Structural and Functional Consequences of Haloenol Lactone Inactivation of Murine and Human Glutathione *S*-Transferase†

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ABSTRACT: Mass spectrometric analysis of proteolysis products of haloenol lactone-modified glutathione *S*-transferase isozyme mGSTP1 indicates that the haloenol lactone 3-cinnamyl-5(*E*)-bromomethylidene-tetrahydro-2-furanone is covalently attached to the protein at Cys-47. Comparisons of the extent of adduct formation with losses in enzymatic activity indicate that mGSTP1 exhibits greatest reactivity toward the haloenol lactone, followed by mGSTM1 and mGSTA3. Activities of mGSTP1 and mGSTM1 decrease in inverse proportion to haloenol lactone concentration, whereas modification had no apparent effect on catalytic activity of mGSTA3. Decreases in activity agree with the extent of protein modification observed in ESI mass spectra for mGSTP1 and mGSTM1 but not for mGSTA3. Kinetic studies employing recombinant human proteins with replacement of cysteine by serine at Cys-47 and Cys-101 indicate that rapid inactivation ($t_{1/2} = 2$ min) occurs only when residue 47 is cysteine. Mass spectra of C47S-hGSTP1 incubated with haloenol lactone demonstrate covalent attachment of a haloenol lactone−glutathione conjugate and suggest that an ester forms between the lactone and Ser-47. Therefore, we propose that initial opening of the lactone ring is promoted by Cys-47 through thioester formation between the lactone carbonyl and the Cys-47 sulfhydryl. Enol−keto tautomerization and enzyme-mediated hydrolytic cleavage of the thioester produces a reactive α-bromoketone which reacts a second time with Cys-47 and inactivates the enzyme. These results suggest that Pi class GSTs have thioesterase activity and that haloenol lactone inactivation occurs through an enzyme-mediated process.

Drug resistance in tumors often limits the effectiveness of cytostatic drugs in cancer treatment *(1).* Multidrug resistance arises from numerous factors including altered expression of bioactivating and detoxifying metabolic enzymes, active export of reactive intermediates from target cells, and repair of modified DNA *(1−5).* Many drug-resistant tumor cells exhibit striking overexpression of glutathione *S*-transferases (GSTs,[1] E.C. 2.5.1.18; for review, see ref *3*). GSTs make up a family of enzymes that catalyze detoxification of electrophilic xenobiotics. Cytosolic GSTs are encoded by six distantly related gene families designated as classes Alpha, Pi, Mu, Theta, Kappa, and Sigma based on their chemical properties, immuno-cross-reactivity, and amino acid sequence homology (*6*). GST isozymes constitute 3−8% of cytosolic proteins in mammalian liver.

Correlations between expression of specific GST isozymes and resistance to chemotherapeutic drugs including adriamycin, chlorambucil, and nitrogen mustards are well established *(2, 7−9).* It has been proposed that increased levels of GST confer resistance through increased capacity to conjugate glutathione (GSH) to reactive intermediates of prodrugs formed in vivo *(10).* Malignancies including carcinoma of breast, colon, lung, kidney, and ovary demonstrate elevated levels of the Pi class GST hGSTP1 *(11−15).* Measurement of hGSTP1 levels within tumors has been proposed as a diagnostic indicator of the progression of cancer with potential clinical value in treating certain malignancies *(16−18).* Although increased levels of hGSTP1 have been found in many human cancers, other factors contribute to multidrug resistance, as some drug-resistant carcinomas do not exhibit overexpression of Pi class GST *(2, 19).*

Successful cancer therapy is based on the toxicity of cancer drugs being greater in tumors than in other tissues, and such selectivity may be enhanced by inhibiting detoxification in tumor cells. The diuretic drug ethacrynic acid and its GSH conjugate are competitive inhibitors of GSTs *(20),* and ethacrynic acid enhances cytotoxicity of several alkylating agents in cultured drug-resistant tumor cells *(21, 22).* Selective inhibition of GST isoenzymes overexpressed in tumor cells offers the prospect of lower effective doses of chemotherapeutic agents and has stimulated interest in

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[1] Abbreviations: GST, glutathione *S*-transferase; GSH, glutathione; haloenol lactone, 3-cinnamyl-5(*E*)-bromomethylidenetetrahydro-2-furanone; HPLC, high-performance liquid chromatography; ESI, electrospray ionization; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; CDNB, 1-chloro-2,4-dinitrobenzene; ACN, acetonitrile; TFA, trifluoroacetic acid; CNBr, cyanogen bromide.

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development of specific and potent GST inhibitors *(23, 24)*. A promising approach for improving specificity of enzyme inactivation resides in enzyme-activated irreversible inhibitors (mechanism-based inhibitors). Mechanism-based inhibitors are substrate analogues that employ the catalytic mechanism of a target enzyme to generate reactive electrophiles which inactivate the enzyme by covalent attachment *(25)*. Mechanism-based inhibitors exhibit great potency owing to high binding affinity of target enzyme for substrate analogues.

Although the catalytic mechanism of Pi class GST is still a subject of study, spectroscopic studies revealed that the cosubstrate GSH exists as a thiolate anion in the active site of the protein *(26)*. In addition, sulfhydryl groups of human Pi class GST are sensitive to alkylating reagents such as iodoacetamide *(27)*. The Pi class GSTs from mammalian species have three highly conserved cysteine residues (Cys-14, Cys-47, and Cys-169), and a fourth cysteine residue (Cys-101) is conserved in all studied mammalian species but mouse *(28)*. Site-directed mutagenesis studies demonstrated that none of these residues are essential for catalysis; however, replacement of Cys-47 or Cys-14 with serine reduces catalytic efficiency *(29, 30)*. The sulfhydryl group of Cys-47 is unusually acidic and has a pK_a of 4.2 *(31)*. On the basis of these observations and studies of Daniels et al. describing mechanism-based inactivation of α-chymotrypsin by haloenol lactone derivatives *(32)*, we postulated that either the thiolate anion of GSH or the reactive sulfhydryl of Cys-47 would be capable of activating haloenol lactone derivatives and would produce enzyme inactivation through formation of either a GSH−haloketone conjugate or a protein−haloketone adduct.

In earlier studies, we demonstrated that a novel haloenol lactone (3-cinnamyl-5(*E*)-bromomethylidenetetrahydro-2-furanone) is a competitive, time-dependent inhibitor of murine mGSTA3, mGSTP1, and mGSTM3, displaying greatest reactivity toward mGSTP1 *(33)*. Time-dependent inhibition studies indicated that 50% inactivation of mGSTP1 was achieved in less than 3 min. In contrast, mGSTM1 and mGSTA3 isozymes were less sensitive to inactivation by the haloenol lactone, and only 60% and 70% enzyme inhibition was achieved, respectively, after 15-min incubations with the haloenol lactone *(33)*. Electrospray ionization (ESI) mass spectra obtained for mGSTP1 incubated with 5 molar equiv of haloenol lactone indicated that inactivation was due to a covalent modification which produced an increase in molecular weight of 230 Da.

The objectives of the present study were to identify the site of haloenol lactone adduct formation, to determine whether haloenol lactone inactivation of mGSTA3 and mGSTM1 is due to covalent modification, and to ascertain the role of reactive Cys-47 side chains as determinants of the specificity of GST inactivation.

EXPERIMENTAL PROCEDURES

Chemicals. The haloenol lactone derivative (3-cinnamyl-5(*E*)-bromomethylidenetetrahydro-2-furanone) was synthesized as described earlier *(33)*. Acetonitrile (ACN, Optima grade) was purchased from Fisher Scientific Corp. (Fair Lawn, NJ). GSH−agarose (attached through sulfur to epoxide-activated 4% cross-linked beaded agarose) was obtained from Sigma Chemical Co. (St. Louis, MO). Dye reagent for protein assays was purchased from Bio-Rad (Richmond, CA). All other chemicals used were of analytical grade.

Animals. Male CD-1 mice, 25−30 g, were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Animals were housed on autoclaved pine shavings in approved animal care facilities and fed Purina rodent chow ad libitum for 1 week. Mice were killed by cervical dislocation, and livers were removed and minced in 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 1 mM each EDTA and DTT. Minced livers were immediately frozen under liquid nitrogen and stored at −80 °C until GSTs were purified.

Purification of Glutathione S-Transferase Isozymes. Murine GST isozymes were affinity purified using a GSH−agarose affinity column (5.5 × 1.0 cm) according to established methods *(34)*. Individual GST subunits were identified by ESI mass spectrometry and determined to be >98% pure by HPLC analysis.

Wild-type and mutant hGSTP1 enzymes were prepared as described *(35)*. Recombinant proteins were dialyzed against two changes (3 L each) of 25 mM sodium phosphate buffer, pH 6.5, and 1 mM dithiothreitol (DTT), frozen, and archived at −80 °C until use.

Glutathione S-Transferase Activity and Kinetic Studies. GST activity was measured at 25 °C using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates according to methods of Habig et al. *(36)*. The rate of product formation was monitored by measuring the change in absorbance at 340 nm using a Shimadzu PC-2101 UV−visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Protein concentration was determined by the method of Bradford (*37*).

GST Modification: (1) Haloenol Lactone Incubations. Affinity-purified isozymes were dialyzed against 4 changes of Nanopure water and lyophilized. Proteins were reconstituted (∼1 mg/mL) in 0.1 M sodium phosphate buffer (pH 6.5) and were incubated for 3 h at 37 °C in a shaking water bath with 0-, 5-, and 10-fold molar equiv of haloenol lactone. Aliquots were removed at several points during incubation to establish the time dependence of enzyme inhibition. After incubation, incubation mixtures were dialyzed against 3 changes (4 L) of Nanopure water. Protein content and activity toward CDNB were measured after dialysis so that changes in specific activities of isozymes that could be attributed to modification could be determined. Proteins were lyophilized, frozen at −80 °C, and archived for characterization using ESI mass spectrometry.

(2) Pyridylethylation. Haloenol lactone-modified GSTs (10 μg) were denatured in 200 μL of 10 mM Tris-HCl buffer (pH 8.5) containing 6 M guanidine-HCl and 1 mM EDTA. A 10% solution of β-mercaptoethanol (10 μL) was added to each incubation. Vessels were purged with nitrogen and incubated in the dark for 2 h at room temperature. To each sample 10 μL of 4-vinylpyridine (neat) was added, and incubations were continued for another 2 h. Modified proteins were dialyzed against 3 changes (1 L each) of Nanopure water and evaporated to dryness under vacuum.

Glutathione S-Transferase Digestion: (1) Trypsin. Tryptic digests were performed using a modified method of Stone et al. *(38)*. GSTs were denatured in 500 mM sodium phosphate buffer (pH 7.4) containing 8 M urea (100 μg of

Table 1: Irreversible Inhibition of Enzymatic Activity of mGSTA3, mGSTP1, and mGSTM1 by Haloenol Lactone[a]

isoenzyme	molar ratio (GST:inhibitor)	% catalytic activity remaining after 3 h incubation[b]
mGSTA3	1:1	97
	1:5	74
	1:10	65
mGSTP1	1:1	47
	1:5	12
	1:10	7.8
mGSTM1	1:1	108
	1:5	68
	1:10	43

[a]Isozymes were incubated with 1-, 5-, and 10-fold molar equiv of haloenol lactone for 3 h, pH 6.5, 37 °C, and control enzymes were treated identically except for omission of haloenol lactone. Proteins were dialyzed against nanopure water, and GST activity was measured by using 1-chloro-2,4-dinitrobenzene as substrate. [b] Relative to control enzyme treated in an identical manner except without haloenol lactone inhibitor.

protein/25 μL of buffer). To each solution 5 μL of 45 mM DTT was added, and proteins were incubated at 50 °C for 15 min in a shaking water bath. Solutions were allowed to cool to room temperature, and 60 μL of Nanopure water was added to each incubation mixture. Samples were transferred to Eppendorf tubes containing 5 μg of trypsin and incubated at 37 °C for 2 h. An additional 5 μg aliquot of trypsin was added to each tube, and incubations were continued overnight. Digestions were stopped by freezing the samples, and digest products were stored at −20 °C until HPLC analysis.

(2) CNBr. Modified GSTs were concentrated under vacuum to near dryness, and 50 μL of a 70% formic acid solution was added. One small crystal of CNBr (∼5 μg) was added, and the tubes were purged with nitrogen and left at room temperature overnight to react. Reaction products were diluted with 200 μL of Nanopure water and concentrated to near dryness under vacuum. An additional 200 μL aliquot of Nanopure water was added to each reaction tube, and proteins were again concentrated to near dryness. CNBr digest fragments were reconstituted in 20/80 ACN/water and analyzed by HPLC.

HPLC Analysis of Proteolytic Digest Fragments. GST digest products were separated using reverse-phase HPLC on a Vydac 214TP54 (25 cm × 4.6 mm i.d., 300 Å pore-size) C-18 column. Chromatographic analyses were carried out using a Varian 9010 solvent delivery system and a Hewlett-Packard series 1050 diode-array detector monitoring absorbance at 214, 280, 242 nm (absorbance maximum of the haloenol lactone). Proteins were separated using a flow rate of 1.0 mL/min with an analysis time of 90 min/sample. The initial mobile phase composition was held at 5/95 ACN/water + 0.1% TFA (v/v) for 5 min followed by a linear gradient to 70/30 ACN/water + 0.1% TFA (v/v) at 65 min, and an additional linear gradient to 100% ACN + 0.1% TFA (v/v) at 90 min. Injections of 200 μL were made using a Rheodyne injector.

Mass Spectrometry. HPLC fractions corresponding to digest fragments of GST isozymes were collected and concentrated under vacuum to approximately 25 μL, and an equal volume of ACN + 0.05% formic acid was added. Molecular masses of modified GST peptides and proteins were determined using a Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, U.K.) as described earlier (*32, 34*). Mathematical transformation of electrospray spectra to true mass scale was attained using the MaxEnt algorithm (Fisons Masslynx software).

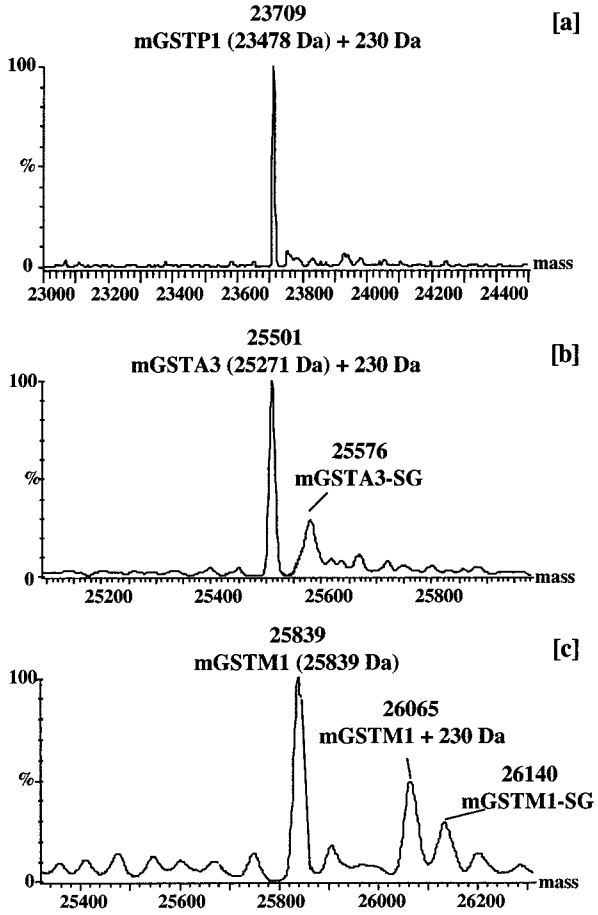

FIGURE 1: (a) Electrospray ionization MaxEnt transformed spectrum of mGSTP1 incubated with 10 molar equiv of haloenol lactone. (b) Electrospray ionization MaxEnt transformed spectrum of mGSTA3 incubated with 10 molar equiv of haloenol lactone. (c) Electrospray ionization MaxEnt transformed spectrum of mGSTM1 incubated with 10 molar equiv of haloenol lactone. Molecular mass addition relative to control protein of 230 Da corresponds to Adduct 1, Figure 2a. Molecular mass addition relative to control protein of 305 Da corresponds to *S*-glutathionylation.

RESULTS

Murine Glutathione S-Transferases. GST isozymes mGSTA3, mGSTP1, and mGSTM1 isolated from CD-1 mouse livers were were incubated in buffer with 0, 5, and 10 molar equiv of the haloenol lactone, without added GSH, for 3 h at 37 °C. After dialysis, specific activities toward CDNB for mGSTA3, mGSTM1, and mGSTP1 were shown in Table 1. Isozyme mGSTP1 displayed the greatest losses in enzymatic activity upon haloenol lactone incubation, losing 92% of original activity after incubation with 10 molar equiv of haloenol. After otherwise identical incubations, 57% of mGSTM1 activity and 35% of mGSTA3 activity was irreversibly lost.

Haloenol lactone-modified GSTs were analyzed using ESI/MS to determine the stoichiometry of protein modification and molecular mass addition resulting from covalent attachment of haloenol lactone to enzyme. ESI mass spectra revealed that 98% of mGSTP1 had undergone covalent modification (Figure 1a). The dominant peak in the trans-

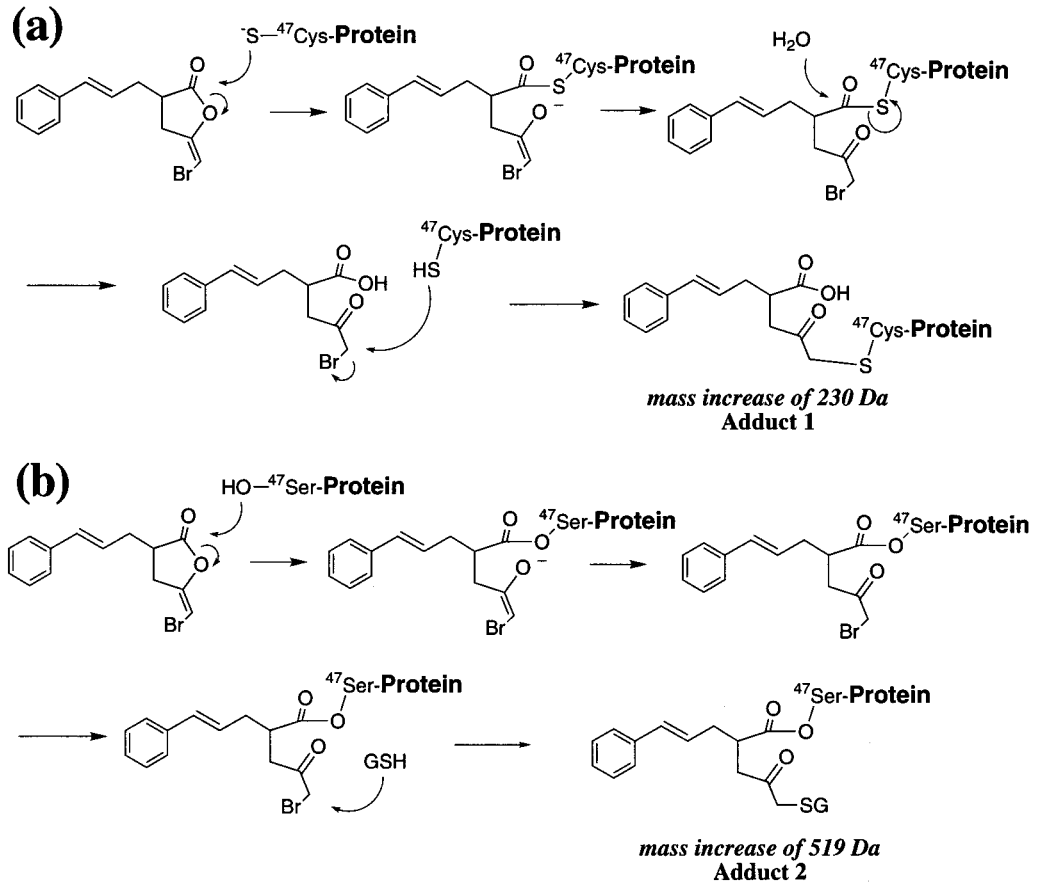

FIGURE 2: Proposed mechanisms for inactivation of (a) murine mGSTP1 and (b) recombinant human C47S-hGSTP1 by haloenol lactone derivative 3-cinnamyl-5-(*E*)-bromomethylidenetetrahydro-2-furanone.

formed ESI mass spectrum (23 708 Da) corresponds to addition of 230 Da to mGSTP1 (23 478 Da). This mass addition indicates displacement of bromide from the haloenol lactone plus addition of one molecule of water molecule to form adduct 1 (Figure 2a). The extent of covalent modification apparent in ESI mass spectra (98% of protein) agrees with losses in enzymatic activity toward CDNB after dialysis (92%). Isozymes mGSTA3 and mGSTM1 also underwent covalent modification by haloenol lactone (panels b and c, respectively, of Figure 1). No unmodified mGSTA3 (25 271 Da) remained after incubation with 10 molar equiv of haloenol lactone. The most abundant component (25 501 Da, 65% of enzyme) corresponds to addition of 230 Da, similar to mGSTP1. ESI mass spectra also demonstrated that 35% of mGSTA3 had been glutathionylated (a glutathione disulfide adduct results in an increase of 305 Da), as evident from the peak of 25 576 ± 5 Da. Since all protein molecules underwent covalent modification but only 10% of enzymatic activity was lost, covalent attachment of either haloenol lactone or glutathione had minimal effects on the catalytic activity of mGSTA3. This isozyme has only one cysteine residue (Cys-211), which is located near the C-terminus of the protein and is the most probable site of modification, though direct evidence of the actual site of attachment is still being pursued.

In an otherwise identical incubation of mGSTM1 (25 839 Da), 45% of protein molecules formed adduct 1 (26 069 Da), and 30% of protein had been glutathionylated (26 144 Da). As with mGSTP1, the extent of protein modification by the haloenol lactone (45%) corresponds to losses seen in enzymatic activity toward CDNB after dialysis (43%). In these and previous studies of murine GST modification, glutathionylation was observed with mGSTA3 and mGSTM1, but not mGSTP1 (*34*). Differences in amounts of glutathionylation are attributed to differences in reactivities of cysteine side chains in the individual isoenzymes because mGSTP1 exhibited a lower K_m for GSH binding (93 μM) than mGSTA3 (156 μM) or mGSTM1 (787 μM), and removal of GSH by dialysis would be at least as difficult for mGSTP1 as for the other isoenzymes. Glutathionylation was prevented in separate incubations by displacement of GSH using extensive dialysis against 1 mM *S*-hexylglutathione prior to incubations with protein modifiers. To determine whether glutathionylation contributed to inactivation of mGSTM1 incubated with haloenol lactone, incubations with haloenol lactone were repeated with mGSTM1 which had been dialyzed against 1 mM *S*-hexylglutathione. ESI mass spectra of these proteins showed no glutathionylation (spectra not shown) and demonstrated that the extent of formation of adduct 1 corresponded to fractional losses in enzymatic activity for mGSTM1; therefore, glutathionylation had no adverse affect on catalytic activity of mGSTM1.

Site of Haloenol Lactone Modification. To identify which amino acids were modified during haloenol lactone inactivation, products of tryptic digestion of haloenol lactone-modified mGSTP1 were isolated using HPLC, detected using spectrophotometric detection at 214 and 242 nm (λ_{max} for the haloenol lactone), collected, and analyzed by ESI/MS. A single new peak showing absorbance at 242 nm at a retention time of 41.5 min (Figure 3) was identified by

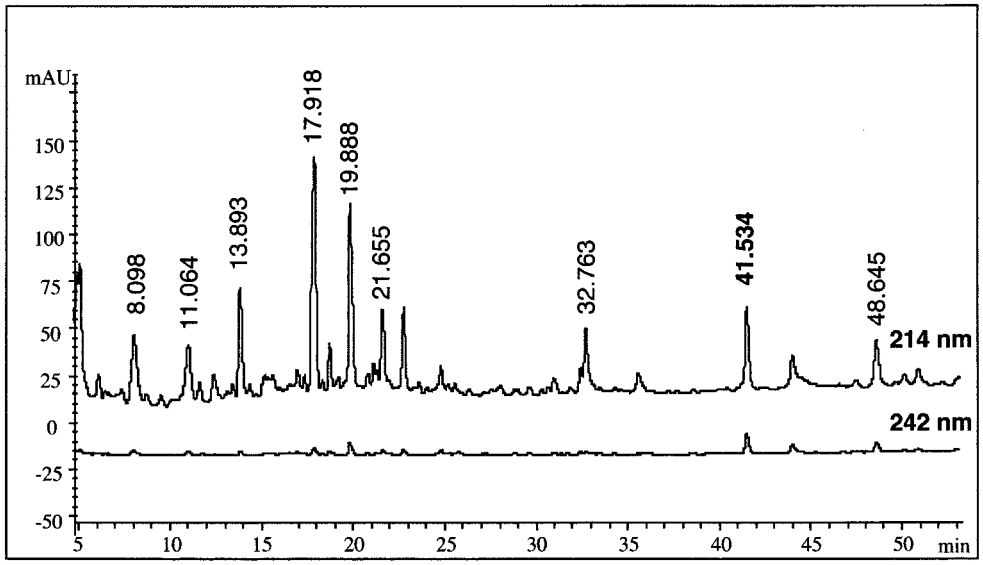

FIGURE 3: Reverse-phase HPLC chromatogram of products from tryptic digestion of mGSTP1 incubated with 10 molar equiv of haloenol lactone. The peak at 41.53 min contains haloenol lactone adduct.

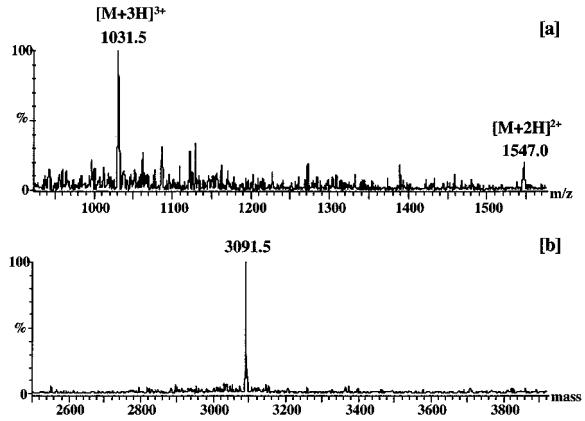

FIGURE 4: (a) Electrospray ionization mass spectrum of haloenol lactone modified T5 fragment of mGSTP1 and (b) MaxEnt transformation showing true mass scale spectrum.

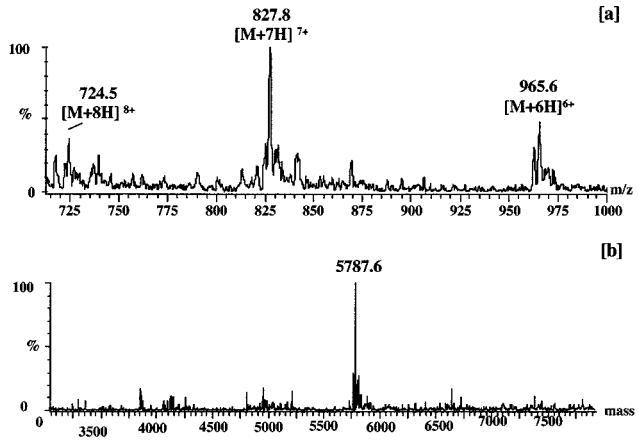

FIGURE 5: (a) Electrospray ionization mass spectrum of haloenol lactone and 4-vinylpyridine-modified CNBr C4 fragment of mGSTP1 and (b) MaxEnt transformation showing true mass scale spectrum.

comparing chromatograms of haloenol lactone-modified digests with a control digest. ESI mass spectra of this peptide showed major peaks at *m*/*z* 1031.5 and 1547.0 (Figure 4a), corresponding to doubly charged ($[M + 2H]^{2+}$) and triply charged ($[M + 3H]^{3+}$) ions of a peptide of molecular mass 3091.5 Da (Figure 4b). The molecular mass of this peptide corresponds to a covalent adduct of haloenol lactone with tryptic fragment T5 (EEVVT IDTWM QGLLK PTCLY GQLPK, 2861 Da unmodified).

The T5 fragment contains two potentially nucleophilic side chains, Cys-47 and Tyr-48, either of which could be alkylated by the α-bromoketone intermediate. The unusual acidity of Cys-47 suggested that it was the likely nucleophile. To elucidate which nucleophile becomes modified during inactivation, haloenol lactone-modified mGSTP1 was alkylated with the sulfhydryl blocking reagent 4-vinylpyridine and digested with cyanogen bromide. The generated CNBr fragments were isolated using HPLC and characterized using ESI mass spectrometry.

The mGSTP1 isozyme has three cysteine residues: Cys-14, Cys-47, and Cys-169, located in CNBr digest fragments C1, C4, and C6, respectively. The ESI spectrum of the HPLC fraction corresponding to peptide C4 (amino acids 40−89) is shown in Figure 5a. Mathematical transformation of this spectrum yields a molecular mass of 5787.6 Da (Figure 5b). This mass corresponds to peptide C4 plus addition of haloenol lactone and indicates that Cys-47 is not modified by 4-vinylpyridine. ESI mass spectra of CNBr fragments C1 and C6 indicated that both Cys-14 and Cys-169 were alkylated by 4-vinylpyridine (2054.2 and 13,145.8 Da, respectively). This indirect evidence points to Cys-47 as the site of haloenol lactone attachment.

Recombinant Human Pi Class GSTs. Recombinant human placental Pi class GSTs hGSTP1 and mutants C47S-hGSTP1, C101S-hGSTP1, and C47S/C101S-hGSTP1 were expressed in *Escherichia coli* and purified (*35*). Purities and identities were determined from ESI mass spectra, which showed hGSTP1 to be composed of a mixture of four components (Figure 6a) with molecular masses 23 224, 23 295, 23 356, and 23 429 Da. The calculated molecular mass of hGSTP1 is 23 225 Da. The additional peaks are attributed to attachment of a molecule of about 70 Da (23 295 Da) and failed removal of N-terminal methionine for both proteins, yielding 23 356 and 23 429 Da, respectively. Earlier studies of this protein demonstrated that removal of N-terminal

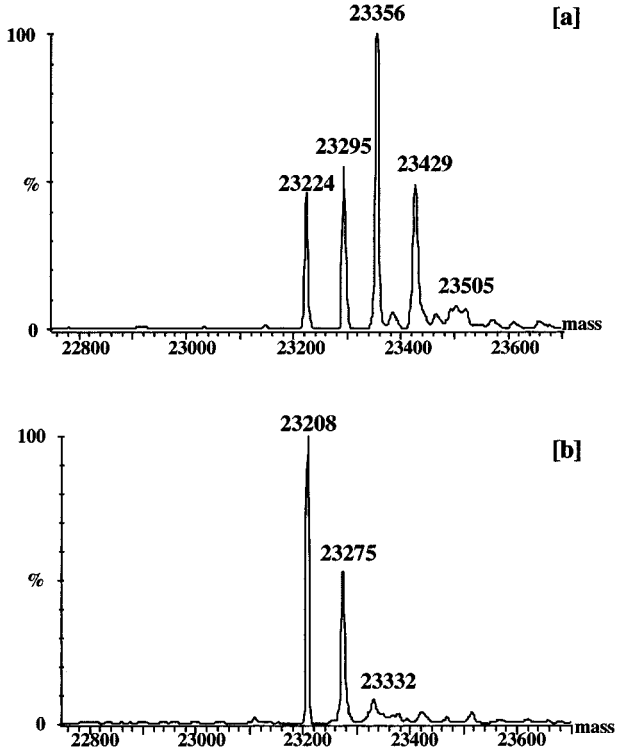

FIGURE 6: Electrospray ionization MaxEnt transformed spectrum of (a) recombinant human placental hGSTP1 and (b) recombinant human placental C47S-hGSTP1 mutant.

methionine by bacterial aminopeptidases was not complete during expression of recombinant hGSTP1 *(39).* ESI mass spectra were obtained under conditions that dissociate noncovalent complexes, and addition of 70 Da does not correspond to adducts formed from the presence of common salts or additives used in recombinant protein purification. In addition, all recombinant proteins underwent extensive dialysis against phosphate buffer containing DTT and against water prior to mass spectrometric characterization. These procedures were found to eliminate adducts arising from thiol modifying agents such as mercaptoethanol. The fraction of protein containing this adduct was expected to have minimal effect on the catalytic function of the enzyme.

ESI mass spectra of the C47S mutant (23 208 Da) show a decrease of 16 Da relative to wild type and verify replacement of cysteine by serine. ESI mass spectra of C47S mutant indicate complete removal of N-terminal methionine; however, a peak was observed at 23 275 Da (Figure 6b). This alteration in molecular mass is similar to the addition of 70 Da observed in recombinant hGSTP1. ESI mass spectra of the C101S mutant (23 209 Da, not shown) also documented replacement of cysteine by serine. Additional peaks observed in ESI mass spectra correspond to the 70-Da addition (23 278 Da) and failed removal of Met from both components (23 339 and 23 409 Da, respectively). ESI mass spectra of the C47S/C101S double mutant (23 196 Da, not shown) verify replacement of both cysteines by serines.

Time-Dependent Inhibition of GSTs by Haloenol Lactone. Time-dependent inhibition studies of recombinant hGSTP1 and C47S, C101S, and C47S/C101S mutants were performed with an excess of haloenol lactone (100 μM) with each protein (1 μM) in phosphate buffer (pH 6.5) at 37 °C. As shown in Figure 7, the haloenol lactone caused selective and rapid inhibition ($t_{1/2}$ = 2 min) of wild-type enzyme and

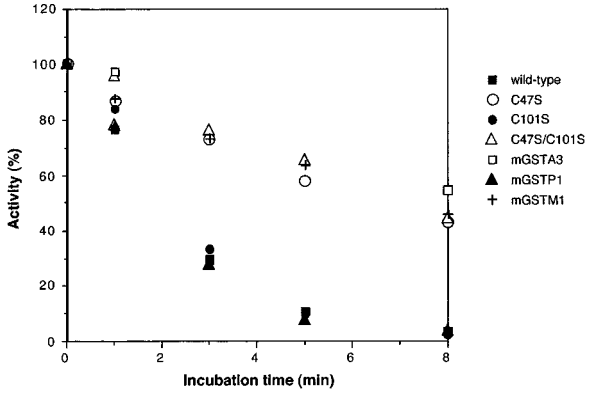

FIGURE 7: Time-dependent inhibition of human recombinant GST and murine GST activities by haloenol lactone. Studies were performed with an excess of haloenol lactone (100 μM) with each isozyme (1 μM) in phosphate buffer (pH 6.5) at 37 °C. GST isoenzymes: (■) wild-type hGSTP1, (○) recombinant hGSTP1 C47S mutant, (●) recombinant hGSTP1 C101S mutant, (△) recombinant hGSTP1 C47S/C101S double mutant, (□) mGSTA3, (▲) mGSTP1, and (+) mGSTM1.

C101S mutant. For both enzymes, less than 3% of original enzymatic activity remained after 8 min. Mutants C47S and C47S/C101S were also inhibited but at a slower rate ($t_{1/2}$ = 8 min). These studies suggest that although Cys-47 is not essential for irreversible inactivation, the reactivity of residue 47 plays a key role in determining the rate of GST inactivation.

Time-dependent inactivations of murine isozymes mGSTA3, mGSTP1, and mGSTM1 by the haloenol lactone were compared to those of human recombinant GSTs and are included in Figure 7. Curves documenting time-dependent inactivation of hGSTP1 and C101S-hGSTP1 were the same as curves generated for mGSTP1 ($t_{1/2}$ = 2 min for all three proteins), further documenting an important role for Cys-47 in the reactivity of protein toward inhibitor. Furthermore, comparisons show that mGSTA3 and mGSTM1 (both lack an acidic Cys such as Cys-47) are inactivated by the haloenol lactone at the same slow rate as C47S-hGSTP1 and C47S/C101S-hGSTP1 ($t_{1/2}$ = 8 min) and suggest that a slower rate predominates for reaction with Cys side chains that are less acidic and less reactive than Cys-47.

DISCUSSION

Mechanisms of GST Inactivation by Haloenol Lactone. Two reactions of the haloenol lactone with nucleophiles are needed to displace bromide. In our preliminary work *(33)* we proposed a two-step mechanism involving enzyme-mediated hydrolysis of the lactone followed by displacement of bromide by a nucleophilic side chain forming a covalent adduct (Figure 2a). In the present study the second nucleophile has been identified as Cys-47, and comparisons of kinetics of inactivation between enzymes containing Cys-47 and C47S mutants suggest that residue 47 is also the first nucleophile.

Incubations of recombinant hGSTP1 with haloenol lactone also formed adduct 1 (addition of 230 Da) as was also observed with murine GST isozymes. In contrast, incubation of recombinant C47S-hGSTP1 with the haloenol lactone yielded a mass increase of 519 Da (Figure 8) corresponding to addition of one haloenol lactone molecule and GSH. This reaction is in accord with opening of the lactone ring by the

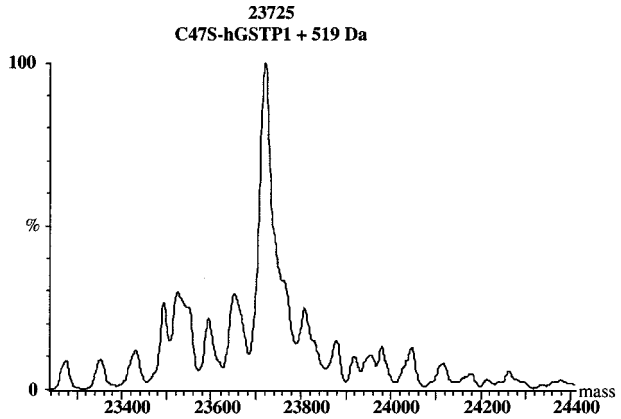

FIGURE 8: Electrospray ionization MaxEnt transformed spectrum of human recombinant placental C47S-hGSTP1 incubated with 10 molar equiv of haloenol lactone. Molecular mass addition of 519 Da corresponds to Adduct 2, Figure 2b.

Ser-47 side chain followed by displacement of bromide by residual GSH (adduct 2, Figure 2b).

These results suggest that the initial step of inactivation of wild-type and mutant Pi class GSTs involves addition of residue 47 to the lactone ring, which is opened in the process to form an α-bromoketone adduct. Enzymes containing Cys-47 form adducts at faster rates than C47S mutants, suggesting that initial attachment of lactone to protein is the rate-determining step. The acidity of Cys-47 confers good leaving group properties, and rapid hydrolysis occurs to generate an α-bromoketoacid intermediate. The reaction may proceed via alkylation of the transient thioester to form a six-membered ring episulfonium ion intermediate which would be yet more reactive toward hydrolysis, with either process leading to the observed mass increase of 230 Da.

When Cys-47 is replaced by serine (C47S mutant), Ser-47 serves as the nucleophile capable of opening the lactone ring, but this reaction is slower than that for Cys-47 owing to differences in nucleophilicities of hydroxyl and sulfhydryl. The Ser side chain is also a poorer leaving group than Cys, and formation of an intermediate analogous to the episulfonium ion is unlikely. As a result, the ester bond between inhibitor and C47S mutant protein is expected to undergo hydrolysis at a slower rate than wild-type adduct. Instead, the α-bromoketone alkylates residual bound GSH, yielding an adduct still linked to protein via an ester bond with a molecular mass increase of 519 Da (adduct 2). Similar behavior involving attachment to serine residues and subsequent modification of covalent adducts has been observed in ESI-MS studies of inhibition of serine β-lactamases *(40, 41).* Because hGSTP1 and C47S-hGSTP1 were purified under identical conditions, concentrations of residual GSH in incubation buffers are expected to be similar and should not influence adduct formation.

Although there is evidence that millimolar concentrations of GSH confer some protection for GST Pi isoenzymes, some, if not all, of the protective effect may be attributed to nonenzymatic reaction of GSH with the lactone. In a companion study *(42),* incubations of the lactone increased cell death in kidney tumor cells at levels that caused only small decreases in GSH levels. These results suggest that cytotoxicity may still occur in the presence of millimolar GSH.

Pi isozymes of GST appear to possess three characteristics that may confer thioesterase activity: (1) Cys-47 is an unusually reactive nucleophile, (2) water molecules are situated near the site of initial adduct formation, and (3) the acidic properties of Cys-47 make it a good leaving group. It is premature to speculate regarding the thioester substrate specificity of GST Pi isoenzymes. However, overexpression of this isoenzyme in many cancers suggests potential exploitation of this activity for selective conversion of prodrugs to active forms for cancer chemotherapy.

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