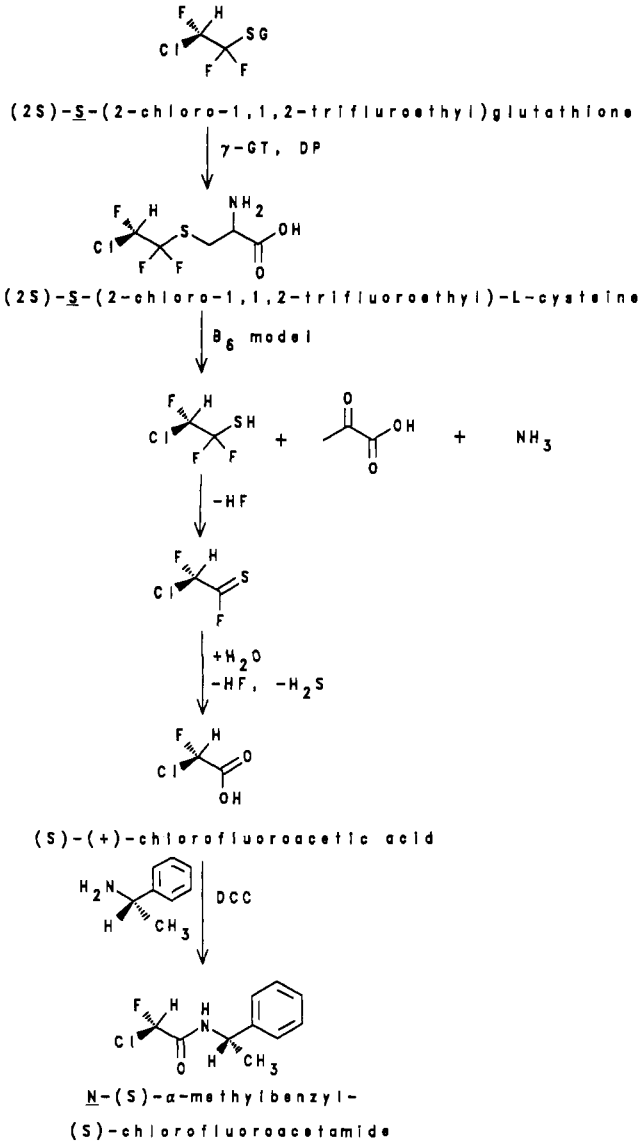

FIGURE 2: Degradation of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione to chlorofluoroacetic acid, followed by derivatization with *S*-(−)-α-methylbenzylamine. γ-GT, γ-glutamyltransferase; DP, dipeptidases; B_6 model, *N*-dodecylpyridoxal in cetyltrimethylammonium chloride; DCC, 1,3-dicyclohexylcarbodiimide.

Degradation of CTFG formed by hepatic microsomal fractions to chlorofluoroacetic acid, followed by derivatization and GC/MS analysis showed that microsomes catalyzed the formation of 81% (2*S*)-CTFG, whereas cytosol yielded (2*R*)- and (2*S*)-CTFG in a 1:1 ratio; chemically synthesized CTFG also yields equal amounts of the diastereomers (Table I).

To investigate the possibility that the observed excess of (2*S*)-CTFG may be due to the selective degradation of (2*R*)-CTFG, microsomes were incubated with chemically synthesized (2*R,S*)-CTFG, which was then isolated, degraded, and derivatized; no selective loss of (2*R*)-CTFG was observed (data not shown). The degradation and derivatization pro-

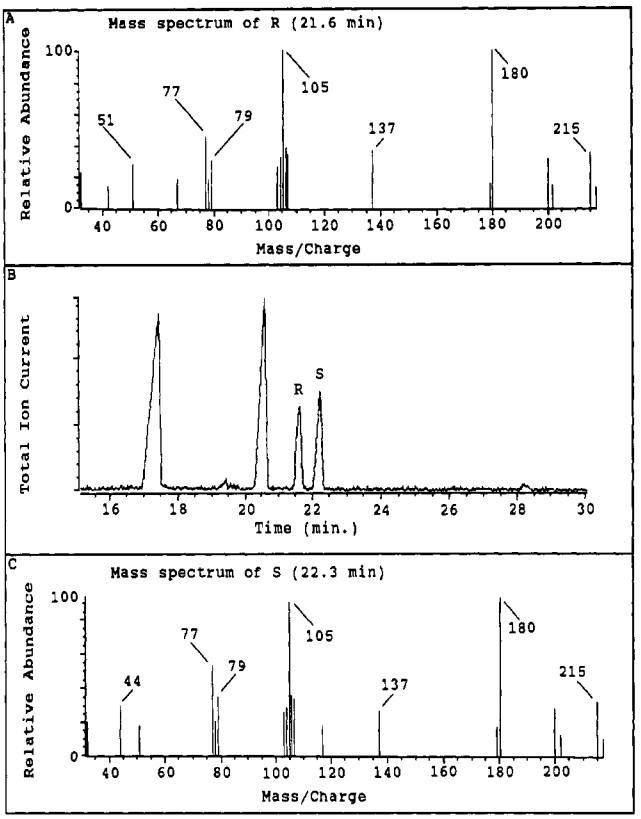

FIGURE 3: Gas chromatographic analysis of *N*-(*S*)-α-methylbenzyl-(*R*)-chlorofluoroacetamide (21.6 min) and *N*-(*S*)-α-methylbenzyl-(*S*)-chlorofluoroacetamide (22.3 min) (panel B); the mass spectra of the respective diastereomers are shown in panels A and C.

Table I: Diastereomeric Composition of *S*-(2-Chloro-1,1,2-trifluoroethyl)glutathione (CTFG) Formed by Various GST Preparations[a]

preparation	(2*S*)-CTFG produced (%)
synthetic	51 ± 1[b]
cytosol	45 ± 1[b]
microsomes	81 ± 1[c]
9000*g* supernatant	79 ± 4[c]
purified GST_m	77 ± 3[c]
N-ethylmaleimide-activated microsomes	79
hepatocytes	75 ± 1[c]

[a] Data are shown as mean ± SEM; $n = 3$. For *N*-ethylmaleimide-activated microsomes, the value shown is the mean of two experiments. [b,c] Values with the same superscripts do not differ significantly ($p > 0.05$), and values with different superscripts are significantly different ($p < 0.05$).

cedures did not change the stereochemical composition of chemically synthesized CTFG (data not shown).

Studies with purified GST_m and with *N*-ethylmaleimide-activated GST_m (Morgenstern et al., 1982) were conducted to determine whether association of the enzyme with microsomal membranes or activation of the enzyme altered the stereochemical course of the reaction. The diastereomeric composition of CTFG formation catalyzed by purified GST_m and by *N*-ethylmaleimide-activated GST_m was the same as that formed by native microsomes (Table I).

Isolated rat hepatocytes were incubated with chlorotrifluoroethene to investigate the diastereomeric composition of CTFG formed in cells. GC/MS analysis showed that 75% (2*S*)-CTFG was formed in hepatocytes (Table I). The initial viability of all hepatocyte preparations was ≥95%; at the end of incubation time, the viability of chlorotrifluoroethene-exposed hepatocytes was about 75%, whereas untreated hepatocytes were ≥85% viable at 75 min. To determine whether GST_c or GST_m released from nonviable cells contributed to CTFG formation, cells (10^6/mL) were lysed by freezing and thawing three times and were then incubated with chlorotrifluoroethene; CTFG formation was not detectable by HPLC, presumably because the glutathione concentration became too dilute to support detectable, extracellular S-conjugate formation.

CTFG formation was also studied in rat liver 9000*g* supernatant fractions, which contain both GST_c and GST_m. The ratio of diastereomers formed by rat 9000*g* supernatant was the same as that found in isolated hepatocytes (Table I).

Discussion

Glutathione S-transferases catalyze nucleophilic vinylic substitution reactions (S_NV) between glutathione and electrophilic alkenes (Boyland & Chasseaud, 1967; Chasseaud, 1979; Keen & Jakoby, 1978). The mechanism of S_NV reactions has been elucidated (Bernasconi, 1989; Modena, 1971; Rappoport, 1985). Briefly, S_NV reactions proceed via addition–elimination routes with alkenes with good nucleofuges (leaving groups) or via addition routes with alkenes with poor nucleofuges; the metabolism of hexachloro-1,3-butadiene to *S*-(1,2,3,4,4-pentachlorobutadienyl)glutathione (Dekant et al., 1988) and of chlorotrifluoroethene to CTFG (Dohn et al., 1985a) provide respective examples of these routes. The formation of glutathione S-conjugates of haloalkenes is the first step in the bioactivation of nephrotoxic haloalkenes via the cysteine conjugate β-lyase pathway (Anders et al., 1988, 1990), and the nephrotoxicity of haloalkenes is related to the rate of S-conjugate formation (Anders et al., 1990). Chlorotrifluoroethene is a prochiral substrate, and CTFG formation catalyzed by hepatic GST_m and GST_c results in the formation of a new chiral center. An excess of one diastereomer is produced with GST_m, but not with GST_c, as the catalyst (Dohn et al., 1985a). This phenomenon was exploited to study the relative contributions of GST_m and GST_c to CTFG formation by subcellular fractions, by purified GST_m, and in rat hepatocytes.

The results reported herein show that intracellular CTFG formation is catalyzed preferentially by GST_m and that GST_c makes a modest contribution to CTFG formation in isolated cells. The results of this study illustrate the importance of GST_m-catalyzed glutathione conjugation in vivo with highly lipophilic substrates; GST_m plays a dominant role in CTFG formation in rat hepatocytes and perhaps in vivo and with other haloalkenes. Although chlorotrifluoroethene has the opportunity to react with GST_c in hepatocytes, it is possible that the lipophilic nature of CTFE results in its rapid partitioning to the endoplasmic reticulum, where it is preferentially available to GST_m. The observation that there was no difference in the diastereomeric composition of CTFG formed by purified GST_m, by either activated or native microsomes, or in isolated hepatocytes and 9000*g* supernatant indicates that the observed stereoselectivity is not a function of cellular architecture but is determined only by substrate binding and attack at the active site of GST_m.

The observed stereoselectivity of CTFG formation by GST_m indicates that the active-site topography or chemical mechanisms, or both, of GST_m and GST_c differ. In general, the detoxication enzymes are noted for their lack of substrate specificity and accept many substrates for processing at relatively low catalytic efficiencies, without requiring a unique enzyme or isoform for each substrate (Armstrong, 1988). Both GST_c and GST_m exhibit stereoselectivity toward prochiral

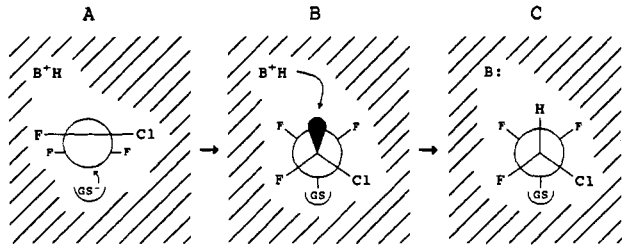

FIGURE 4: Schematic representation of the microsomal glutathione S-transferase catalyzed formation of (2*S*)-*S*-(2-chloro-1,1,2-trifluoroethyl)glutathione. The figure shows the binding of chlorotrifluoroethene to the active site of GST_m so as to facilitate the regiospecific attack of glutathione on the *si* face of the substrate (panel A), development and protonation of the incipient carbanion (panel B), and product before release from the enzyme (panel C). Although a stepwise reaction is shown, it is not known whether the enzymatic reaction is stepwise or concerted.

substrates (Cobb et al, 1983; Kubo & Armstrong, 1989). The possibility that individual isozymes of GST_c catalyze diastereoselective CTFG formation was not addressed in the present study.

Although GST_c metabolizes chiral substrates at different rates (Armstrong, 1987; Cobb et al., 1983; Mangold & Abdel-Monem, 1983; Ridgewell and Abdel-Monem, 1986; Te Koppele et al., 1988) and catalyzes the stereoselective formation of glutathione conjugates of para-substituted 4-phenyl-3-buten-2-ones (Kubo and Armstrong, 1989), the selective formation of (2*S*)-CTFG is the only reported example of diastereoselective glutathione conjugate formation catalyzed by GST_m (Dohn et al., 1985a).

The observed formation of an excess of the diastereomeric product (2*S*)-CTFG by GST_m reflects a difference in the free energies of activation for the two diastereomeric transition states formed during the addition of glutathione to chlorotrifluoroethene. Although the available data do not allow an explanation of the differences between the two transition states, some contributing factors can be noted: One, the orientation of chlorotrifluoroethene at the active site of the enzyme must favor preferential attack of glutathione from the *si* face at the carbon bearing two fluorines. Two, the enzyme must facilitate protonation of the incipient carbanion before inversion of the carbanion occurs. The regiospecific and stereoselective formation of (2*S*)-CTFG by GST_m is shown schematically in Figure 4.

The catalytic mechanism and active-site topography of GST_m have apparently not been elaborated, and such information is necessary for a detailed understanding of the stereochemistry of GST_m-catalyzed reactions.

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