Alkylation and Inactivation of Human Glutathione Transferase Zeta (hGSTZ1-1) by Maleylacetone and Fumarylacetone

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Glutathione transferase zeta (GSTZ1-1) catalyzes the *cis*−*trans* isomerization of maleylacetoacetate or maleylacetone (MA) to fumarylacetoacetate or fumarylacetone (FA), respectively. GSTZ1-1 also catalyzes the glutathione-dependent biotransformation of a range of α-haloacids, including dichloroacetic acid. The objective of this study was to investigate the mechanism of inactivation of hGSTZ1-1 by MA and FA and to determine the covalent modification of hGSTZ1-1 by MA and FA in the presence and absence of glutathione. MA and FA (0.01−1 mM) inactivated all hGSTZ1-1 polymorphic variants in a concentration- and time-dependent manner, and this inactivation was blocked by glutathione. The C16A mutant of hGSTZ1c-1c was partially inactivated by MA and FA. Electrospray ionization-tandem mass spectrometry and SALSA (Scoring Algorithm for Spectral Analysis) analyses of tryptic digests of hGSTZ1 polymorphic variants revealed that the active site (SSC*SWR) and C-terminal (LLVLEAFQVSHPC*R) cysteine residues of hGSTZ1-1 were covalently modified by MA and FA. MA and FA adduction resulted in diagnostic 156-Da shifts in the masses of the modified peptide ions and in their MS-MS fragment ions. Alkylation of the active-site cysteine residues, but not of the C-terminal cysteine, was relatively less intense when hGSTZ1-1 polymorphic variants were incubated with MA or FA in the presence of *S*-methyl glutathione. These data indicate that MA and FA are substrate and product inactivators of hGSTZ1-1 and covalently modify hGSTZ1-1 at the active-site cysteine residue in the absence of glutathione. The observation that inactivation was blocked by glutathione indicates that binding of glutathione to the active site prevents reaction of MA or FA with the active-site cysteine residue. These data also indicate that MA and FA may covalently modify and inactivate other proteins that have accessible cysteine residues and may, thereby, contribute to dichloroacetic acid-induced or hypertyrosinemia type-I-associated toxicities.

Introduction

Glutathione transferases (GSTs)[1] are members of a superfamily of Phase-II drug metabolizing enzymes that catalyze the conjugation of glutathione with a range of electrophilic substrates (*1*, *2*). GSTs are expressed in plants, microorganisms, and animals. Cytosolic GSTs include alpha, mu, pi, theta, omega, sigma, and zeta classes of GSTs (*3*−*5*). Microsomal and mitochondrial GSTs have been also been identified (*1*, *6*). The GST-catalyzed conjugation of glutathione with electrophilic substrates is the first step in the mercapturic acid pathway that is associated with the detoxication, excretion, or bioactivation of xenobiotics (*7*−*9*). The conjugation of glutathione with chemotherapeutic agents may be associated with increased drug resistance. Some GSTs also show activities with endogenous substrates, such as 4-hydroxyalkenals (*10*), Δ^5-3-ketosteroids in the prostaglandin and leukotriene synthetic pathways (*11*), and maleylacetoacetate (MAA) in the tyrosine degradation pathway (*12*, *13*).

Glutathione transferase zeta (GSTZ1-1), which is identical with maleylacetoacetate isomerase, catalyzes the *cis*−*trans* isomerization of MAA to fumarylacetoacetate (FAA), the penultimate step in the tyrosine degradation pathway (*4*, *13*, *14*). In many GST-catalyzed reactions, glutathione is incorporated into the product glutathione *S*-conjugates; in contrast, with GSTZ1-1 and with DCA and MA as substrates, glutathione is required for activity but is not consumed (*15*, *16*, *17*). MAA is labile and undergoes decarboxylation to the more stable maleylacetone (MA) (*12*, *18*), which is a substrate for GSTZ1-1 and is converted to fumarylacetone (FA) (*15*, *16*, *19*). GSTZ1-1 also catalyzes the glutathione-depend-

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[1] Abbreviations: MAA, maleylacetoacetate; FAA, fumarylacetoacetate; MA, maleylacetone; FA, fumarylacetone; SA, succinylacetone; DCA, dichloroacetic acid; CFA, chlorofluoroacetic acid; NEM, *N*-ethylmaleimide; GST, glutathione transferase; GSTZ1-1, glutathione transferase zeta; SALSA, Scoring Algorithm for Spectral Analysis.

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ent biotransformation of dichloroacetic acid (DCA), chlorofluoroacetic acid (CFA), and other dihaloacetic acids to glyoxylate (*17*, *20*). DCA is a mechanism-based inactivator of GSTZ1-1 (*21*, *22*).

DCA-induced inactivation of GSTZ1-1 results in the loss of activity and degradation of GSTZ1-1 (*21*, *22*) with the resultant perturbation of the tyrosine degradation pathway such that the excretion of MAA-derived compounds, MA, FA, and their saturated analogue, succinylacetone (SA), is elevated (*23*). Plasma and urine concentrations of FA and SA are also elevated in patients suffering from hypertyrosinemia type-I (*24*, *25*). Hypertyrosinemia type-I is a metabolic disorder that is associated with a loss-of-function mutation in fumarylacetoacetate hydrolase, which catalyzes the terminal step in the tyrosine degradation pathway (*26*). The metabolic stability and toxic effects of MA and FA, which are α,β-unsaturated ketones, have not been investigated (*27*).

MA and FA are mixed inhibitors of hGSTZ1-1 with CFA as substrate.[2] These kinetic experiments also showed that the specific activities of hGSTZ1-1 variants with MA as substrate declined at MA concentrations greater than 1 mM (unpublished observations). These data indicated that MA and FA were substrate and product inhibitors of GSTZ1-1, respectively. Wong and Seltzer observed that MA covalently modifies maleylacetone isomerase purified from *Escherichia coli* by a non-Schiff-base mechanism (*28*). Hence, we hypothesized that MA and FA covalently modify and inactivate GSTZ1-1 by a Michael-addition reaction with the active-site cysteine residue (*4*, *29*, *30*).

The objective of this study was to determine the mechanism of inactivation of hGSTZ1-1 polymorphic variants by MA and FA in the presence and absence of glutathione. Substitution mutation analysis and LC-MS-MS analysis were undertaken to determine the location and type of MA- and FA-induced modifications. The data show that MA and FA covalently modify cysteine residues in hGSTZ1-1. These findings have implications with respect to possible alkylating effects of MA and FA, which are elevated in hypertyrosinemia-type-I patients or after DCA-induced perturbations in tyrosine metabolism.

Experimental Procedures

Materials. Maleylacetone and fumarylacetone were a gift from Dr. Peter Dedon, MIT, and were synthesized by the method of Fowler and Seltzer (*18*); their properties were confirmed by ^{1}H NMR and UV spectroscopic analyses. Dichloroacetic acid (>99% pure) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Chlorofluoroacetic acid (99% pure) was prepared by hydrolysis of ethyl chlorofluoroacetate (Lancaster Synthesis, Inc., Windham, NH), as described previously (*20*). Glutathione, succinylacetone (4,6-dioxoheptanoic acid), phenylhydrazine, potassium ferricyanide, and mono- and dibasic potassium phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were obtained from commercial suppliers.

Expression and Purification of Recombinant hGSTZ1-1 Polymorphic Variants and the hGSTZ1c-1c C16A Mutant. Recombinant *N*-terminal His-tagged hGSTZ1-1 polymorphic variants and the C16A mutant of hGSTZ1c-1c were expressed in *E. coli* M15[pREP4] cells (Qiagen Inc., Valencia, CA) and purified with nickel affinity columns, as described previously (*22*, *31*, *32*). The purified enzymes were stored at −20 °C in 20 mM potassium phosphate buffer (pH 7.4) containing 50 mM sodium chloride, 0.5 mM EDTA, 1.5 mM DTT, and 10% glycerol.

Activities of hGSTZ1-1 with DCA and CFA as Substrates. The formation of glyoxylate from DCA and CFA was measured spectrophotometrically, as previously described (*20*). Reaction mixtures contained 1−2 μg of purified hGSTZ1-1 variants, 1 mM glutathione, and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 1 mL and were incubated at 37 °C. The reactions were initiated by addition of CFA (1 or 2 mM) or 1 mM DCA and were quenched by addition of 50 μL of neat trifluoroacetic acid. Specific activities (nmol/min/mg protein) are reported as the mean ± SEM for three experiments.

Activities with MA as Substrate. The activities of hGSTZ1-1 were determined by measuring the rate of formation of FA from MA by HPLC analysis, as described previously (*31*). Reaction mixtures contained enzyme (0.1−0.3 μg), 1 mM glutathione, and 0.01 M potassium phosphate buffer (pH 7.4) in a final volume of 0.5 mL. The reaction mixtures were incubated for 5 min at 25 °C; the reaction was initiated by addition of 1 mM MA and was quenched after 30 s by addition of 50 μL of concentrated HCl; 50 μL of a salicylic acid solution (1.37 mg in 1 mL methanol) was also added to each sample as an internal standard. Samples (50 μL) were analyzed on a Hewlett-Packard 1090 liquid chromatograph equipped with a μBondapak C_{18}-column (3.9 mm × 300 mm, 10-μm particle size; Waters, Milford, MA). The column was eluted with a 0 to 30% methanol gradient at a flow rate of 0.75 mL/min over 30 min; solvent A contained 0.075% acetic acid in water, and solvent B contained 0.075% acetic acid and 60% methanol in water. The absorbances of MA and FA in the eluate were measured with a diode-array detector at 312 nm. Concentrations of FA in the reaction mixtures were quantified with a calibration curve prepared with known concentrations of FA in the absence of glutathione. The retention times of MA, FA, and salicylic acid were 9.6, 22.3, and 23.5 min, respectively. The rate of nonenzymatic conversion of MA to FA (0.021 nmol/min/mg of protein) was determined by analysis of a solution containing MA (1 mM), glutathione (1 mM), and heat-inactivated enzyme in 0.01 M potassium phosphate buffer (pH 7.4) and was subtracted from each sample. After acidification with HCl, the nonenzymatic conversion of MA to FA and the nonenzymatic reaction of glutathione with FA in 10 mM potassium phosphate buffer containing 1 mM glutathione and heat inactivated enzyme were undetectable permitting reproducibility that was >95%. Specific activities (nmol/min/mg of protein) are reported as the mean ± SEM for three experiments.

Inactivation of hGSTZ1-1 by MA or FA. To investigate the inactivation of hGSTZ1-1, 0−250 μM MA or FA, 0−100 μM succinylacetone, or 0−100 μM *N*-ethylmaleimide were incubated with 50−100 μg of hGSTZ1-1 in 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 1 mL at 37 °C for 0−30 min in the absence of glutathione. The reaction mixtures were diluted 10-fold with ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 1.5 mM DTT, 0.5 mM EDTA, and 10% glycerol, and the protein was recovered by three concentration-dilution cycles with Centricon-10 concentrators (YM-10, 10 kDa molecular mass cutoff; Millipore, Bedford, MA). Because of significant losses of protein by this procedure, the reaction mixtures were sometimes diluted 100−1000-fold in phosphate buffer after inactivation before assaying for activity; this permitted a quantitative recovery of protein for determination of specific activities.

To determine whether glutathione and CFA block the MA- and FA-induced inactivation of hGSTZ1-1, 0−1 mM glutathione or CFA was added to the reaction mixtures, which were incubated for 0−30 min; the activities were determined after protein recovery. The kinetic data were analyzed by nonlinear regression analysis (GraphPad Software, Inc., San Diego, CA).

Preparation of Protein Digests of Unmodified and Modified hGSTZ1-1. hGSTZ1-1 (100 μg) was incubated with 0, 0.1, or 1 mM MA or FA in the presence or absence of 1 mM glutathione or *S*-methyl glutathione (Sigma, St. Louis, MO) in

[2] Lantum, H. B. M., Board, P. G., and Anders, M. W. (2002) Kinetics of the biotransformation of maleylacetone and chlorofluoroacetic acid by polymorphic variants of human glutathione transferase zeta (hGSTZ1-1) *Chem. Res. Toxicol.* (submitted for publication).

0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 1 mL for 1 h at 37 °C. The protein was recovered by three concentration-dilution cycles as described above and suspended in 100 μL of a denaturing solution containing 8 M urea, 0.4 M ammonium bicarbonate, and 4 mM tris(2-carboxyethyl)phosphine (Pierce, Rockford, IL). The mixture was heated at 50 °C for 45 min and diluted 4-fold with double-distilled water. The proteins were then digested with 2 μg (~1:50, w/w, enzyme: protein ratio) of Sequencing Grade Modified Porcine Trypsin (stock made by dissolving 20 μg in 200 μL of trypsin buffer according to the manufacturer's instructions; Promega, Madison, WI) and incubated overnight at 37 °C. The digest was then lyophilized and suspended in 200 μL of 50% acetonitrile/water (v/v) solution.

Electrospray Ionization Tandem Mass Spectrometric Analysis of hGSTZ1-1-Derived Peptides. Samples of the peptide digests were analyzed by reversed-phase LC-MS-MS, as described previously (*33*,*34*) on a ThermoFinnigan LCQ mass spectrometer in positive electrospray ionization mode. Briefly, samples of the peptide digest (10−20 μL) were analyzed on a ThermoSeparation Products liquid chromatograph equipped with a Vydac 218TP51 C18, column (250 × 1.0 mm; 5 μm particle size; Vydac, Hesperia, CA). The column was eluted with a 0−95% gradient at a flow rate of 0.1 mL/min over 150 min; solvent A contained 0.01% trifluoroacetic acid (Pierce) and 0.1 M formic acid (J. T. Baker, Phillipsburg, NJ) in water (Burdick & Jackson, Muskegon, MI), and solvent B contained 0.0085% trifluoroacetic acid and 0.1 M formic acid in acetonitrile (Burdick & Jackson). LC-MS data were acquired by data-dependent scanning with automatic MS-MS analyses of precursor ions with intensities above 35000 absolute ion counts. The LC-MS and LC-MS-MS data were analyzed with ThermoFinnigan Xcalibur software.

Sequest analysis of the LC-MS-MS data was done to determine the peptide coverage of the protein, to compare collision-induced dissociation spectra of peptides with predicted spectra from sequences in the database, and to screen for modifications. The analysis of the data was done with TurboSequest, version 27, running under the Sequest Browser (ThermoFinnigan, San Jose, CA); the Sequest searches used the NCBI nonredundant FASTA human database; defining analysis parameters included the endoprotease (trypsin) and possible modifications (±156 Da for Cys or Lys residues).

To facilitate identification and characterization of unmodified and modified hGSTZ1-1-derived peptides, peptide-sequence motif searches of the LC-MS-MS spectral data were analyzed with the Scoring Algorithm for Spectral Analysis (SALSA) (*35*,*36*). MS-MS scans with high scores were analyzed by matching the identified y-ions with theoretical y-ions generated with GPMAW software version 5.0 (Lighthouse Data, Odense, Denmark).

Results

MA- and FA-Induced Inactivation of hGSTZ1-1. MA and FA were incubated with hGSTZ1-1 polymorphic variants in the absence of glutathione, and the specific activity of the protein recovered at different times was determined with CFA as substrate. MA inactivated hGSTZ1c-1c and all other polymorphic variants (data not shown) in a time- and concentration-dependent manner (Figure 1); maximal inactivation was observed within 20 for all variants and activities were undetectable after the hGSTZ1-1 had been incubated with MA or FA at concentrations greater than 250 μM for 20 min. The time-dependent effects showed second-order kinetics with respect to residual activities ($r^2 > 0.99$) for all concentrations. These data indicated MA was a nonmechanism-based inactivator of hGSTZ1-1 variants and that the inactivating effects were determined by a second-order reaction that was independent of the concentration of MA.

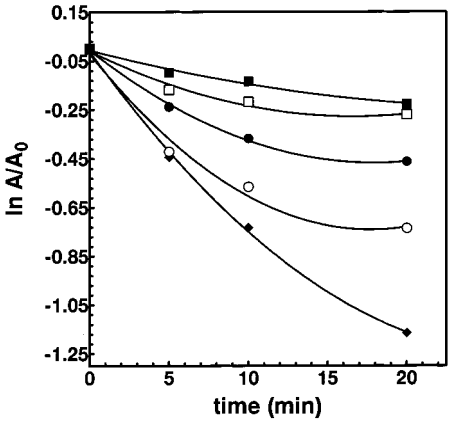

Figure 1. Kinetics of inactivation of hGSTZ1c-1c by MA. hGSTZ1c-1c (50−100 μg/mL) was incubated with 25 (■), 40 (□), 60 (●), 100 (○), and 250 (◆) μM MA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 0−30 min in the absence of glutathione. hGSTZ1c-1c incubated in the absence of MA was subjected to the same conditions and served as a control. The reaction mixtures were placed on ice and diluted 100−1000-fold, as described in Experimental Procedures. Activities with CFA as substrate were determined with 2−5 μg of protein; residual activities of the treated (A) were compared with those of the untreated preparations (A_0). The data were analyzed by nonlinear regressions fitted to the second-order polynomial equation. The data shown are the means of three experiments.

To determine whether this second-order reaction was associated with the nonenzymatic conversion of MA to FA that occurs in the absence of glutathione, the inactivating effects of FA were also determined. FA also inactivated GSTZ1-1 in a concentration- and time-dependent manner; the time-dependent inactivation kinetics for different concentrations of FA was also nonlinear (data not shown). These data indicated that MA and FA were nonmechanism based substrate and product inactivators of hGSTZ1-1, that the rates of inactivation were not linear with time, and that the data fit a second-order rate equation.

Dependence on the Olefinic Bond for Inactivation of hGSTZ1-1. MA and FA are α,β-unsaturated compounds that may react nonenymatically with thiols (*16*). Their diketo moieties may mediate the formation of Schiff-base adducts with proteins (*28*). Hence, the inactivating effects of MA and FA were compared with those of *N*-ethylmaleimide (NEM) and succinylacetone SA. NEM is an α,β-unsaturated imide, and SA is the saturated diketo analogue of MA and FA.

MA, FA, and NEM inactivated hGSTZ1c-1c in a concentration-dependent manner after 30 min incubation; in contrast, the activity of hGSTZ1c-1c was not reduced by SA (Figure 2). These data indicated that the α,β-unsaturated carbonyl group, and less likely the oxo-moiety, was required for MA- and FA-induced inactivation of hGSTZ1-1. Thus, the mechanism of inactivation is likely to be associated with the reactivity of MA and FA with cysteine residues of hGSTZ1-1.

Protection of MA- and FA-Induced Inactivation of hGSTZ1-1 by Glutathione. The glutathione binding site in the active site of hGSTZ1-1 has a highly conserved sequence with a cysteine residue (^{14}SSCSWRVIAL^{23}) (*4*, *29*, *31*). We hypothesized that MA and FA would react with the Cys-16 residue and, thereby, inactivate hGSTZ1-1 and that glutathione, which is a substrate for

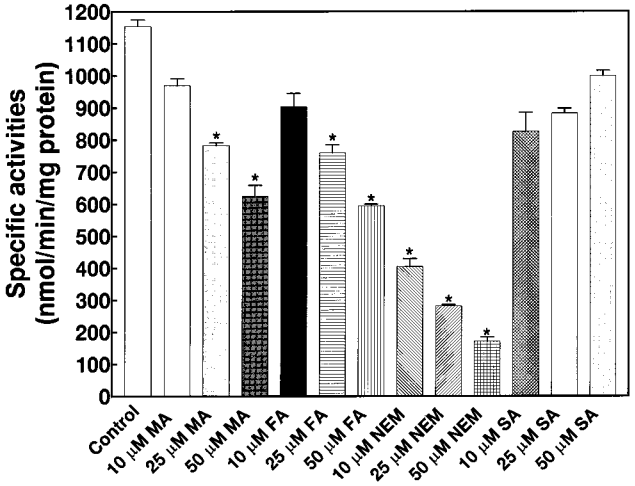

Figure 2. Concentration-dependent effects of MA, FA, NEM, and SA on hGSTZ1c-1c activities. hGSTZ1c-1c (100 μg/mL) was incubated with the indicated concentrations of MA, FA, NEM, and SA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 30 min in the absence of glutathione. The reaction mixture was diluted 100-fold, and protein was recovered by three concentration-dilution cycles; the residual activities were then determined with CFA as substrate, as described in Experimental Procedures. (*) $p < 0.05$, nonparametric repeated measures ANOVA with Dunnett's posttest to compare activities of inhibitor with control, $n = 3$.

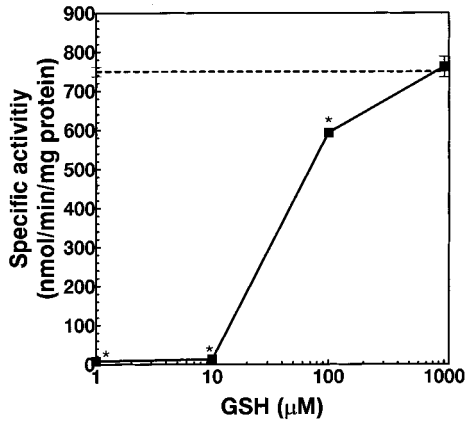

Figure 3. Concentration-dependent protective effects of glutathione on MA-induced inactivation of hGSTZ1c-1c. hGSTZ1c-1c (50 μg/mL) was incubated with 0−1 mM glutathione in 0.1 M phosphate buffer (pH 7.4, 37 °C) for 5 min prior to adding 100 μM MA. After 30 min, the reaction mixture was diluted, and the activities of the recovered protein were determined with CFA as substrate, as described in Experimental Procedures. The broken line indicates activities of hGSTZ1c-1c incubated with 1 mM glutathione for 35 min in the absence of MA. Data are shown as means ± SEM, $n = 3$; (*) $p < 0.05$, unpaired two-tailed *t*-test to compare activities after inactivation for each concentration of glutathione with control.

GSTZ1-1, would block these MA- and FA-induced inactivation. Hence, the effects of glutathione on MA- and FA-induced inactivation of hGSTZ1-1 were determined.

Incubation of hGSTZ1c-1c with 0.1−1 mM glutathione before adding MA or FA protected hGSTZ1c-1c from inactivation (Figure 3). The partial protective effects of glutathione from inactivation by MA and FA were also determined for the four polymorphic variants of hGSTZ1-1; some variants were partially inactivated by MA and FA in the presence of glutathione, and NEM inactivated the polymorphic variants to different extents (Figure 4). The inactivating effects of MA and FA were not blocked by incubating hGSTZ1-1 with 1 mM CFA during the

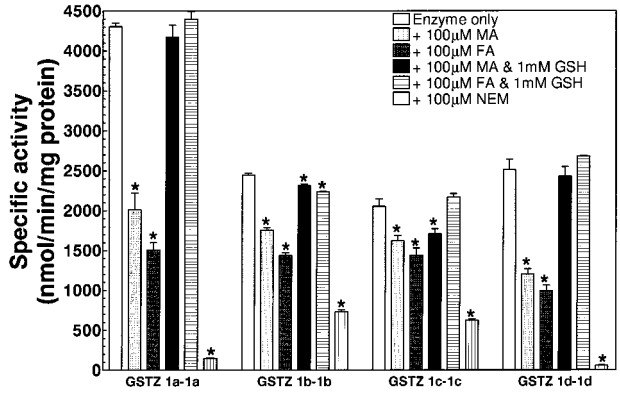

Figure 4. Protection of polymorphic variants of hGSTZ1-1 from MA- and FA-induced inactivation. hGSTZ1-1 variants (100 μg/mL) were incubated with 0 or 100 μM MA or FA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 30 min in the absence or presence of 1 mM glutathione. The residual activities with CFA as substrate were determined for each variant after recovering protein by three dilution-concentration cycles, as described in Experimental Procedures. The effects of NEM on each variant are also shown. Data are shown as means ± SEM, $n = 3$; (*) $p < 0.05$, nonparametric repeated measures ANOVA with Dunnett's posttest to compare activities at each concentration of inhibitor with control.

inactivation reaction (data not shown). These data indicated that glutathione prevented MA and FA from reacting with the active-site cysteine residue but do not exclude the reaction of these compounds with the other residues, particularly the four non-active-site cysteine residues of hGSTZ1-1. Cys-16 is likely to be the residue that was modified in the absence of glutathione.

Partial Inactivation of the hGSTZ1c-1c C16A Mutant by MA and FA. To obtain additional information about the possible reaction of MA and FA with the active-site cysteine residue, the MA- and FA-induced inactivation of the hGSTZ1c-1c C16A mutant was investigated. The activity of the *N*-His tagged C16A mutant with DCA and CFA as substrate was higher than that of the wild-type enzyme, whereas its activity with MA as substrate was less than that of the wild-type enzyme (Figure 5). The reason for these differences in activities between the wild-type and C16A mutant hGSTZ1c-1c is not known.

The inactivation of the wild-type and the C16A mutant of hGSTZ1c-1c by MA and FA was compared (Figure 6). With CFA as substrate, the wild-type enzyme had 3% or 0.5% residual activity after 30 min incubation with 100 μM MA or FA, respectively, whereas the C16A mutant had 73% and 35% residual activity after 30 min incubation with 100 μM MA or FA, respectively (Figure 6A). With MA as substrate, the wild-type enzyme had 17% and 26% residual activity after 30 min incubation with 100 μM MA or FA, respectively; the C16A mutant had 86% and 60% residual activity after 30 min incubation with 100 μM MA or FA, respectively (Figure 6B). Although 1 mM glutathione blocked the MA- and FA-induced inactivation of the wild-type enzyme by 80% as shown above, 1 mM glutathione did not protect the C16A mutant from inactivation by MA or FA (data not shown). These data indicated that the C16A mutant enzyme was partially resistant to inactivation by MA and FA and that a residue other than the active-site Cys-16 residue was a target for MA and FA.

Alkylation of hGSTZ1-1 Polymorphic Variants by MA and FA. Covalent modification of cysteine residues

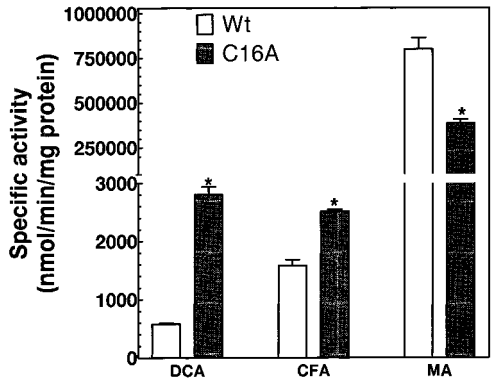

Figure 5. Activities of recombinant N-terminal His-tagged wild-type and C16A mutant hGSTZ1c-1c with DCA, CFA, and MA as substrates. Wild-type or mutant hGSTZ1c-1c was incubated with 1 mM glutathione in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C, and activities were determined with 1 mM DCA, CFA, or MA as substrates, as described in Experimental Procedures. Data are shown as means ± SEM, $n = 3$; (*) $p < 0.05$, unpaired two-tailed t-test to compare activities of the C16A mutant hGSTZ1c-1c with wild-type.

of hGSTZ1-1 polymorphic variants by MA or FA was determined by LC-MS-MS analyses of tryptic peptides. Polymorphic variants of hGSTZ1-1 were incubated with 100 μM MA or FA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 30 min, after which time the reaction mixtures were diluted 20-fold and the proteins were recovered as described in Experimental Procedures to prevent aberrant modifications during the protein denaturing and digestion steps. The assumption was made that MA or FA would modify only solvent-accessible residues under these conditions.

The sequences of the expected cysteine-containing tryptic peptides of hGSTZ1-1, their precursor ion masses, and types of MA- and FA-induced modifications are shown in Table 1. The three cysteine-containing tryptic peptide fragments of hGSTZ1-1 are hereafter referred to as peptides 1, 2, and 3 for the N-terminal-active site peptide, the hydrophobic region peptide, and the C-terminal peptide, respectively.

Peptide 1 ionized relatively poorly (precursor ion count = $\sim 1.03 \times 10^5$ ions), and the peak intensities after collision-induced dissociation were of low abundance, which limited interpretation of the b- and y-ion series of the spectra (Figures 7 and 8). The base peak of the unmodified peptide was at *m*/*z* 691.3, which corresponds to the neutral loss of hydrogen sulfide (Figure 7) from the precursor ion $(M + H)^+ = 725$ amu). LC-MS spectra of the MA-modified peptide 1 showed a 156-amu shift in the precursor ion mass $(M + H)^+ = 881$ amu, and the LC-MS-MS base peak was at *m*/*z* 725, which indicates the retro-Michael loss of 156 amu from the precursor ion; the *m*/z 691.3 amu peak was also intense and could correspond to the neutral loss of hydrogen sulfide (34 amu) from 725 amu or the loss of 2-mercaptosuccinylacetone (2-mercapto-4,6-dioxo-heptanoic acid; 190 amu) from *m*/z 881 (Figure 8). The expected diagnostic shift (Cys-residue mass + 156 amu) in the b_3 (i.e., *m*/z 278 to 434) and y_4 (i.e., *m*/z 551.2 to 707.3) ions was not identified by visual inspection of the spectra of the modified peptides. Sequest analysis failed to identify peptide 1, and, hence, these data on peptide 1 are inconclusive. SALSA analyses were, therefore, performed to facilitate the identification of MS-MS scans corresponding to modified peptides. SALSA analysis identified

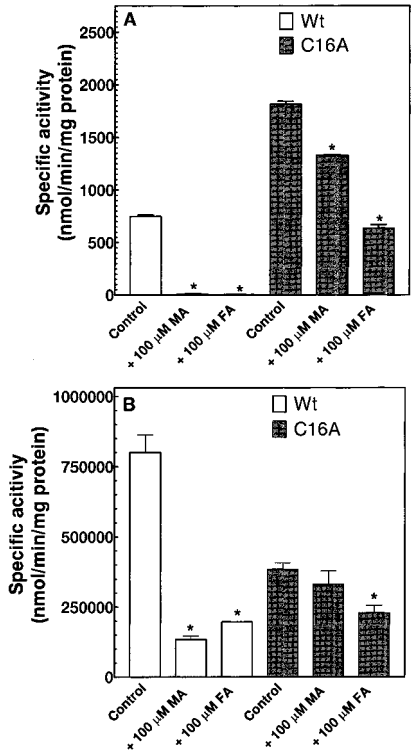

Figure 6. Inactivation of wild-type and mutant C16A hGSTZ1-1 by MA and FA. Wild-type and mutant C16A hGSTZ1c-1c (50 μg/mL) were incubated with 100 μM MA and FA in the absence of glutathione, and the residual activities were determined with (A) 1 mM CFA or (B) 1 mM MA as substrate. Data are shown as means ± SEM, $n = 3$; (*) $p < 0.05$, unpaired two-tailed t-test to compare activities after inactivation with controls.

MS-MS scans for the unmodified and modified peptide 1 for all hGSTZ1-1 variants incubated in the absence or presence of MA or FA, respectively (Table 1). The identified y-ions were diagnostic of MA- or FA-induced modification of the active-site cysteine residue and were observed in all polymorphic variants. The Xcalibur spectra and the Sequest and SALSA data for FA-modified hGSTZ1-1 peptides were similar to those observed with MA (data not shown).

Peptide 3 was readily identified from the LC-MS Xcalibur raw data. Peptide 3 was doubly charged, and the absolute ion count for the *m*/z 806.5 and 886 precursor ions for the unmodified and modified peptides, respectively, was greater than 1.2×10^6. The relative abundances of the b- and y-ion series after collision-induced dissociation were above 10%, thereby permitting manual sequencing by visual inspection of the spectra. The diagnostic 156-amu shifts of the b_{13}- and y_2-ions were visible in the LC-MS-MS spectra; all the y-ions above y_2 and only the b_{13} ion of the b-series shifted by the mass of the adduct compared with the unmodified peptide, indicating a Michael-addition adduct at the Cys-205 residue (Figure 9). Sequest analyses showed MA- and FA-associated modifications at Cys-205, which were seen in all hGSTZ1-1 variants (data not shown). Sequest searches also showed inconsistent modifications of Lys-191. SALSA analyses also confirmed the 156-amu modification at Cys-205 by providing diagnostic y-ions for the unmodified and modified peptides; this modification was observed in all variants. SALSA analysis failed to identify an MS-MS scan corresponding to modification of Lys-191, and its occurrence was, therefore, equivocal (Table 1).

Table 1. Sequence and Diagnostic MA- and FA-Induced Modifications of Three Cysteine-Containing Tryptic Peptides of hGSTZ1-1[a]

peptide	sequence[a]	mass of tryptic peptides, $[M + H]^+$ ($[M + 2H]^{2+}$)	Sequest-identified modified peptides[b]	SALSA-identified peptides[c] (precursor ion, *m/z*; diagnostic y-ions)
peptide 1	^{14}SSCSWR^{19}	725.8 (363.4)	ND[f]	($[M + H]^+ = 881.5$ *m/z*; 725, 794.4, 707.3, 448.2, 361.1, 638.2)[d] ($[M + H]^+ = 725$ *m/z*; 361.2, 448.2, 551.3, 638.33)[e]
peptide 2	^{121}QVGEEMQLTWAQNAITCG FNALEQILQSTAGIYCVGD EVTMADLCLVPQVANAER^{175}	5946 (2973)	ND	ND
peptide 3	^{193}LLVLEAFQVSHPCR^{206}	1613 (807)	LLVLEAFQVSHPC*R[g]	($[M + 2H]^{2+} = 885.6$ *m/z*; 531.2, 668.4, 755.3, 854.2, 982.3, 1129.5, 1200.5, 1329.6, 1442.6, 1541.6)[d,e]
other	^{178}FKVDLTPYPTISSINK^{191}		FKVDLTPYPTISSINK*[g]	ND

[a] hGSTZ1-1 protein sequences were obtained by simulated digestion and fragmentation with GPMAW software; the precursor masses of the tryptic peptides and the b- and y-ions for the MS-MS spectra of the unmodified and modified peptides were determined and compared with the experimental spectra from Xcalibur files or after Sequest and SALSA analyses, as described in Experimental Procedures. [b] LC-MS-MS data in Xcalibur files were analyzed under the Sequest browser, as described in Experimental Procedures; more than 50% of the hGSTZ1-1 sequence was covered; only the sequence of MA/FA-associated modified peptides are shown. [c] LC-MS-MS data in Xcalibur files were analyzed and scored by SALSA with "SCSW" or "LVLEAFQVSHPC" sequences for the motif search. Precursor ions with high scores were identified and their corresponding y-ions series were compared with theoretical data to determine whether the peptide was modified by MA or FA. [d] Precursor ion and corresponding diagnostic ions of tryptic peptides of MA- or FA-inactivated hGSTZ1-1 in the absence of 1 mM *S*-methylglutathione. [e] Precursor ion and corresponding diagnostic ions of tryptic peptides of MA- or FA-inactivated hGSTZ1-1 in the presence of 1 mM *S*-methylglutathione. [f] ND, not detected. [g] (*) 156 amu modified residue.

The effects of *S*-methylglutathione on MA- and FA-induced modification of Cys-16 and Cys-205 were also determined. *S*-Methylglutathione (1 mM) partially protected Cys-16, but not Cys-205, from MA- or FA-induced modifications based on the intensities of the precursor ion peaks of the LC-MS spectra (data not shown).

These data indicated that MA and FA formed Michael-addition adducts with the cysteine residues in peptides 1 and 3. Cys-16, but not Cys-205, was protected from MA- and FA-induced alkylation by *S*-methylglutathione. The Sequest analyses provided equivocal evidence that MA and FA may form Schiff-base adducts with Lys-191.

These data do not exclude the formation of adducts with peptide 2, which was not identified. Polymorphic variants of hGSTZ1-1 were, therefore, digested with endoproteinase Glu-C (Sigma, St. Louis, MO) to obtain smaller fragments of peptide 2 to facilitate identification of modifications of cysteine residues. Sequest analyses showed that the peptide coverage of GLU-C digests of hGSTZ1-1 variants ($n = 4$) was less than 5%, and SALSA motif searches failed to identify any fragments of peptide 2 (data not shown). Hence, the Glu-C digests were not investigated in more detail.

Reactivity of MA and FA with Glutathione. FA was apparently a more potent inactivator of hGSTZ1-1 variants than MA (Figures 2, 4, and 6). This may be associated with relative differences in the reactivities of MA and FA with thiols. To investigate this point, the reactivities of MA and FA with 10 mM glutathione in 0.1 M potassium phosphate buffer (pH 8.5) were studied by continuous UV-spectral analysis over 10 min at room temperature. The absorbance of 400 μM FA at 312 nm decreased rapidly after addition of glutathione, whereas the decrease in the absorbance of 400 μM MA at 312 nm was much slower (Figure 10). These data indicate differences in the reactivities of MA compared with FA with thiols. Furthermore, some of the observed effects of MA may be associated with FA that is formed nonenzymatically during the incubations.

Discussion

This study describes the inactivation and alkylation of hGSTZ1-1 by its substrate and product analogues, MA and FA. The data indicated that the mechanism of alkylation and, hence, inactivation was attributable to a Michael-addition reaction of MA and FA with at least two cysteine residues (Cys-16 and Cys-205) of hGSTZ1-1 variants. A two-site binding mechanism of inactivation was initially indicated by the nonlinearity of the inactivation kinetics and confirmed by substitution mutation analysis and LC-MS analysis. These data explain why MA and FA are mixed inhibitors of hGSTZ1-1 activities with CFA as substrate and why the hGSTZ1-1 activities are lower at concentrations of MA greater than 1 mM (unpublished observations).

The finding that MA and FA formed Michael-addition adducts with cysteine residues does not exclude other mechanisms of covalent modification. Sequest analysis of the LC-MS-MS data showed inconsistent modification of a lysine residue (Table 1). The inhibition data showed that SA did not inhibit hGSTZ1c-1c (Figure 2). Therefore, although the formation of Schiff-base adducts may be another mechanism of alkylation and inactivation, it does not appear to be the primary mechanism. Previous studies have shown that SA nonenzymatically forms stable adducts with proteins and free amino acids, especially lysine, and that the stability of the adducts is enhanced by treatment with sodium borohydride, indicative of Schiff-base formation (*37*). Wong and Seltzer showed, however, that sodium borohydride does not enhance labeling of GSTZ1-1 by [^{14}C]MA (*28*). These findings and observations reported herein indicate that Schiff-base formation of GSTZ1-1 by MA and FA is a less important mechanism of inactivation than a Michael-addition mechanism.

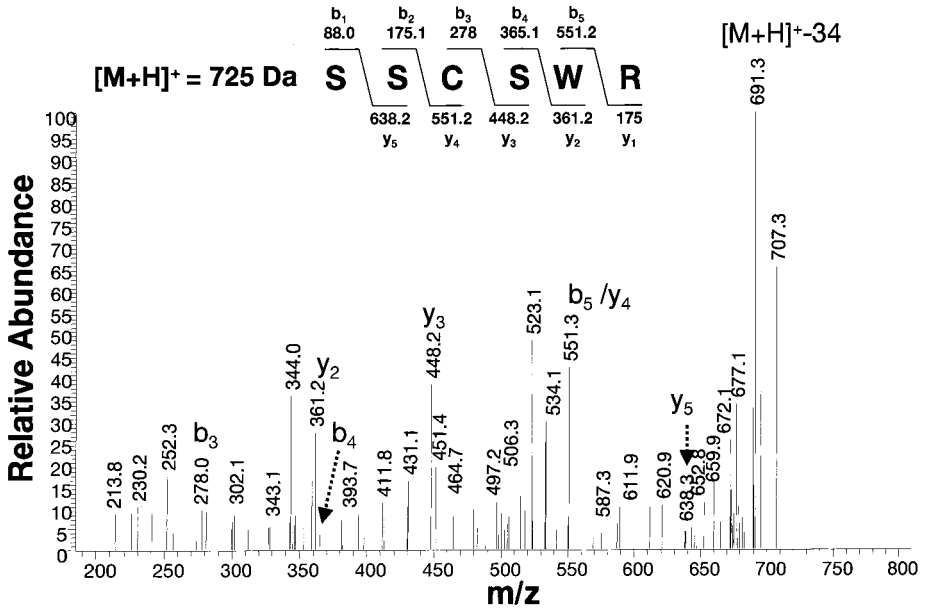

Figure 7. LC-MS-MS spectra of the unmodified active-site peptide (peptide 1) of hGSTZ1c-1c. hGSTZ1c-1c (50 μg/mL) was incubated in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 30 min in the absence of MA, FA, and glutathione. The protein was recovered by three dilution-concentration cycles, digested with trypsin, and analyzed by LC-MS-MS, as described in Experimental Procedures. Inserted text shows the mass of the precursor ions ($[M + H]^+$) and the sequence of peptide 1 with its corresponding b- and y-ions for the unmodified peptide.

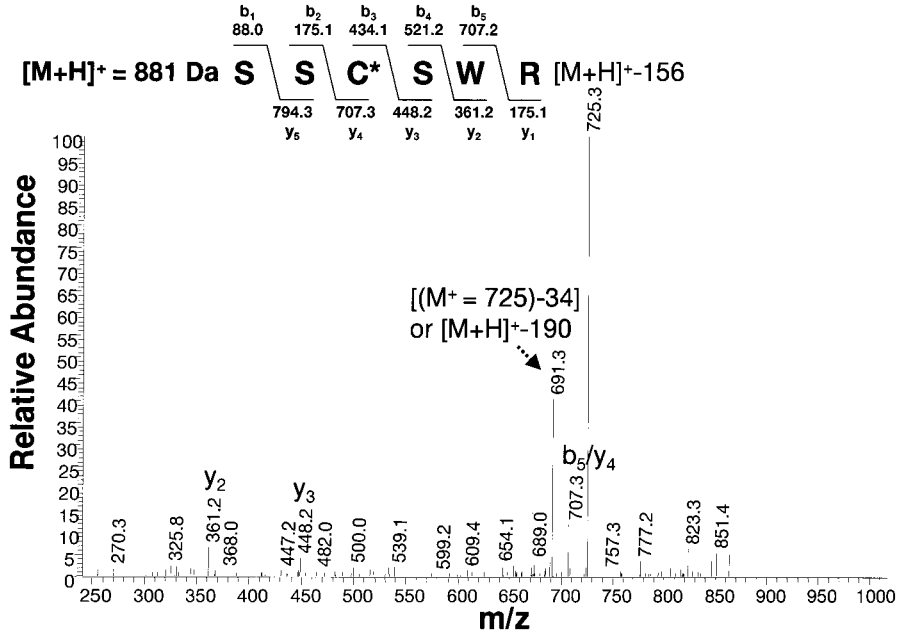

Figure 8. LC-MS-MS spectra of the MA-modified active-site peptide (peptide 1) of hGSTZ1c-1c. hGSTZ1c-1c (50 μg/mL) was incubated for 30 min with 100 μM MA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 30 min in the absence of glutathione. The protein was recovered by three dilution-concentration cycles, digested with trypsin, and analyzed by LC-MS-MS, as described in Experimental Procedures. Inserted text shows the mass of the precursor ions ($[M + H]^+$) and the sequence of peptide 1 with corresponding b- and y-ions for the MA-modified peptide; (*) MA-adducted cysteine residues.

Although complete inactivation of hGSTZ1-1 by MA and FA was observed in the absence of glutathione, the inactivation of wild-type hGSTZ1-1 variants by MA and FA was blocked by glutathione in a concentration-dependent manner. Furthermore, the C16A mutant of hGSTZ1c-1c underwent less inactivation than the wild-type enzyme, indicating that MA and FA modified Cys-16. These data indicate that Cys-16 is a target for alkylation and that alkylation of Cys-16 by MA and FA may interfere with binding of glutathione at the active site of the enzyme. Alternatively, glutathione may be bound to the Cys-16 modified enzyme but not activated to the reactive thiolate that is required for catalytic activity.

The partial inactivation of C16A hGSTZ1c-1c and some variants of wild-type hGSTZ1-1 by MA and FA in the presence of glutathione indicated that another cysteine residue was modified. LC-MS analysis showed that Cys-205 was also modified. The 3D structure of hGSTZ1-1, which was solved in a glutathione- and DTT-containing buffer, shows that Cys-205 lies in a mobile loop (H_2) in the C-terminal domain. Cys-205, but none of the other cysteine residues, formed a mixed disulfide with DTT, indicating that Cys-205 is solvent accessible; the Cys-

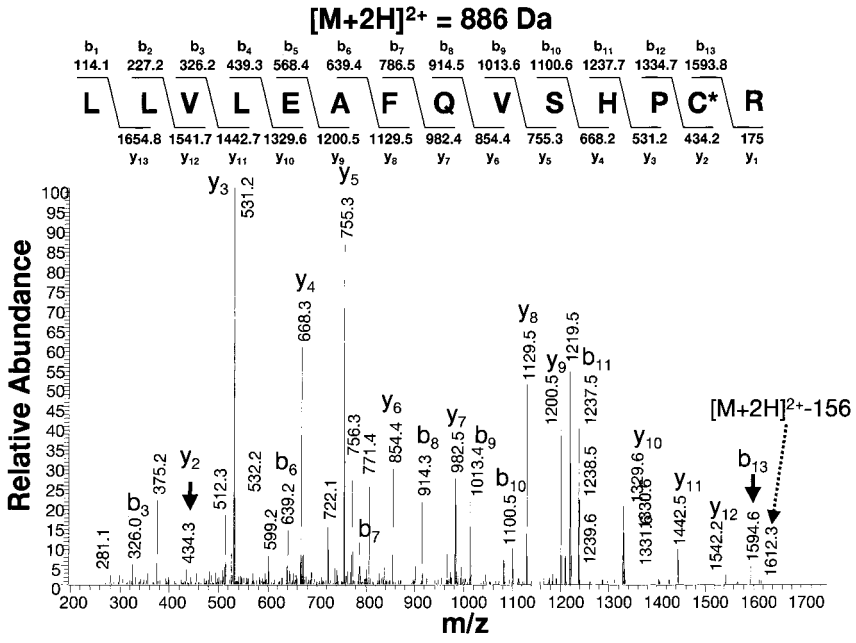

Figure 9. LC-MS-MS spectra of the MA-modified C-terminal peptide (peptide 3) of hGSTZ1c-1c. hGSTZ1c-1c (50 μg/mL) was incubated with 100 μM MA or FA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 30 min in the absence of glutathione. The protein was recovered by three dilution-concentration cycles, digested with trypsin, and analyzed by LC-MS-MS as described in Experimental Procedures. Inserted text shows the mass of the precursor ions ($[M + 2H]^{2+}$) and the sequence of peptide 3 with corresponding b- and y-ions for the MA-modified peptide; (*) MA-adducted cysteine residue.

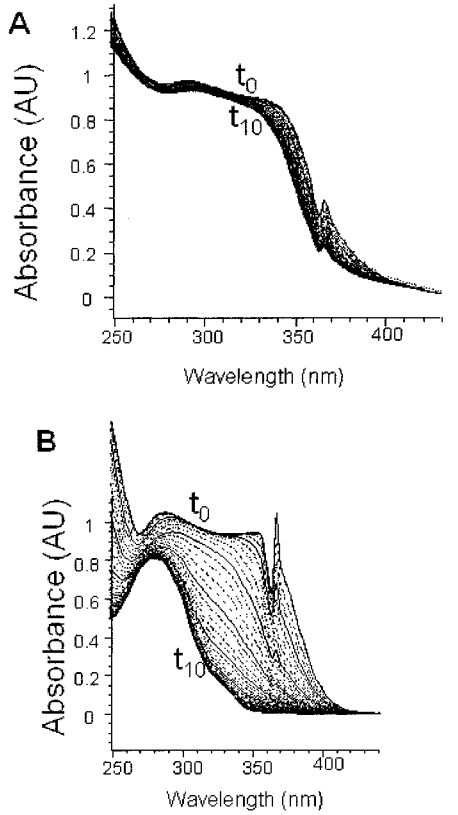

Figure 10. Reactivity of MA and FA with glutathione. MA (panel A) and FA (panel B) were diluted in 1 mL 0.1 M potassium phosphate buffer (pH 8.5) to give a final concentration of 400 μM, and glutathione was added to give a final concentration of 10 mM. The changes in absorbance were measured by UV-spectral analysis. The beginning spectra are marked t_0, and the spectra recorded after 10 min are marked t_{10}.

205-containing loop is located near the active site of hGSTZ1c-1c (*29*). These structural data and the present data indicate that alkylation of Cys-205 is associated with inactivation of hGSTZ1-1, perhaps by steric effects that impede substrate access to the active site.

The mechanism by which glutathione protected hGSTZ1-1 from inactivation is not known. Structural analysis of hGSTZ1-1 shows that the thiol of Cys-16 points toward the active site and is shielded by glutathione with respect to the substrate-binding site (*29*). Glutathione may, therefore, prevent inactivation by the substrate or product during the catalytic cycle. This proposal is supported by the data from LC-MS experiments, which showed that MA or FA did not modify the Cys-16 when incubated with *S*-methylglutathione, whereas Cys-205 was modified in the presence and absence of *S*-methyl glutathione. The data do not, however, exclude the possibility that the mechanism of protection by glutathione may be associated with the nonenzymatic reaction of glutathione with MA and FA (*12*, *16*). This is highly unlikely to be the only mechanism of protection from inactivation, particularly for Cys-205, because MA and FA inactivated the C16A mutant in the absence and presence of glutathione.

Given that MA and FA completely inactivated hGSTZ1-1 in the absence of glutathione, the data support the view that alkylation of the active-site Cys-16 is the major mechanism of inactivation in the absence of glutathione. Certain diseases, such as hereditary tyrosinemia type-I and glutathione synthetase deficiency, are associated with low hepatic glutathione concentrations (*38*). Many xenobiotics form glutathione-conjugates and, thereby, reduce cellular glutathione concentrations (*39*). Thus, alkylation of the active-site peptide may occur in these disease states. Physiologically, however, glutathione should be constantly bound to hGSTZ1-1 making adduction of MA and FA to Cys-205 the most likely mechanism that will occur in the cell.

Inactivation of hGSTZ1-1 by FA indicates a mechanism of feedback inhibition of GSTZ1-1 activities in patients

with fumarylacetoacetate hydrolase (FAH) deficiency. FAH catalyzes the hydrolysis of fumarylacetoacetate to fumarate and acetoacetate, the terminal step in the tyrosine degradation pathway. Hypertyrosinemia type-I patients (*40*, *41*) and mice with FAH-deficiencies (*42*, *43*) excrete elevated amounts of FAA-derivatives, notably succinylacetoacetate and SA (*44*), and the mercapturic acid of fumarylacetone (*S*-2-fumarylacetone *N*-acetylcysteine) (*45*) indicating a build-up of FAA (*24*). FAA, like FA, may also covalently modify and inactivate GSTZ1-1. A mechanism of competitive feedback inhibition of GSTZ1-1 by an FAA-glutathione conjugate has also been proposed (*45*).

Inactivation of GSTZ1-1 by FAA and the ensuing accumulation of MAA-derivatives may be associated with the disorders characteristic of hypertyrosinemia type-I, such as hepatocellular neoplasia. Indirect support for this contention is based on the observation that DCA, a mechanism-based inactivator of GSTZ1-1 (*22*), causes hepatic neoplasia in rats and mice (*46*, *47*) and systemic toxicities (*48*−*51*) in rodents similar to those described in patients with hypertyrosinemia type-I (*26*, *40*). Previous studies have shown that DCA-treated rats have reduced GSTZ1-1 activities and protein concentrations (*21*), and these effects result in the elevated excretion of MA and SA (*23*). The alkylating properties of MA and FA demonstrated in this study indicate that MA- and FA-induced alkylation of macromolecules may contribute to the multiorgan disorders associated with hypertyrosinemia type-I and prolonged exposure to DCA (*26*, *48*, *50*−*52*).

The mechanism and sequence of substrate binding to GSTs have been characterized with a range of substrates, including as 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride, iodomethane, and ethacrynic acid (*1*, *2*). Although experimentally substrates and glutathione bind to GSTs in a random order (*1*, *2*), the high physiological concentrations of glutathione in most tissues favor the binding of glutathione before the second substrate. The physiological implication is that the high intracellular concentrations of glutathione prevent inactivation of hGSTZ1-1 by MA and FA. Under pathological conditions in which intracellular glutathione concentrations are reduced, MA and FA may inactivate hGSTZ1-1.

The active-site peptide of GSTZ1-1 is highly conserved among species and all contain the Cys-16 residue. In contrast, the peptide that contains the Cys-205 residue is highly variable among species; as indicated by the published sequences. Cys-205 is found in all polymorphic variants of hGSTZ1-1 (*4*, *31*), but not in mouse GSTZ1-1 (*14*). The LC-MS data confirmed that all polymorphic variants of hGSTZ1-1 contain Cys-205. Humans may, therefore, be more vulnerable to the inactivating effects of MA and FA associated with modification of Cys-205 than other species. The polymorphic variants of hGSTZ1-1 were inactivated by MA, FA, and NEM to differing extents, and some variants were not completely protected from inactivation by glutathione (Figure 4). These data also indicate that humans may have varying susceptibilities to the GSTZ1-1 inactivating effects of MA and FA.

In conclusion, MA and FA are substrate and product inactivators of hGSTZ1-1. These effects are associated with the formation of Michael-addition adducts with at least Cys-16 and Cys-205. The FA-induced inactivation of GSTZ1-1 may inhibit tyrosine degradation under pathological conditions, but the observed prevention of inactivation by glutathione indicates that FA and MA likely produce little inactivation under physiological conditions. The alkylating effects of MA and FA may contribute to disorders associated with DCA-induced perturbations of tyrosine metabolism and disorders of hypertyrosinemia type-I.

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