Structural and Functional Consequences of Inactivation of Human Glutathione *S*-Transferase P1-1 Mediated by the Catechol Metabolite of Equine Estrogens, 4-Hydroxyequilenin†

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ABSTRACT: The inactivation mechanism(s) of human glutathione *S*-transferase P1-1 (hGST P1-1) by the catechol metabolite of Premarin estrogens, 4-hydroxyequilenin (4-OHEN), was (were) studied by means of site-directed mutagenesis, electrospray ionization mass spectrometric analysis, titration of free thiol groups, kinetic studies of irreversible inhibition, and analysis of band patterns on nonreducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The four cysteines (Cys 14, Cys 47, Cys 101, and Cys 169 in the primary sequence) in hGST P1-1 are susceptible to electrophilic attack and/or oxidative damage leading to loss of enzymatic activity. To investigate the role of cysteine residues in the 4-OHEN-mediated inactivation of this enzyme, one or a combination of cysteine residues was replaced by alanine residues (C47A, C101A, C47A/C101A, C14A/C47A/C101A, and C47A/C101A/C169A mutants). Mutation of Cys 47 decreased the affinity for the substrate GSH but not for the cosubstrate 1-chloro-2,4-dinitrobenzene (CDNB). However, the Cys 47 mutation did not significantly affect the rate of catalysis since V_{max} values of the mutants were similar or higher compared to that of wild type. Electrospray ionization mass spectrometric analyses of wild-type and mutant enzymes treated with 4-OHEN showed that a single molecule of 4-OHEN-*o*-quinone attached to the proteins, with the exception of the C14A/C47A/C101A mutant where no covalent adduct was detected. 4-OHEN also caused oxidative damage as demonstrated by the appearance of disulfide-bonded species on nonreducing SDS−PAGE and protection of 4-OHEN-mediated enzyme inhibition by free radical scavengers. The studies of thiol group titration and irreversible kinetic experiments indicated that the different cysteines have distinct reactivity for 4-OHEN; Cys 47 was the most reactive thiol group whereas Cys 169 was resistant to modification. These results demonstrate that hGST P1-1 is inactivated by 4-OHEN through two possible mechanisms: (1) covalent modification of cysteine residues and (2) oxidative damage leading to proteins inactivated by disulfide bond formation.

Glutathione *S*-transferases (GSTs;[1] EC 2.5.1.18) are a family of enzymes which play an important role in the detoxification of both physiological and xenobiotic electrophilic compounds by catalyzing conjugation with glutathione (GSH) (*1*, *2*). The human pi class GST (hGST P1-1) is the most widely distributed nonhepatic isozyme present in the placenta, erythrocytes, breast, lung, and prostate (*3*, *4*). In addition, hGST P1-1 is known to be overexpressed in several human tumors such as cancers of the breast, colon, esophagus, lung, pancreas, and uterus (*5*, *6*). As a result, the level of GST P1-1 has been proposed as one of the diagnostic indicators of chemical carcinogenesis (*7*−*9*).

Several structural studies on GST P1-1 showed that there are three highly conserved cysteine residues (Cys 14, Cys 47, and Cys 169) in all mammalian species examined, and a fourth cysteine residue (Cys 101) is conserved in most species with the exception of the mouse (*10*). It has been demonstrated that none of these cysteines are crucial for catalysis since the enzymatic activity was only partly reduced upon replacement of cysteine with either serine or alanine (*11*, *12*). Sulfhydryl groups of GST P1-1 are sensitive to

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[1] Abbreviations: GST, glutathione *S*-transferase; GSH, glutathione; GST P, pi class GST; hGST P1-1, human pi class GST; NEM, *N*-ethylmaleimide; ANM, *N*-(4-anilino-1-naphthyl)maleimide; ABD-F, 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole; 4-OHEN, 4-hydroxyequilenin [3,4-dihydroxy-5(10),6,8-estrapentaen-17-one]; equilenin, 1,3,5(10),6,8-estrapentaen-3-ol-17-one; equilin, 1,3,5(10),7-estratetraen-3-ol-17-one; *o*-quinone, 3,5-cyclohexadiene-1,2-dione; P450, cytochrome P450; GST M1-1, mu class GST; His_6-hGST P1-1, hexahistidine-tagged human pi class GST; CDNB, 1-chloro-2,4-dinitrobenzene; ESI-MS, electrospray ionization mass spectrometry; IPTG, isopropyl β-D-thiogalactopyranoside; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, superoxide dismutase; β-ME, β-mercaptoethanol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn-HCl, guanidine hydrochloride; JNK, c-jun N-terminal kinase.

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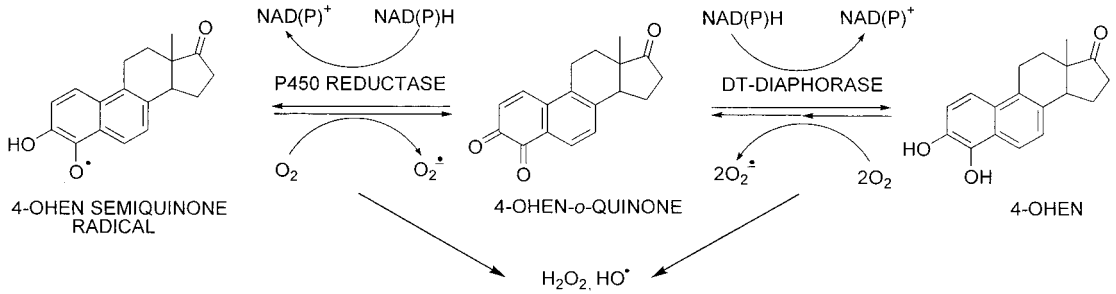
Scheme 1: Formation of 4-OHEN Quinoids



thiol-modifying agents such as *N*-ethylmaleimide (NEM) (*13*), iodoacetamide (*14*), *N*-(4-anilino-1-naphthyl)maleimide (ANM) (*15*), and 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole (ABD-F) (*16*). Cys 47, which has a pK_a value of approximately 4.2, is considered to be the most reactive thiol group among the four cysteines in hGST P1-1 (*17*). Conditions that favor formation of intra- and/or interdisulfide bonds between Cys 47 and Cys 101 led to inactivation of the enzyme (*18*−*21*). The hGST P1-1 is also inactivated by oxidized glutathione, cystamine, or cystine, all of which react preferentially with Cys 47 (*16*). As a result, cysteine residues appear to play an important role in the structural and functional integrity of hGST P1-1, although they do not appear to be directly involved in enzyme catalysis. Taken together, oxidation and/or covalent modification of cysteine residues in hGST P1-1 may impair enzyme activity. As a result, electrophilic and/or redox-active compounds could be potential inhibitors of hGST P1-1.

Estrogen replacement therapy has been correlated with an increased risk of developing hormone-dependent cancers (*22*, *23*). Premarin (Wyeth-Ayerst) is the most widely prescribed estrogen replacement formulation, and yet there is very little information on the potential cytotoxic/genotoxic effects of the 10 different estrogens present in Premarin. 4-Hydroxyequilenin (4-OHEN) is the major phase I metabolite of the Premarin estrogens equilenin and equilin. In previous studies, we showed that 4-OHEN rapidly autoxidized to 4-OHEN-*o*-quinone, which in turn formed a redox couple with the semiquinone radical catalyzed by NAD(P)H, P450 reductase, or DT-diaphorase (*24*, *25*) (Scheme 1). We also showed that the 4-OHEN-semiquinone radical formed unusual cyclic adducts with deoxynucleosides and DNA which might represent one mechanism for equine estrogen carcinogenesis (*26*). In addition, 4-OHEN also induced DNA damage and apoptosis in breast cancer cell lines (*27*). Finally, we demonstrated that 4-OHEN is a potent irreversible inhibitor of human GST M1-1, which is the most abundant GST isozyme in the human liver (*28*). Inhibition of phase II enzymes, such as GST, might indirectly contribute to the cytotoxic effects of estrogens by decreasing the GST-mediated detoxification of xenobiotic as well as endogenous carcinogens.

In the present study, we examined the relative ability of 4-OHEN to inhibit recombinant hexahistidine-tagged hGST P1-1 (His_6-hGST P1-1)-catalyzed conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) by oxidative damage and/or covalent modification of critical amino acid residues. Site-directed mutagenesis experiments, where each cysteine residue was replaced by alanine, revealed the selectivity of the cysteine residues with respect to reactivity with 4-OHEN. Electrospray ionization (ESI) mass spectrometric analyses of the wild type and mutants incubated with 1−3 molar equiv of 4-OHEN indicated that the 4-OHEN-*o*-quinone caused covalent modification of the enzymes and favored reaction with a single amino acid residue. Reactive oxygen species, generated from redox cycling of 4-OHEN, were also shown to be involved in inactivation of this enzyme since intra- and/or intersubunit disulfide bonds were observed in hGST P1-1 treated with 4-OHEN using nonreducing SDS−PAGE. Thiol group titration experiments showed that the free thiol groups were selectively modified by 4-OHEN in a concentration-dependent fashion. Kinetic experiments using the wild-type as well as the mutant enzymes showed that Cys 47 was the most reactive residue toward 4-OHEN, followed by Cys 101 and Cys 14. The significance of 4-OHEN-mediated hGST P1-1 inhibition is discussed in terms of possible carcinogenic mechanism(s) of this equine estrogen metabolite.

EXPERIMENTAL PROCEDURES

Caution: 4-OHEN was handled in accordance with NIH Guidelines for the Laboratory Use of Chemical Carcinogens (*29*).

Materials. All chemicals and reagents were purchased from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), Fischer Scientific (Itasca, IL), or Fischer Biotech (Fair Lawn, NJ) unless otherwise indicated. Restriction endonucleases and T4 DNA ligase were purchased from Fermentas MBI (Amherst, NY). *Pfu* polymerase was obtained from Stratagene (La Jolla, CA). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Co^{2+} affinity resin was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). 4-OHEN was synthesized by treating equilin with Fremy's salt as described previously (*26*, *30*) with minor modifications (*24*). 4-OHEN stock solutions were freshly prepared in DMSO.

Bacterial Strains and Plasmids. Plasmid pET15b was purchased from Novagen (Madison, WI). The cDNA encoding for hGST P1-1 carried in the plasmid pGEM-4 was purchased from ATCC (Manassas, VA). The *Escherichia coli* strain TG1 was used for cloning and amplification of recombinant expression plasmids. For protein production, the *E. coli* strain BL21(DE3) (Novagen, Madison, WI) was used.

Construction of hGST P1-1 and Mutants. Standard recombinant DNA techniques were carried out as described by Sambrook et al. (*31*). Wild-type hexahistidine-tagged human recombinant glutathione *S*-transferase P1-1 (His_6-hGST P1-1) was prepared as described previously (*32*). Site-directed mutagenesis for the Cys 47, Cys 101, Cys 47/Cys

Table 1: Sequences of the Primers for Site-Directed Mutagenesis[a]

C14A	+	5′-GTTCGAGGCCGCGCTGCGGCCCTGCGC-3′
	−	5′-GCGCAGGGCCGCAGCgcgGCCTCGAAC-3′
C47A	+	5′-CTCAAAGCCTCCGCTCTATACGGGCAG-3′
	−	5′-GGGGAGCTGCCCGTATAGAGCGGAGGCTTTGAG-3′
C101A	+	5′-GAGGACCTCCGCGCTAAATACATCTCC-3′
	−	5′-GATGAGGGAGATgtaTTTAGCGCGGAGGTCCTC-3′
C169A	+	5′-CTAGCCCCTGGCGCTCTGGATGCGTTCCC-3′
	−	5′-GGGAACGCATCCAGAGCGCCAGGGGCTAG-3′

[a] Positions mutated are underlined. (+) = sense strand; (−) = antisense strand.

101, and Cys 47/Cys 101/Cys 169 positions was carried out by overlapping PCR (*33*) using pairs of mutually complementary primers containing mismatching bases for the codons to be mutated (Table 1). The C47A/C101A mutant was obtained using the cDNA-encoding C47A mutant as the template DNA in PCR. The cDNA for C47A/C101A mutant was used as the template DNA during PCR to create both C14A/C47A/C101A and C47A/C101A/C169A mutants.

The primers to amplify the cDNA-encoding mutants were the upstream primer 5′-CCGCTCGAGCCGCCGTACAC-CGTGGTC-3′ and the downstream primer 5′-CGGGATC-CCCCTCACTGTTTCCCGTTGC-3′. The 650 bp PCR fragment was digested with *Xho*I and *Bam*HI (restriction sites underlined in the primers) and then subcloned into the pET15b plasmid vector which had been digested with the same restriction enzymes. Preparation of large amounts of enzymes from the expression plasmids was performed using Co^{2+} affinity column chromatography as described previously (*32*). Aliquots of purified GST in either 50 mM ammonium bicarbonate buffer (pH 8.0) and 1 mM DTT or 0.1 M potassium phosphate buffer (pH 7.4), 1 mM EDTA, and 1 mM DTT were stored at −80 °C until further use. Before all modification experiments, the enzymes were reduced by incubation with 10 mM DTT for 30 min at 37 °C. Removal of DTT and exchange of buffers were performed using gel filtration on a NAP-5 column (Biotech, Piscataway, NJ).

Analysis of Purified Proteins. Protein concentration was determined either by the method of Bradford (*34*) using bovine serum albumin as a standard or by absorbance at 280 nm with molar absorptivity of 26 980 M^{-1} cm^{-1} for the wild-type His_6-hGST P1-1, 26 860 M^{-1} cm^{-1} for the C47A, C101A, and C47A/C101A mutants, and 26 740 M^{-1} cm^{-1} for both C14A/C47A/C101A and C47A/C101A/C169A triple mutants. The purity and molecular weights of the proteins were determined using SDS−PAGE by the method of Laemmli (*35*) with a Bio-Rad Mini-Protean III (Hercules, CA), size-exclusion chromatography as described previously (*32*) and by electrospray ionization mass spectrometry (ESI-MS).

Enzyme Activity Assay. The initial rates of GST-catalyzed conjugation of GSH with CDNB were determined spectrophotometrically according to the method of Habig et al. (*36*). Reactions contained GST (1−2 μg/mL), GSH (2.5 mM), and CDNB (1 mM) in a total volume of 1.0 mL of 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA (assay buffer). The absorbance differences were recorded using a Hewlett-Packard (Palo Alto, CA) model 8452A diode array UV/vis spectrophotometer at 340 nm, 25 °C.

Electrospray Ionization Mass Spectrometry (ESI-MS). For analysis of the proteins covalently modified by 4-OHEN, 10 μM (as monomer) protein in 50 mM ammonium bicarbonate (pH 8.0) was incubated with 4-OHEN for 15 min at 37 °C. These solutions were diluted in 50% methanol/0.2% formic acid to give a final concentration of 2.5 pmol/μL. Each protein was analyzed by ESI-MS using a Micromass (Manchester, UK) Quattro II triple quadrupole mass spectrometer equipped with a Waters (Milford, MA) 2960 HPLC system. The protein samples were analyzed by flow injection using an injection volume of 10 μL and a carrier solvent of methanol/water/formic acid (50:49.8:0.2 v/v/v). Typical operating parameters were capillary voltage 3.6 kV, source temperature 80 °C, and cone voltage 41 V. The spectra were scanned over the range of *m*/*z* 620−1350 at 5 s/scan. The deconvoluted spectra were obtained using maximum entropy software (Micromass Co., Manchester, U.K.).

Analysis of Tryptic Digests. Complete or limited proteolysis by trypsin of wild-type hGST P1-1 (0.25 mg/mL) and enzyme treated with 30 μM 4-OHEN for 15 min (37 °C, pH 8.0, 50 mM ammonium bicarbonate buffer) was carried out according to the method of Stone et al. (*37*). The proteolytic fragments were analyzed by either LC/ESI-MS or sequencing analysis (*38*, *39*).

Effect of Scavengers of Reactive Oxygen Species on the Inactivation of GST by 4-OHEN. Initial rates of conjugation of GSH and CDNB catalyzed by wild-type His_6-hGST P1-1 were determined spectrophotometrically in the presence of scavengers of reactive oxygen species. Incubations contained wild-type His_6-hGST P1-1 (1 μg/mL), GSH (2.5 mM), CDNB (1 mM), 4-OHEN (5 μM), and various radical scavengers in assay buffer. The control incubations contained 10 μL of DMSO without 4-OHEN. The concentrations of scavengers were as follows: catalase (880 units/mL), superoxide dismutase (SOD) (210 units/mL), mannitol (10 mM), and sodium benzoate (10 mM).

Electrophoretic Analyses of GST Treated with 4-OHEN. Wild-type His_6-hGST P1-1 inactivated by 4-OHEN was analyzed using nonreducing SDS−PAGE. Briefly, the protein (10 μM) was incubated with several concentrations of 4-OHEN for various times in 50 mM ammonium bicarbonate buffer (pH 8.0) at 37 °C. Samples (200 μL) were mixed in sample buffer (40 μL) without the reducing agent β-mercaptoethanol (β-ME), followed by incubation at 100 °C for 5 min, and then subjected to SDS−PAGE.

Thiol Group Titration. The number of free thiol groups in His_6-hGST P1-1 was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to Riddles et al. (*40*) with minor modifications. Briefly, approximately 250 μg of each enzyme was incubated with either DMSO or various concentrations of 4-OHEN in 0.1 M potassium phosphate (pH 8.0) containing 1 mM EDTA in a total volume of 1 mL

at 37 °C for 15 min. Protein samples were then denatured by gel filtration on a NAP-5 column which had been equilibrated with buffer containing 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, and 6 M Gdn-HCl. The absorbance change at 412 nm was monitored for 20 min after addition of 0.1 mM DTNB to the denatured protein solution. Total thiol concentration was calculated using a molar absorptivity of 14 000 M^{-1} cm^{-1} obtained using GSH as a standard under the same buffer conditions.

Kinetics of Irreversible Inhibition. The proteins (100 μg/mL) were preincubated at 25 °C with different concentrations of 4-OHEN. Aliquots (10 μL) were removed at various times and diluted 100-fold into the assay buffer containing 2.5 mM GSH and 1 mM CDNB in a total volume of 1.0 mL. The kinetic experiments under anaerobic conditions were performed with enzymes prepared by nitrogen purge. Incubations were carried out in a tube purged with nitrogen and sealed with a rubber septum. 4-OHEN-*o*-quinone was prepared by autoxidation of 4-OHEN in the assay buffer at 25 °C for 30 s, and 10 μL of this *o*-quinone solution was injected into a sealed tube containing the enzyme. The conversion of the catechol to quinone was confirmed by following the increase in absorbance at 390 nm (chromophore for 4-OHEN-*o*-quinone) as described in ref *24*. Aliquots were removed using a syringe and diluted in the assay buffer. Initial rates were determined spectrophotometrically, and irreversible kinetic parameters were obtained as described previously (*28*, *41*).

RESULTS AND DISCUSSION

Recombinant hGST P1-1 and Cysteine Mutants. The studies with site-directed fluorescent reagents such as *N*-(4-anilino-1-naphthyl)maleimide (ANM) showed that Cys 47 is the most susceptible sulfhydryl group to covalent modification in hGST P1-1 (*15*). It has been shown that both Cys 47 and Cys 101 were selectively labeled by 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole (ABD-F), which specifically reacts with thiol groups, and that formation of a bulky adduct on Cys 47 prevented the substrate GSH from binding in the active site (*16*). Several other research groups have also shown that hGST P1-1 is sensitive to thiol group modifiers and that Cys 47 is the likely target of chemical modification leading to rapid inactivation (*19*, *42*). To study whether cysteine residues in hGST P1-1 are involved in the mechanism of enzyme inactivation mediated by 4-OHEN, we prepared a series of cysteine mutants. The five mutant proteins (C47A, C101A, C47A/C101A, C14A/C47A/C101A, and C47A/C101A/C169A mutants) were successfully overexpressed in *E. coli* as hexahistidine-tagged proteins and were purified by Co^{2+} affinity column chromatography. The hexahistidine tag eased purification since the proteins could be isolated without GSH contamination which has been shown to form glutathionyl−cysteine disulfide bonds during GSH affinity chromatography (*43*). We have previously shown that the hexahistidine tag had no effect on the specific activity of the enzyme (*32*). The purity of each mutant was determined to be over 95% by SDS−PAGE (data not shown). All mutant proteins eluted as dimers on size-exclusion chromatography as reported previously for the wild-type enzyme (*32*).

Kinetic Properties of Wild-Type and Mutant Enzymes. Substitution of Cys 47 with alanine in hGST P1-1 did not dramatically affect the affinity toward CDNB or V_{max} values (Table 2). The C47A mutant displayed a higher V_{max} when compared to the wild type. The affinity for GSH decreased by 9-fold in the C47A or C47A/C101A mutants and 13-fold in the C47A/C101A/C169A mutant (Table 2). In contrast, the kinetic parameters for the C101A mutant are similar to that of wild type. These results indicate that none of these cysteine residues are essential for the catalytic mechanism (*44*). The crystal structure of the hGST P1-1 complexed with *S*-hexylglutathione showed that Cys 47 is located at the end of the binding site for GSH (G-site) of α helix 2 (residues 37−46) (*45*, *46*). Site-directed mutagenesis studies as well as the crystal structure of the enzyme established that Cys 47 is not directly involved in the catalytic mechanism but rather in the correct spatial arrangement of the G-site (*47*, *48*) through ionic interactions with Lys 54. Therefore, substitution of Cys 47 to alanine results in an enzyme that exhibits lower affinity for GSH. These data are consistent with previous reports which confirmed the nonessential character of Cys 47 in the catalytic mechanism (*11*, *12*).

Table 2: Kinetic Constants for Mutants of His_6-hGST P1-1

enzyme	specific activity[a] (units/mg)	V_{max}[b] (μmol mg^{-1} min^{-1})	K_m^{GSH}[b] (mM)	K_m^{CDNB}[c] (mM)
wild type	124 ± 7.4	123 ± 14	0.14 ± 0.01	1.09 ± 0.06
C47A	82.3 ± 1.9	185 ± 27	1.24 ± 0.04	1.15 ± 0.05
C101A	161.5 ± 7.3	117 ± 6.0	0.23 ± 0.01	0.83 ± 0.02
C47A/C101A	93.8 ± 3.1	109 ± 21	1.35 ± 0.1	1.09 ± 0.03
C47A/C101A/C169A	55.9 ± 2.2	118 ± 6.0	1.82 ± 0.02	0.66 ± 0.02
C14A/C47A/C101A	6.20 ± 1.5	ND[d]	ND	ND

[a] Specific activities were measured at 25 °C with 2.5 mM GSH and 1 mM CDNB in 0.1 M phosphate buffer (pH 6.5) containing 1 mM EDTA. [b] Apparent V_{max} and K_m values for GSH were obtained at 1 mM CDNB by Lineweaver−Burk analysis. [c] K_m values for CDNB were calculated at 2.5 mM GSH using Lineweaver−Burk analysis. [d] Not determined.

ESI-MS Analyses of 4-OHEN-Modified Proteins. Wild-type His_6-hGST P1-1 and mutants incubated with a 1−3 molar excess of 4-OHEN were analyzed using ESI-MS to determine if the enzyme had been covalently modified by 4-OHEN-*o*-quinone. The deconvoluted ESI mass spectra showed that wild-type His_6-hGST P1-1 incubated with a 1:1 molar ratio of 4-OHEN was partially alkylated (Figure 1A), while complete modification was achieved with a 3 molar excess of 4-OHEN (Figure 1B). The two major peaks in the mass spectrum (Figure 1A) correspond to the unmodified wild type (25 630 Da) and the protein plus 4-OHEN-*o*-quinone (25 910 Da), respectively. Monoadducts alkylated with 4-OHEN-*o*-quinone were also detected in the ESI mass spectra of C47A, C101A, C47A/C101A, and C47A/C101A/C169A mutants after similar experiments (Figure 1C−F). No peak corresponding to modified protein was observed in the ESI mass spectrum of the C14A/C47A/C101A mutant following incubation with 4-OHEN-*o*-quinone (Figure 1G), suggesting that the Cys 169 might be the only cysteine residue that does not react with 4-OHEN-*o*-quinone under these conditions.

Attempts were made to identify the specific modified cysteine residue by trypsin digestion of the 4-OHEN-treated

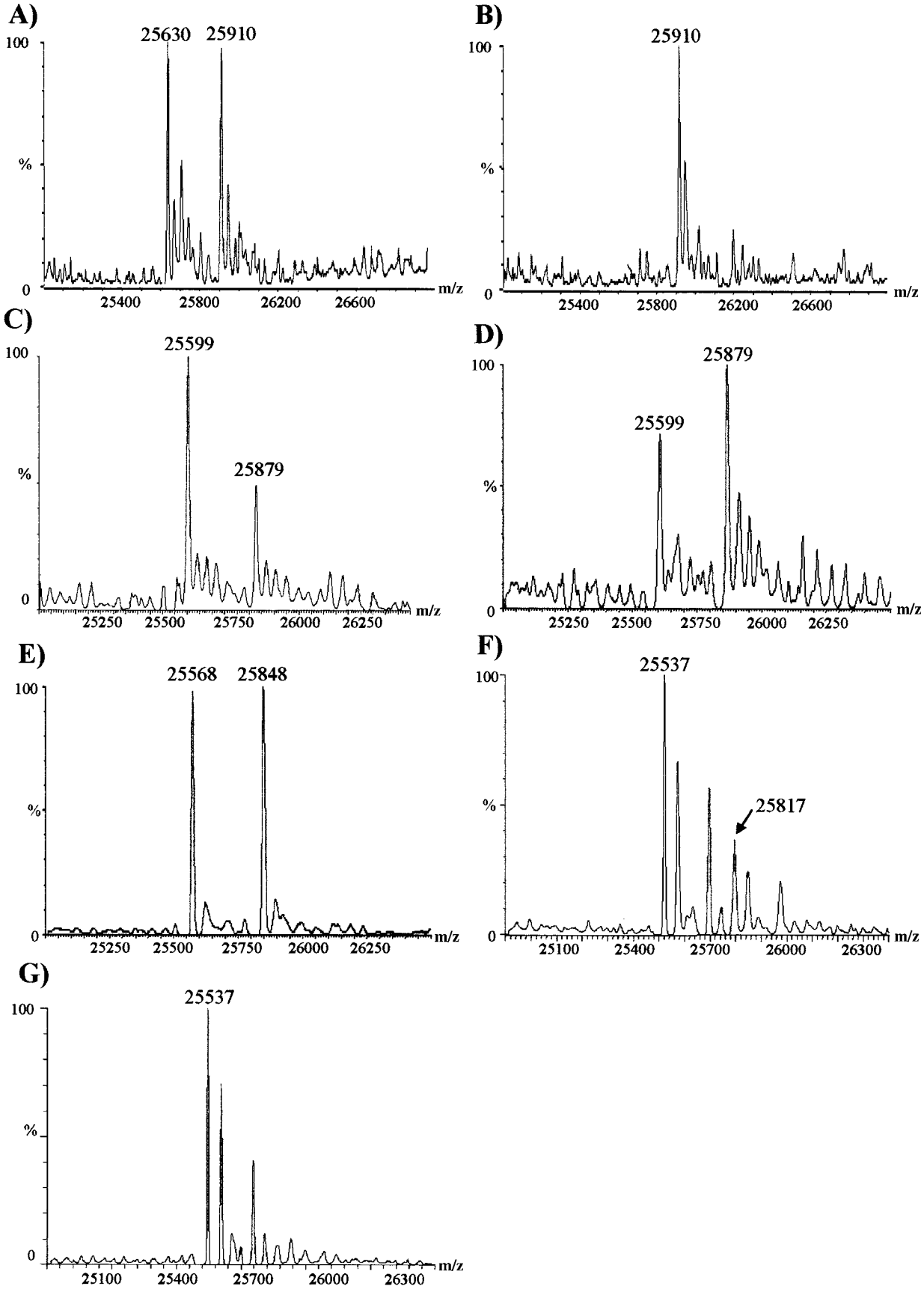


FIGURE 1: Electrospray mass spectra of His_6-hGST P1-1 modified by 4-OHEN. The proteins were analyzed by ESI-MS in positive ion mode as described in Experimental Procedures. The protein (10 μM) was incubated with 1 molar equiv of 4-OHEN unless otherwise specified. Spectra: (A) wild type, (B) wild type incubated with 3 molar equiv of 4-OHEN, (C) C47A mutant, (D) C101A mutant, (E) C47A/C101A mutant, (F) C47A/C101A/C169A mutant, and (G) C14A/C47A/C101A mutant.

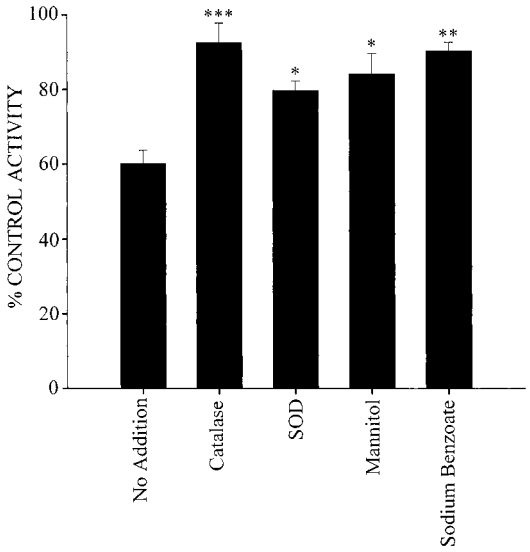


FIGURE 2: Effect of scavengers of reactive oxygen species on the inactivation of GST activity by 4-OHEN. Incubations contained wild-type His_6-hGST P1-1 (1 μg/mL), GSH (2.5 mM), CDNB (1 mM), 4-OHEN (5 μM), and various radical scavengers in 0.1 M potassium phosphate (pH 6.5) containing 1 mM EDTA. The control incubations contained 10 μL of DMSO without 4-OHEN. The concentrations of scavengers were as follows: catalase (880 units/mL), superoxide dismutase (SOD) (210 units/mL), mannitol (10 mM), and sodium benzoate (10 mM). Asterisks represent values significantly different from no addition of scavengers: *, $p \leq 0.001$; **, $p \leq 0.0005$; ***, $p \leq 0.0001$.

enzyme followed by peptide mapping. However, these experiments gave equivocal results likely due to the instability of the cysteine conjugates. We have previously shown that 4-OHEN GSH conjugates have half-lives of less than 3 h in acidic solution and they are considerably more labile under physiological conditions (*28*). In addition, it is known that quinone thioethers are much more redox active than their corresponding unmodified *o*-quinones due to the electron-donating thiol residue which makes these conjugates very labile (*49*, *50*). As a result, it was not possible to identify the specific cysteine residue(s) modified by 4-OHEN due to the length of time required for enzymatic digestion during which time the protein cysteine conjugates degraded.

Inhibition of His_6-hGST P1-1 by 4-OHEN and Effects of Radical Scavengers. hGST P1-1 is known to undergo oxidative damage by hydrogen peroxide or copper ion, causing a loss of enzymatic activity (*13*, *21*, *51*). As a result, it was of interest to determine whether reactive oxygen species generated from redox cycling of 4-OHEN (Scheme 1) are involved in oxidative inactivation of hGST P1-1. We investigated if the inhibitory effect of 4-OHEN on His_6-hGST P1-1 could be modulated by scavengers of reactive oxygen species. The data presented in Figure 2 showed that superoxide dismutase, catalase, mannitol, and sodium benzoate which are scavengers of $O2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$, respectively, protected His_6-hGST P1-1 from inhibition by 4-OHEN. These data suggest that reactive oxygen species play a role in 4-OHEN-mediated His_6-hGST P1-1 inhibition.

Band Patterns of Wild-Type His_6-hGST P1-1 Inactivated by 4-OHEN on Nonreducing SDS−PAGE. The formation of internal disulfide bonds in proteins often results in the generation of intermediates that can be distinguished from reduced forms by their altered migration on nonreducing SDS−PAGE which can be reversed under reducing conditions (*52*−*54*). In addition, disulfide-linked aggregates can be induced by thiol/disulfide exchange through thiol groups in the modified protein (*55*). Shen et al. proposed that H_2O_2 caused intrasubunit disulfide bond formation in rat pi class GST between Cys 47 and Cys 101, Cys 47 and Cys 169, or Cys 14 and Cys 169 (*20*). In addition, Cys 47 participated in interdisulfide bond formation, and Cys 101 was responsible for polymer formation. Since rat pi class GST conserves the four cysteines present in the pi class of human GST, hGST P1-1 might be expected to form intra/intersubunit disulfide bonds in a similar fashion. However, Ricci et al. reported that only one intradisulfide bond between Cys 47 and Cys 101 was formed in hGST P1-1 after metal ion catalyzed oxidation with no evidence of intersubunit disulfide bonds (*21*).

To address the effect of 4-OHEN on formation of disulfide bonds, wild-type His_6-hGST P1-1 was treated with 4-OHEN and analyzed using nonreducing SDS−PAGE. Figure 3 showed that 4-OHEN treatment produced more than three different species of oxidized enzyme due to both intrasubunit disulfide bond formation and oligomerization through intersubunit disulfide bond formation. The SDS−PAGE experiments performed with other cysteine mutants suggest that band B (23.5 kDa) was the result of intrasubunit disulfide bond formation between Cys 14 and Cys 169 since band B was not observed in experiments with C14A/C47A/C101A or C47A/C101A/C169A mutants but was detected in studies with C47A, C101A or C47A/C101A mutants. Band C (21.5 kDa) resulted from intradisulfide bond formation between Cys 47 and Cys 101 similar to what was previously reported by Ricci et al. (*21*). In addition, band D (43 kDa) was likely a dimeric enzyme formed by disulfide bonds between subunits. It has been shown that protein aggregates/conglomerates can be induced by reactive oxygen species (*56*, *57*). Similar types of aggregates/conglomerates were also observed with hGST P1-1 treated with 4-OHEN (designated as E in Figure 3), implying oxygen radicals generated from redox cycling of 4-OHEN induced protein damage. These changes in tertiary structure of His_6-hGST P1-1 induced by 4-OHEN were time (Figure 3A) and concentration (Figure 3B) dependent with preferred formation of protein aggregates/conglomerates at higher concentrations of 4-OHEN and longer incubation times.

Titration of Thiol Groups in 4-OHEN-Modified His_6-hGST P1-1 with DTNB. The reduction in the number of protein thiols could be due to either covalent modification or oxidation to form disulfides or mixed disulfides. Since 4-OHEN forms electrophilic/redox-active quinoids, the number of accessible thiols in hGST P1-1 are expected to decrease with increasing concentration of 4-OHEN. Thiol group titration experiments were carried out using the DTNB assay to investigate whether the nucleophilic thiol group of cysteine was modified by 4-OHEN through either disulfide bond formation or covalent modification and to determine the selectivity of the cysteine residues in hGST P1-1. To take into account all of the free cysteine residues present in the enzyme after modification, thiol groups were titrated under denaturing conditions. All GSTs showed a decrease in thiol groups after 4-OHEN treatment in a concentration-dependent fashion (Figure 4). Both wild type and the C101A mutant (10 μM) treated with 2 μM 4-OHEN showed that

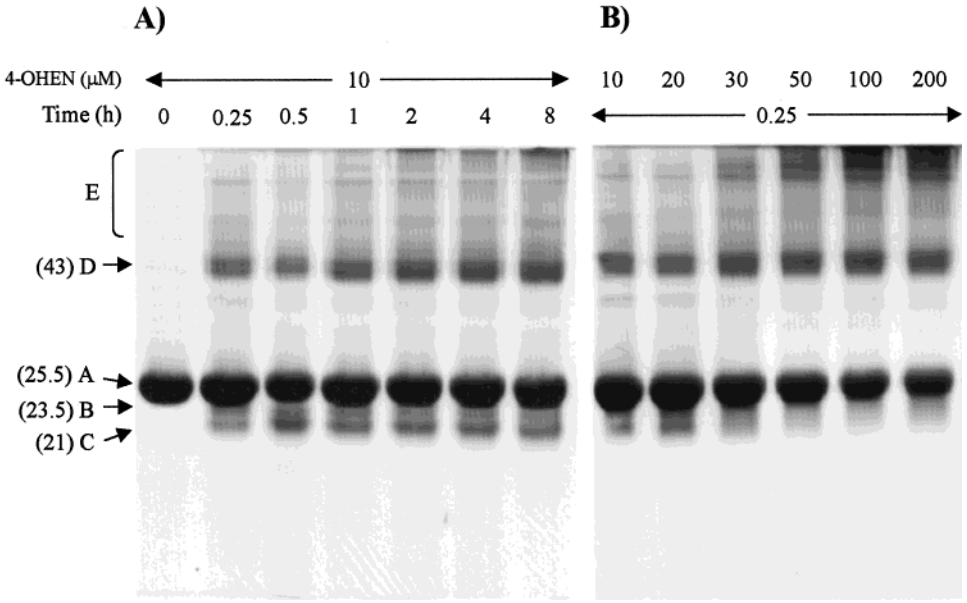


FIGURE 3: Nonreducing SDS−PAGE of wild-type His_6-hGST P1-1 modified by 4-OHEN. (A) Aliquots (200 μL) from an incubation mixture containing 10 μM enzyme and 10 μM 4-OHEN in 50 mM ammonium bicarbonate buffer (pH 8.0) at 37 °C were removed at different time points. (B) Protein (10 μM) was incubated with various concentrations of 4-OHEN in 50 mM ammonium bicarbonate buffer (pH 8.0) at 37 °C for 15 min. Samples were diluted 5-fold in sample buffer without the reducing agent β-ME. Each lane contained 10 μg of protein, and the protein bands were visualized with Coomassie blue.

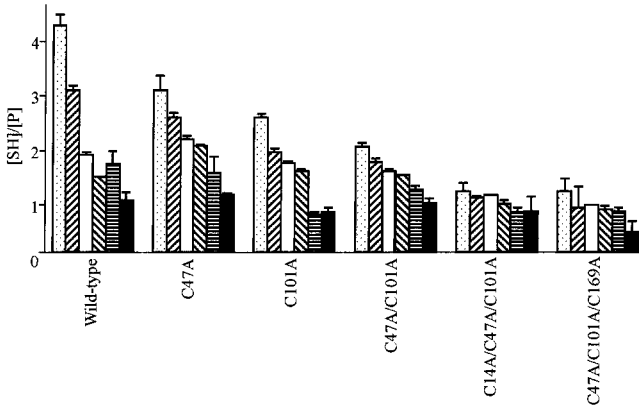


FIGURE 4: Thiol group titration of 4-OHEN-modified wild type and mutants with DTNB. The enzymes (250 μg) were incubated with either DMSO or various concentrations of 4-OHEN in 0.1 M potassium phosphate (pH 8.0) containing 1 mM EDTA at 37 °C for 15 min. The absorbance change at 412 nm was monitored for 20 min after addition of 0.1 mM DTNB to the solution of denatured protein in 0.1 M potassium phosphate (pH 8.0), 1 mM EDTA, and 6 M Gdn-HCl. Total thiol concentration was calculated with a molar extinction coefficient of 14 000 M^{-1} cm^{-1} obtained from GSH as a standard under the same buffer conditions: bar with black dots, DMSO; bar with slashes, 1 μM; open bar, 2 μM; bar with back-slashes, 3 μM; bar with horizontal stripes, 6 μM; solid bar,10 μM 4-OHEN.

two thiol groups had been modified, whereas 10 μM 4-OHEN was required to modify two thiol groups in C47A and C47A/C101A mutants. No complete loss of free thiols was observed even at high concentrations of 4-OHEN. Even after treatment of wild type as well as other mutants with concentrations higher than 10 μM 4-OHEN, approximately one thiol group remained unmodified, implying that a single cysteine remains resistant to 4-OHEN modification under these conditions. For example, in the C14A/C47A/C101A triple mutant free thiol concentration did not significantly decrease after incubation with high concentrations of 4-OHEN, implying that Cys 169, the only cysteine left in that

Table 3: Kinetic Parameters of Irreversible Inhibition of His_6-hGST P1-1 by 4-OHEN[a]

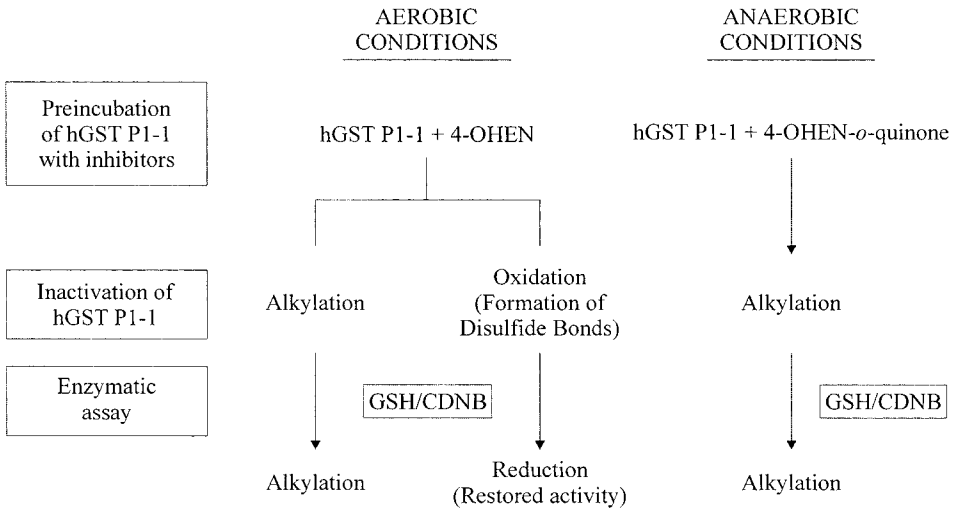
	aerobic conditions		anaerobic conditions	
enzyme	K_i (μM)	k_2 ($\times 10^{-3}$ s^{-1})	K_i (μM)	k_2 ($\times 10^{-3}$ s^{-1})
wild type	18.3 ± 0.3	1.72 ± 0.02	34.0 ± 0.6	2.15 ± 0.03
C47A	109 ± 1.4	3.64 ± 0.04	74.2 ± 4.0	4.23 ± 0.23
C101A	11.9 ± 0.2	1.32 ± 0.02	21.8 ± 1.4	1.20 ± 0.07
C47A/C101A	502 ± 1.3	22.2 ± 0.06	78.4 ± 1.5	4.00 ± 0.07
C47A/C101A/C169A	84.2 ± 2.8	13.1 ± 0.43	97.3 ± 0.8	9.00 ± 0.08
C14A/C47A/C101A	ND[b]	ND	ND	ND

[a] The dissociation constant K_i and the rate constant for irreversible inhibition k_2 were obtained for wild type as well as mutants as described in Experimental Procedures. [b] Not determined.

mutant, was resistant to 4-OHEN modification. On the other hand, 10 μM 4-OHEN reduced the molar ratio of thiols to protein from 1.1 to 0.38 in the C47A/C101A/C169A mutant, which suggests that Cys 14 reacts with 4-OHEN. These results are supported by the ESI-MS data in which no modification was detected in the C14A/C47A/C101A triple mutant treated with 4-OHEN. Taken together, these data indicate that Cys 169 is resistant to reaction with 4-OHEN.

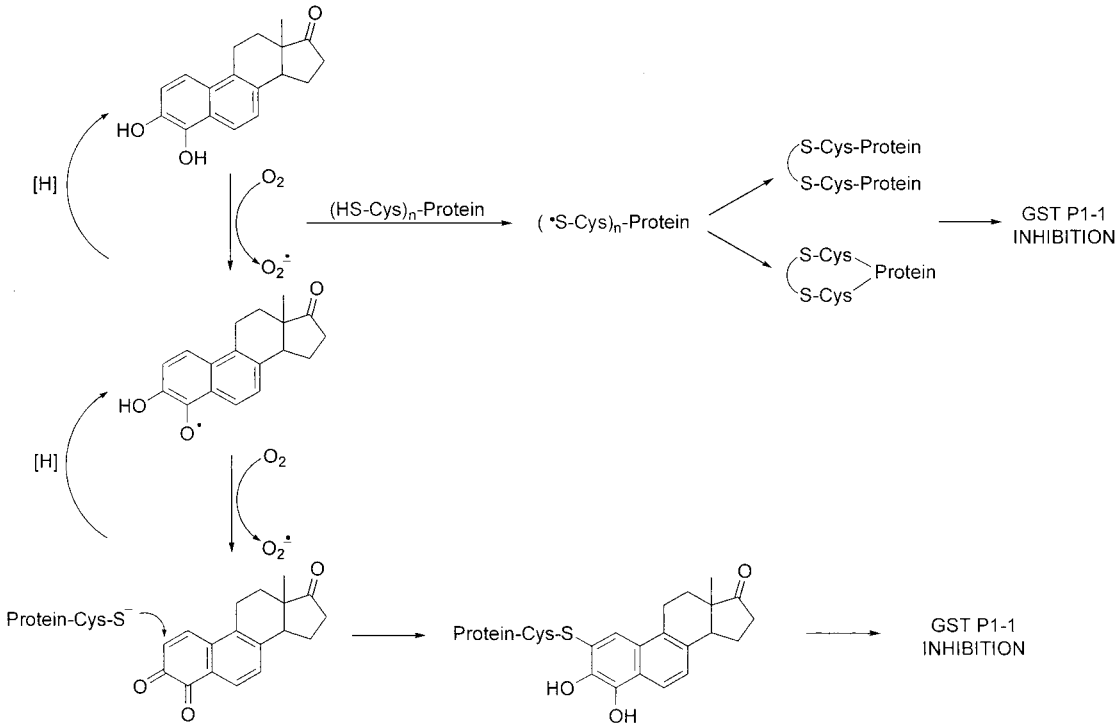
Kinetics of Irreversible Inhibition. Inhibition kinetic studies were performed according to Kitz and Wilson (*41*), since enzyme activity could not be restored after gel filtration, inhibition of enzyme was not reversible by 100-fold dilution, and the inhibition progressed exponentially with time. Since GSH, which was added as one of the enzyme substrates, reduces disulfide bonds, the inactivation of the enzyme due to oxidative damage was at least partially negated upon addition of GSH to the assay buffer, resulting in higher enzymatic activity. Therefore, kinetic experiments were carried out under anaerobic conditions, which prevents formation of reactive oxygen species produced by redox cycling of 4-OHEN (Scheme 2), thereby differentiating covalent modification from oxidative damage in the mech-

Scheme 2: Scheme Showing Kinetic Experiments under Aerobic or Anaerobic Conditions[a]



[a] The enzymes were preincubated with 4-OHEN in the presence of air, and the aliquots were diluted 100-fold into the assay buffer containing GSH (2.5 mM) and CDNB (1 mM). For kinetic experiments under anaerobic conditions, the enzymes were preincubated with 4-OHEN-*o*-quinone under nitrogen, and 4-OHEN-*o*-quinone was prepared by autoxidation of 4-OHEN in the assay buffer (pH 6.5) at 25 °C for 30 s. The initial rates were determined spectrophotometrically as described in Experimental Procedures.

Scheme 3: Proposed Mechanisms for Inactivation of HGST P1-1 Mediated by 4-OHEN[a]



[a] *n* is the number of cysteine residues in HGST P1-1 and [H] refers to any reducing agent.

anism(s) of 4-OHEN-mediated enzyme inactivation. The dissociation constants for the reversible enzyme−inhibitor complex (K_i) and the rate constants for the conversion of the reversible enzyme−inhibitor complex to the irreversibly inhibited enzyme (k_2) were obtained for the wild type and mutants (Table 3). Kinetic parameters could not be obtained with C14A/C47A/C101A, since the control activity (without inhibitor) for this mutant was very low (6% of the wild-type activity; Table 2), resulting in no appreciable values of initial rates. The data showed that the wild type and the C101A mutant had much lower K_i values than the other proteins containing the alanine at position 47, implying that Cys 47 is critical in terms of determining the affinity of 4-OHEN. However, the rate of the conversion of the enzyme−inhibitor complex to the irreversibly inhibited enzyme was found to be higher in the mutant enzymes that contain no Cys 47. Two different kinetic experiments under aerobic compared to anaerobic conditions with one enzyme resulted in different kinetic parameters (Table 3). Interestingly, a large decrease in the K_i value for the C47A/C101A mutant from aerobic ($K_i = 502 \pm 1.3$ μM) to anaerobic conditions ($K_i = 78.4 \pm 1.5$ μM) was observed, unlike similar experiments with the wild-type and other mutant enzymes where the K_i values did not vary significantly. These

results suggest that disulfide bond formation may play an important role in the inhibition mechanism(s) in the C47A/C101A mutant, while covalent modification may mainly contribute to the inactivation mechanism(s) in the other mutants.

It has recently been demonstrated that the monomeric form of hGST P1-1 elicits inhibitory activity toward c-Jun N-terminal kinase (JNK; stress activated kinase), implying hGST P1-1 may play a role as an endogenous regulator of JNK activity (*58*). The activation of JNK can also be influenced by additional exogenous oxidants or antioxidants (*59*, *60*), suggesting a role of redox status regulation in the kinase-mediated stress response pathways. Changes in JNK activity could impact cell growth, apoptosis, and cellular transformation (*61*). Given the physiological roles of hGST P1-1 in stress response signaling pathways linked to apoptosis or proliferation and the cytotoxic/genotoxic effects exhibited by 4-OHEN, it is of interest to explore whether the mechanism(s) of induction of apoptosis or transformation by 4-OHEN quinoids proceed(s) through GST P1-1 inactivation in vivo, and these studies will be the subject of future work.

In conclusion, the potential mechanism(s) of GST P1-1 inhibition by 4-OHEN are presented in Scheme 3. 4-OHEN-*o*-quinone could alkylate critical amino acid residues such as cysteines in hGST P1-1, leading to inactivation of the enzyme. Reactive oxygen species generated from redox cycling of 4-OHEN are responsible for disulfide bond formation as well as changes in the tertiary structure of the enzyme leading to enzyme inactivation. Such events occur with differing selectivity among the various cysteine residues in hGST P1-1 with respect to reactivity with 4-OHEN. We are currently investigating whether alkylation/oxidation of proteins and GSTs in particular could occur in vivo and how protein inactivation may contribute to the cytotoxic/genotoxic effects induced by 4-OHEN.

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