Photoaffinity Labeling of Rat Liver Glutathione *S*-Transferase, 4-4, by Glutathionyl *S*-[4-(Succinimidyl)-benzophenone]†

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ABSTRACT: Glutathionyl *S*-[4-(succinimidyl)benzophenone] (GS-Succ-BP), an analogue of the product of glutathione and xenobiotic substrate, was synthesized and shown to act as a photoaffinity label of rat liver glutathione *S*-transferase, 4-4. A time-dependent photoinactivation occurs upon irradiation at long wavelength UV light of the complex of enzyme and GS-Succ-BP. The rate of inactivation exhibits nonlinear dependence on [GS-Succ-BP], characterized by an apparent K_I of 115 μM and k_{max} of 0.469 min^{-1}. Effective protection against photoinactivation by 150 μM GS-Succ-BP is provided by dinitrophenol, nitrobenzene, ethacrynic acid, and S-hexylglutathione, analogues of xenobiotic substrates and product. These results suggest that GS-Succ-BP reacts with the enzyme within the active site, probably in the xenobiotic substrate-binding site. Upon complete inactivation, reagent incorporation of about 1 mol/mol of enzyme dimer is measured by radioactivity and MALDI-TOF mass spectrometry. Isolation of modified peptides followed by gas-phase sequencing and mass spectrometry indicates that Met-112 is the only reaction target of GS-Succ-BP. Although only one subunit of the enzyme dimer is modified, catalytic activity of both subunits is lost. Molecular modeling suggests that the benzophenone moiety of the compound binds in the cleft between the two enzyme subunits and modification of Met-112 on one subunit excludes reaction of the corresponding methionine on the other subunit. It is proposed that the new compound, glutathionyl *S*-[4-(succinimidyl)benzophenone], may have general applicability as a photoaffinity label of other enzymes with glutathione binding sites.

The glutathione *S*-transferases (EC 2.5.1.18) constitute a group of isoenzymes involved in the metabolism of both endogenous and xenobiotic compounds. They catalyze the conjugation reaction of glutathione with various electrophilic substrates including aryl halides, alkyl halides, sulfate esters, nitrate esters, epoxides, and α,*β*-unsaturated ketones (*1*−*6*). The resulting glutathione adducts can be degraded and transported out of the cell, thus providing a detoxification pathway for these electrophiles. The cytosolic enzymes, which have been grouped into at least six classes (α, *μ*, *π*, *θ*, *κ*, and *σ*), can exist as either homo or heterodimers. Each enzyme subunit contains a glutathione and a xenobiotic substrate-binding site that can accommodate diverse hydrophobic substrates. Crystal structures have been reported for representative enzymes from each gene class (*7*−*13*). All structures share similar topology, although the structural basis of the distinct substrate specificity for each isoenzyme is still not well understood (*4*).

Various affinity labels have been used to probe the hydrophobic-binding site of the enzyme (*14*). Most of these affinity labels have been alkylating reagents which require proximity to a nucleophilic side chain of an enzymic amino acid in order for covalent reaction to occur; these reagents would be incapable of reacting with the hydrophobic amino acids which are most likely to contribute to binding of the xenobiotic substrates of glutathione *S*-transferase. We have designed glutathionyl *S*-[4-(succinimidyl)benzophenone] (GS-Succ-BP)[1] as a new photoaffinity label for glutathione *S*-transferase. This reagent, shown in Figure 1, retains the glutathione moiety, which is expected to direct it to the active site of glutathione *S*-transferase. In addition, it features the photoreactive benzophenone group which can react with C−H bonds of either side chain or enzyme backbone (*15*−*17*). In this paper, we demonstrate that GS-Succ-BP reacts specifically with Met-112 of the *μ* class glutathione *S*-transferase, isoenzyme 4-4,[2] and present evidence that modification of a methionyl residue on one subunit prevents reaction at a second methionine on the other subunit of the dimer. A preliminary version of this study has been presented (*19*).

EXPERIMENTAL PROCEDURES

Materials. Frozen Sprague−Dawley rat livers were purchased from Pel Freez Biologicals. Glutathione, *S*-

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[1] Abbreviations: BP-MAL, benzophenone-4-maleimide; GS-Succ-BP, glutathionyl S-[4-(succinimidyl)benzophenone]; GST 4-4, glutathione *S*-transferase, isoenzyme 4-4; CDNB, 1-chloro-2,4-dinitrobenzene; PTH, phenylthiohydantoin; DMF, *N*,*N*′-dimethylformamide; mBBr, monobromobimane; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; MALDI TOF, matrix-assisted laser desorption ionization time-of-flight; TFA, trifluoroacetic acid.

[2] Glutathione *S*-transferase, isoenzyme 4-4, is designated as the rGSTM2,2 isoenzyme in the nomenclature proposed by Hayes and Pulford (*18*). Glutathione *S*-transferase, isoenzyme 3-3, is designated as the rGSTM1,1 isoenzyme.

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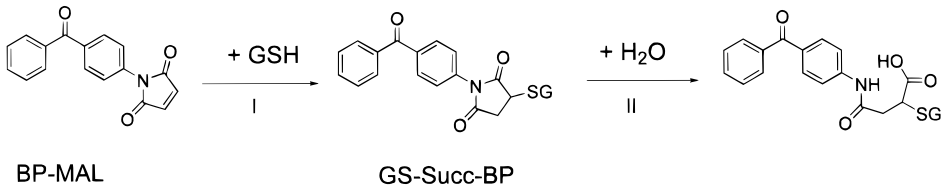

FIGURE 1: Scheme of synthesis of photoaffinity label glutathionyl *S*-[4-(succinimidyl)benzophenone] (GS-Succ-BP) from benzophenone-4-maleimide (BP-MAL) and glutathione (GSH) (step I). Under certain conditions, such as those used for peptide isolation, GS-Succ-BP is hydrolyzed (step II).

methylglutathione, *S*-hexylglutathione, *S*-hexylglutathione-Sepharose, Sephadex G-50, *N*-ethylmaleimide (NEM), 2,4-dinitrophenol, nitrobenzene, benzophenone-4-maleimide (BP-MAL), sinapinic acid, ethacrynic acid, 17β-estradiol-3,17-disulfate, lactic dehydrogenase from porcine muscle (Type XXIS-S), cytochrome *c* from pig heart, insulin B chain oxidized, trypsin, and α-chymotrypsin were obtained from Sigma Chemical Co. 1-Chloro-2,4-dinitrobenzene (CDNB) was supplied from Aldrich Chemical Co. Polybuffer exchanger PBE 118 and Pharmalyte, pH 8−10.5, were purchased from Pharmacia Fine Chemicals. Bio-Rad protein assay dye reagent was supplied by Bio-Rad Laboratories. Liquiscint was purchased from National Diagnostics, and ultrapure urea was from Schwartz/Mann Biotech. Monobromobimane (mBBr) was obtained from Molecular Probes, Inc. Steraloids, Inc., Wilton, New Hampshire, provided Δ^5-androstene-3,17-dione, Analtech supplied Cellulose TLC plates (MN300F, 250 microns), and Dupont NEN supplied glutathione, [glycine-2-^{3}H].

Enzyme Preparation. Rat liver glutathione *S*-transferase, 4-4 (GST 4-4) was purified from Sprague−Dawley rat livers by a simplified procedure using only affinity column chromatography on *S*-hexylglutathione-Sepharose followed by chromatofocusing on PBE 118 resin in the pH range of 10.8−7 (*20*). The 4-4 isoenzyme was eluted last from the chromatofocusing column after α class isoenzymes (1-1, 1-2, 2-2) and μ class isoenzymes (3-3, 3-4). In a typical experiment, about 16 mg of 4-4 isoenzyme was isolated from 160 g of rat liver. The protein concentration was measured using $\epsilon_{270\ nm}$ of 36 700 $M^{-1}cm^{-1}$ (*21*) and a M_r of 26 500 per subunit (*22*). The purity of the final preparation was evaluated by HPLC on a Vydac C_4 reversed-phase column using a 50 min gradient of 30 to 60% acetonitrile containing 0.1% trifluoroacetic acid. On the basis of the absorbance at 220 nm, the purity was more than 95% for each final preparation.

Synthesis of Glutathionyl S-[4-(Succinimidyl)benzophenone] (GS-Succ-BP). As shown in Figure 1, step I, the GS-Succ-BP was synthesized by addition of the −SH of glutathione to the maleimide moiety of benzophenone-4-maleimide (BP-MAL). BP-MAL (1 molar equivalent) and GSH (1.1 molar equivalent) were mixed in acetonitrile and water (7:2) solution. To synthesize radioactive GS-Succ-BP, 50 μCi of [^{3}H]glutathione was mixed with 50 μmol of unlabeled glutathione. The dithiothreitol (present in the commercial radioactive glutathione) was first removed by extraction with ethyl acetate, using the method of Butler et al. (*23*). The reaction of BP-MAL and GSH was carried out in the dark at room temperature for 4 h. The resulting solution, containing about 50 μmol GS-Succ-BP, was applied to a preparative cellulose TLC plate (MN300F) (250 μm thickness). The plate was then developed at room temperature in *n*-butanol/acetic acid/water (20:5:8). The GS-Succ-BP could be visualized by its UV absorbance and by reaction of its α-amino-group with ninhydrin. This band was scraped from the plate, and the product was eluted from cellulose with dimethylformamide (DMF). The concentration of GS-Succ-BP was determined by the reaction of its primary amino group with 2,4,6-trinitrobenzenesulfonic acid (*24*). In the determination, reduced glutathione was used as the standard.

Enzymatic Assays. Enzyme activity was routinely measured using a Gilford model 240 spectrophotometer by monitoring the formation of conjugate between 2.5 mM glutathione and 1.0 mM 1-chloro-2,4-dinitrobenzene at 340 nm ($\Delta\epsilon$ = 9.6 mM^{-1} cm^{-1}) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C according to the method of Habig et al. (*25*). Enzyme activity toward monobromobimane (mBBr) was measured according to the method of Hulbert and Yakubu (*26*) using a Perkin-Elmer MPF-3 fluorescence spectrophotometer (excitation at 395 nm and emission at 480 nm) by monitoring the formation of the conjugate between 600 μM glutathione and 100 μM mBBr in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C.

Reaction of GS-Succ-BP with Glutathione S-Transferase, Isoenzyme 4-4. Glutathione *S*-transferase (0.4 mg/mL) was incubated with various concentrations of GS-Succ-BP in 0.1 M potassium phosphate buffer, pH 6.5, containing 15% dimethylformamide (DMF). The solution (100−250 μL/well) on ice was irradiated with Spectroline model ENF-24 UV lamp at long wavelength (365 nm). Aliquots (20 μL) were diluted 500-fold and assayed for residual activity after various times of irradiation. When the effect of ligands on the rate of inactivation was studied, the ligands were preincubated with enzyme for 10 min prior to the addition of GS-Succ-BP.

Incorporation of [^{3}H]GS-Succ-BP into Glutathione S-Transferase. Enzyme was incubated with 100 μM [^{3}H]GS-Succ-BP and irradiated with long-wavelength UV light, as described above. At various times, an aliquot of the reaction mixture (500 μL) was withdrawn. Excess reagent was removed using the gel centrifugation method of Penefsky (*27*). The reaction mixture aliquot was applied to one 5 mL Sephadex G-50 column equilibrated with 0.1% trifluoroacetic acid, pH 2.0. The protein concentration in the eluate was determined by the Bio-Rad protein assay, which is based on the Bradford dye binding method (*28*), using unmodified glutathione *S*-transferase as standard. A Bio-Rad 2550 RIA plate reader equipped with a 600 nm filter was used to measure the absorbance. The amount of reagent incorporated into the enzyme was determined from the radioactivity measured using a Packard Tri-Carb liquid scintillation counter, model 1500.

Proteolysis of GS-Succ-BP-Modified Glutathione S-Transferase. Glutathione *S*-transferase (0.4 mg/mL) was incubated

with 100 μM [^{3}H]GS-Succ-BP and irradiated with long wavelength UV light (250 μL/well) on ice for 15 min. The reaction mixture was then divided into 500 μL aliquots and excess reagent was removed by applying the aliquot to a 5 mL Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The sulfhydryl groups of free cysteine residues of the enzyme were blocked by addition of *N*-ethylmaleimide to a final concentration of 5 mM. After incubation under nondenaturing conditions at 25 °C for 5 min, solid urea was added to the solution to yield a final concentration of 8 M in order to completely denature the enzyme. After incubation under denaturing conditions at 25 °C for another 30 min, the solution was dialyzed against 6 L of 50 mM ammonium bicarbonate, pH 8.0, at 0 °C, with one change for a total of 20 h. The enzyme solution was lyophilized and redissolved in 250 μL of 50 mM ammonium bicarbonate solution, pH 8.0. Solid urea was added to a concentration of 8 M and the solution was incubated at 25 °C for 30 min. A 50 mM ammonium bicarbonate solution (750 μL) was added to dilute the urea concentration to 2 M. Trypsin (5% w/w) was added and digestion was conducted at 37 °C for 1 h, followed by another 1 h after a second addition of 5% trypsin. The solution was filtered with no loss of radioactivity and was immediately subjected to HPLC.

Separation of Modified Peptides by HPLC. After proteolysis, the tryptic digest was separated by HPLC on a Varian 5000 LC equipped with a Vydac C_{18} column (0.46 $\times$ 25 cm). The detector was set at 220 nm to monitor the elution of peptides. Solvent system I used to initially separate modified peptide from most other peptides was 16 mM ammonium acetate in H_2O, pH 6.0, (solvent A), and 16 mM ammonium acetate, pH 6.0, in 60% acetonitrile (solvent B). After elution with 0% solvent B for 10 min, a linear gradient was run to 70% solvent B at 185 min followed by a linear gradient to 100% solvent B at 240 min. The flow rate was 1 mL/min, and 1 mL fractions were collected. Aliquots of the fractions were mixed with 5 mL of Liquiscint, and the samples were monitored for radioactivity.

When further purification of peptides was needed, peaks from solvent system I were pooled, lyophilized, and separated using solvent system II: 0.1% trifluoroacetic acid (TFA) in H_2O as solvent A and 0.075% TFA in acetonitrile as solvent B. After elution with 0% solvent B for 10 min, a linear gradient was run to 10% of solvent B at 20 min followed by successive linear gradients to 40% B at 140 min and 100% at 160 min at a flow rate of 1 mL/min.

In some cases, purified peptides from HPLC solvent system II were lyophilized and redigested with 5% chymotrypsin at 37 °C for 1 h in 50 mM ammonium bicarbonate, pH 8.0. After redigestion, the peptides were fractionated by HPLC using solvent system II.

Sequence Determination of Separated Peptides. The amino acid sequences of purified peptides were determined on an Applied Biosystems gas-phase protein (peptide) sequencer, model 470, equipped with a phenylhydantoin analyzer, model 120, and a model 900A computer. Typically, 20−100 pmol samples of peptide were analyzed.

MALDI-TOF Mass Spectrometry. Reagent incorporation was also determined by mass spectrometry. Modified enzyme was prepared as described above. Excess reagent was removed by HPLC with a Vydac C_4 column using solvent system III: 0.1% TFA in H_2O as solvent A and 0.075% TFA in acetonitrile as solvent B. After elution with 30% solvent B for 10 min to remove excess reagent, a linear gradient was run to 60% solvent B at 40 min followed by a linear gradient to 100% B at time 50 min. The peak with modified enzyme was lyophilized and redissolved in 0.1% TFA/H_2O solution. Enzyme solution (2 μL) was mixed with 2 μL of saturated sinapinic acid solution (in 70% H_2O, 30% acetonitrile), and 1 μL of this mixture (about 30 pmol protein) was applied to the sample plate and air-dried. The mass of the protein was determined by MALDI-TOF mass spectrometry using a Voyager DE BioSpectrometry Workstation of PerSeptive Biosystems. Measurement was carried out using the linear mode with accelerating voltage at 25 kV, grid voltage at 92%, guide wire voltage at 0.1%, laser step 2150 and delay time at 300 ns. Cytochrome *c* (MW = 12 360) and lactate dehydrogenase from porcine muscle (MW = 36 487) were used as protein mass standards.

Pure modified peptides from HPLC solvent system II were lyophilized and redissolved in 0.1% TFA/H_2O. The peptide solution (5 μL) was mixed with 5 μL of saturated sinapinic acid solution (in 70% H_2O and 30% CH_3CN), and 1 μL of the mixture (about 30 pmol peptide) was applied to the sample plate and air-dried. The mass of peptide was determined using the linear mode with accelerating voltage at 20 kV, grid voltage at 94.7%, guide wire voltage at 0.05%, laser step 2050, and delay time at 50 ns. GS-Succ-BP (MW = 584.6) and insulin B chain, oxidized (MW = 3495.9), were used as mass standards.

Determination of Affinity of Modified Enzyme for Glutathione and S-Hexylglutathione. The affinities of the enzyme for glutathione and *S*-hexylglutathione were determined by the ability of the bound ligand to quench the enzyme's tryptophan fluorescence, according to the method of Zhang and Armstrong (*29*). Modified enzyme, free of reagent, was prepared as described above. A Perkin-Elmer MPF-3 fluorescence spectrophotometer (excitation at 280 nm with 5 nm slit width and emission at 355 nm with 10 nm slit width) was used. Small volumes of 1.5 mM glutathione or *S*-hexylglutathione solution were added to 500 μL of a 0.92 μM enzyme solution in 0.1 M potassium phosphate buffer, pH 6.5, and the decrease in fluorescence intensity was measured as a function of glutathione or *S*-hexylglutathione concentration.

Determination of the Ability of GS-Succ-BP-Modified Enzyme to Bind Monobromobimane. Monobromobimane (3 mM) was incubated with 0.4 mg/mL of modified enzyme (prepared as described above) or unmodified enzyme in 0.1 M potassium phosphate buffer, pH 7.5, containing 10% DMF, at 25 °C for 1 h. The reaction mixture was applied to one 5 mL Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The protein concentration in the eluate was determined by the Bio-Rad protein assay as described above. The amount of reagent incorporated into the enzyme was determined from the absorbance at 390 nm (*20*).

Molecular Modeling. Molecular modeling was conducted on Indigo workstations from Silicon Graphics using the Insight II program package from Biosym Technologies. The model of glutathione *S*-transferase (isoenzyme 4-4) was based on the known crystal structure of the isoenzyme 3-3[2] (5GST) deposited in the Brookhaven Protein Data Bank. A 78%

identity plus 12% similarity exists between the amino acid sequences of these two isoenzymes. The Homology module was used to substitute the amino acids of the 4-4 isoenzyme for those in 3-3 isoenzyme. Once the substitutions were completed, the structure was submitted for global energy minimization by the Discover 3 module using the steepest descent and conjugated gradient methods to optimize the structure for 4-4 isoenzyme.

The glutathione portion of the GS-Succ-BP structure was based on the structure of the glutathione moiety of 1-[*S*-(glutathionyl)-2, 4-dinitrobenzene] in the crystal structure of isoenzyme 3-3. The BP-Succ portion of the label was constructed using the Builder module. Docking studies were conducted using the Docking module which monitors both the van der Waals and electrostatic potential between the ligand and enzyme. After docking, the entire enzyme−reagent complex was again submitted to the Discover 3 module for global energy minimization.

RESULTS

Characterization of Glutathionyl S-[4-Succinimidyl)benzophenone]. The synthesis of GS-Succ-BP from glutathione and BP-MAL proceeded readily with a yield of about 40% based on the initial amount of BP-MAL. The product purity was evaluated by thin-layer chromatography on cellulose plates with a fluorescent indicator, using *n*-butanol/acetic acid/water (20:5:8) as the solvent system. A single spot, which was ninhydrin positive, was observed with an R_f value of 0.52. The spot of the product is distinct from those of the precursors, reduced glutathione ($R_f = 0.29$) and BP-MAL ($R_f = 0.95$), and that of oxidized glutathione ($R_f = 0.09$).

GS-Succ-BP exhibits an ultraviolet absorption peak at 260 nm. The extinction coefficient at 260 nm was measured as 17 800 $M^{-1}cm^{-1}$ in ethanol as based on the GS-Succ-BP concentration determined from the primary amine contributed by the glutathione. This value compares well with the characteristic ϵ_{264nm} of 18 000 for BP-MAL itself and ϵ_{260nm} of 20 000 for the adduct of mercaptoethanol and BP-MAL (*15*).

Inactivation of Rat Liver Glutathione S-Transferase 4-4 *by GS-Succ-BP*. A solution of GS-Succ-BP (100 μM) and glutathione *S*-transferase (0.4 mg/mL) in 0.1 M potassium phosphate buffer, pH 6.5, containing 15% DMF, was irradiated with long wavelength UV light (365 nm) at 0 °C. The enzyme activity, assayed by the conjugation of CDNB and glutathione, decreased as a function of time of irradiation (Figure 2). As a control, irradiation of the enzyme under the same conditions but without GS-Succ-BP caused no significant loss of activity. Similarly, incubation of the enzyme plus GS-Succ-BP in the dark did not produce inactivation.[3] The inactivation reaction clearly requires irradiation of enzyme in the presence of GS-Succ-BP. The inactivation exhibits first-order kinetics with a rate constant of 0.253 ± 0.008 min^{-1}.

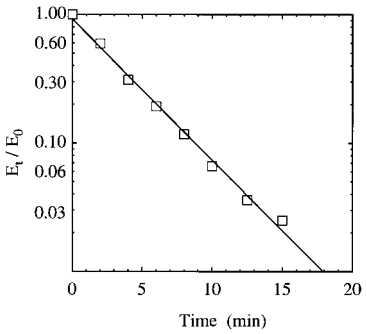

FIGURE 2: Inactivation of glutathione *S*-transferase, isoenzyme 4-4, by GS-Succ-BP. A solution of (0.4 mg/mL) and 150 μM GS-Succ-BP in 0.1 M potassium phosphate buffer, pH 6.5, containing 15% DMF, was irradiated with long wavelength UV light (365 nm) at 0 °C. Residual activity, E_t/E_0, was measured using CDNB and glutathione as substrates, as described in Experimental Procedures. The apparent reaction rate constant (k_{obs}) for the reaction was determined from the slope of $\ln(E_t/E_0)$ versus time, where E_0 and E_t are the enzyme activity at time 0 and t, respectively. In this case, k_{obs} was found to be 0.253 ± 0.008 min^{-1}.

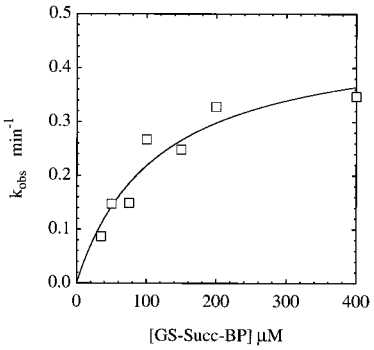

FIGURE 3: Concentration dependence of the k_{obs} for the inactivation of glutathione *S*-transferase by GS-Succ-BP. Solutions of enzyme with various concentrations of GS-Succ-BP were irradiated under the same conditions as in Figure 2. At each concentration of GS-Succ-BP, k_{obs} was calculated as exemplified by Figure 2. A least squares curve fit of the data with $k_{obs} = k_{max}/(1 + K_I/[I])$ yields values of 115 ± 39 μM and 0.469 ± 0.068 min^{-1} for K_I and k_{obs}, respectively.

Dependence on the Concentration of GS-Succ-BP of the Reaction Rate with Glutathione S-Transferase. Glutathione *S*-transferase, isoenzyme 4-4, with various concentrations of GS-Succ-BP (35−400 μM) was irradiated with long-wavelength UV light (365 nm) at 0 °C to determine the dependence of the rate of inactivation on the reagent concentration. As seen in Figure 3, k_{obs} exhibits a nonlinear dependence on the GS-Succ-BP concentration. This result suggests that a reversible enzyme−reagent complex is formed prior to the irreversible modification of the enzyme. This saturation curve is typical of an affinity label and can be described by the equation $1/k_{obs} = 1/k_{max} + K_I/k_{max}$ (1/[GS-Succ-BP]), where K_I is the apparent dissociation constant of the enzyme−reagent complex and k_{max} is the maximum rate of inactivation at saturating concentrations of the reagent. A least-squares curve fit of the observed data yields K_I = 115 ± 39 μM and k_{max} = 0.469 ± 0.068 min^{-1}.

Effect of Substrate Analogues on the Inactivation of Glutathione S-Transferase by GS-Succ-BP. Table 1 shows the effect of substrate analogues on the inactivation of glutathione *S*-transferase by 150 μM of GS-Succ-BP. The results are expressed as the ratio of the enzyme activity after 10 min of irradiation to the enzyme activity at 0 min, in the

[3] Although GS-Succ-BP behaves in the assay as a reversible inhibitor competitive with respect to glutathione, when removed by gel filtration, the enzyme is fully active. When tested in the standard assay (1 mM CDNB and 2.5 mM GSH), the initial velocity is decreased 50% by 27 μM of GS-Succ-BP. However, in assaying the enzyme during the irradiation process, the reaction mixture was diluted 500-fold so that the GS-Succ-BP concentration was never more than 0.8 μM. Under these conditions, there was negligible inhibition in the assay.

Table 1: Effects of Enzyme Ligands on the Photoinactivation of Glutathione *S*-Transferase by 150 μM GS-Succ-BP[a]

ligand added	residual activity[b]
none	0.08
S-methylglutathione (5 mM)	0.62
S-hexylglutathione (5 mM)	0.95
dinitrophenol (5 mM)	1.01
nitrobenzene (5 mM)	1.02
ethacrynic acid (5 mM)	0.97
Δ^5-androstene-3,17-dione (500 μM)	0.33
Δ^5-androstene-3,17-dione (1 mM)	0.36
17β-estradiol-3,17-disulfate (500 μM)	0.18
17β-estradiol-3,17-disulfate (1mM)	0.22

[a] Inactivation reaction was conducted at 0 °C in 0.1 M phosphate buffer, pH 6.5, containing 15% DMF, using long wavelength UV. [b] Residual activity was the ratio of enzyme activity at 10 min of photolysis to that at 0 min.

absence or presence of a particular ligand. Glutathione analogues protect the enzyme well against inactivation. *S*-Hexylglutathione protects better than *S*-methylglutathione, probably because the longer S-hexyl substituent occupies a larger part of the xenobiotic substrate site than does the S-methyl group, leading to tighter binding.

The electrophilic substrate ethacrynic acid, as well as the CDNB analogues dinitrophenol and nitrobenzene, affords complete protection against inactivation, suggesting that GS-Succ-BP occupies the xenobiotic substrate-binding site of the enzyme in causing photoinactivation. The protection afforded by these ligands is not due simply to a decreased incident radiation level under the experimental conditions: the average light intensity in the reaction solution with ligand at 365 nm is 65, 88, and 92% of that without ligand for dinitrophenol, nitrobenzene, and ethacrynic acid, respectively.

Several glutathione *S*-transferase isoenzymes are known to bind steroids, both at a substrate and a nonsubstrate steroid site (*18*). Steroid 17β-estradiol-3,17-disulfate is an inhibitor of this enzyme ($K_I = 10$ μM) and of several other isoenzymes with, respectively, inhibition constants of 2.1 μM for GST 1-1 (*30*) and of 1.6 μM for GST 3-3 (Bhardwaj, S., and R.F.C., unpublished data). Steroid Δ^5-androstene-3, 17-dione has been shown to be a competitive inhibitor for GST 1-1 with respect to CDNB, with an apparent K_I of 20 μM (*30*). The steroids Δ^5-androstene-3,17-dione and 17β-estradiol-3,17-disulfate at a concentration of 500 μM (high with respect to their K_I values) do not afford substantial protection against inactivation (Table 1). Furthermore, increasing the steroid concentrations to 1 mM does not improve the protection against inactivation. These results suggest that the GS-Succ-BP probably binds or reacts with glutathione *S*-transferase outside the steroid-binding site(s).

Incorporation of GS-Succ-BP into Glutathione S-Transferase. Glutathione *S*-transferase, isoenzyme 4-4, was incubated with 100 μM of [^{3}H]GS-Succ-BP and irradiated with long-wavelength UV light as described under Experimental Procedures. A time-dependent incorporation of [^{3}H]-GS-Succ-BP was observed concomitant with the decrease in enzyme activity. A plot of percentage of residual activity versus incorporation (Figure 4, open squares) extrapolates to 0.48 $\pm$ 0.02 mol of radioactive GS-Succ-BP incorporated/mol of enzyme subunit upon complete inactivation.

To confirm that, upon complete inactivation, only half of the enzyme subunits are modified, the mass of the enzyme

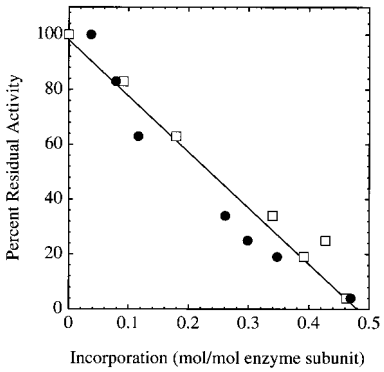

FIGURE 4: Percent residual activity as a function of GS-Succ-BP incorporated. The incorporation of GS-Succ-BP was either determined by radioactivity (open squares) or by MALDI-TOF mass spectroscopy (filled circles). Extrapolation to 0% residual activity gives a maximum incorporation of 0.48 $\pm$ 0.02 mol of reagent/mol of enzyme subunit.

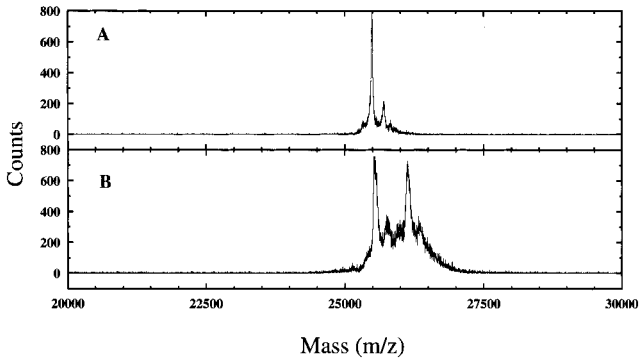

FIGURE 5: MALDI TOF mass spectra of unmodified and modified enzymes. Enzyme was modified by GS-Succ-BP as described in Experimental Procedures. (A) Mass spectrum of unmodified enzyme gives a major peak centered at *m*/*z* 25493 and a minor peak centered at *m*/*z* 25703, attributable to the adduct of matrix and enzyme. (B) Mass spectrum of modified enzyme gives two major peaks centered at *m*/*z* 25547 and 26138 respectively, and two minor peaks corresponding to the matrix adducts of the major peaks.

subunits was determined for unmodified and modified enzyme using MALDI TOF mass spectrometry, as described in the Experimental Procedures. Figure 5 shows the MALDI TOF mass spectra of control (panel A) and of modified enzyme with 2% residual activity (panel B). Control enzyme exhibits a major peak at *m*/*z* 25 493 and a minor peak at *m*/*z* 25 703 which corresponds to a photochemically generated adduct of the enzyme with the matrix (sinapinic acid). This result is frequently observed for enzymes when sinapinic acid (mass = 206) is used as matrix (*31*). Modified enzyme exhibits two major peaks, one at *m*/*z* 25 547, due to the unmodified subunit and the other at *m*/*z* = 26 138, corresponding to the modified subunit. The difference in mass between these two major peaks gives a value of 591, which corresponds well with the mass of GS-Succ-BP (585). These two peaks are each accompanied by a minor peak which is attributable to the sinapinic adduct of the major peak. The mass spectrum was obtained for modified enzyme at various time points during the reaction. The area corresponding to the peaks of modified and unmodified subunits, respectively, was calculated, and the incorporation was determined from the ratio of modified subunit to total subunits. The resulting incorporation versus residual activity is plotted in Figure 4 (filled circles) and is in reasonable agreement with the data

obtained from incorporation measured from the radioactivity.

Isolation of Tryptic Peptides from Modified Glutathione S-Transferase. Photoinactivated glutathione *S*-transferase (0.4 mg/mL) with 10% residual activity was prepared as described in Experimental Procedures, the free −SH groups were blocked by treatment with NEM, and the enzyme was digested with trypsin. The digest was fractionated by HPLC at pH 6.0 using a reversed-phase column (C_{18}) and solvent system I as described in Experimental Procedures. The major region of radioactivity was found between fractions 100 and 125 (at about 24% acetonitrile). This region was pooled, lyophilized, and fractionated again at pH 2.0 using solvent system II as described in Experimental Procedures. Two distinct radioactivity peaks were found this time, peak I and peak II, which elute at 27 and 29% of acetonitrile, respectively.

Characterization of Modified Peptides. Peaks I and II from solvent system II contain the same pure peptide as indicated by the amino acid sequences. The sequence Leu-Gln-Leu-Ala-X-Val-NEMCys-Tyr-Ser-Pro-Asp-Phe-Glu-Arg[4] corresponds to residues 108−121 in the known amino acid sequence (*32*, *33*). The X at cycle 5 indicates that no phenylthiohydantoin derivative was detected in this cycle. Since the known amino acid sequence contains a Met at this position, it seems likely that Met-112 is the modified residue. All the radioactivity from the reagent remained bound to the filter of the peptide sequencer, rather than eluting in fractions with the PTH-amino acids. This observation probably results from the fact that the derived amino acid is too hydrophilic to be extracted by the nonpolar solvent used in the sequencer. To better localize the target amino acid, the peptide in peak II was further digested with chymotrypsin, and purified by HPLC. The sequence of the radioactive redigested peptide shows that the residue modified by GS-Succ-BP is within the peptide 108−115, which includes Met-112.

Additional evidence supporting the identification of Met 112 as the only modified residue comes from mass spectrometry. The masses of the peptides found in peaks I and II were determined by MALDI-TOF mass spectroscopy. The peak I peptide gives a signal at *m*/*z* 2417.5, about 34.8 mass units higher than the calculated mass (masses for GS-Succ-BP, NEM and unmodified peptide are 584.6, 125.1 and 1672.0, respectively; MH^+ (*m*/*z*) for the modified peptide is thus calculated to be 2382.7). The peptide in peak II yields a signal at *m*/*z* 2401.6, about 18.9 mass unit higher than the calculated mass. Since it has been reported that the sulfur atom of the methionine group can be oxidized to the sulfoxide during protein purification and isolation of peptides (*35*), we suspected that such an oxidation could account for the difference in mass between peptides in peak I and peak II (about 16 mass unit).

Reduction of methionine sulfoxide to methionine can be accomplished using DTT (*36*). Lyophilized pure peptide from peak I was redissolved in 50 mM ammonium acetate buffer, pH 8.0 with 70 mM DTT. After incubation at 37 °C for 2 h, the peptide solution was mixed with sinapinic acid and the mass of the resulting peptide was determined, as described above. About 40% of the peptide in peak I (*m*/*z* 2417.5) has been reduced and yields a signal at *m*/*z* 2401.6, the same position as exhibited by the peak II peptide.

[4] PTH-NEM-Cys peak appears as a doublet on the HPLC between the PTH derivatives of Pro and Met (*34*).

Table 2: Dissociation Constant for Enzyme−Ligand Complex for GS-Succ-BP Modified and Unmodified Enzymes

	ligand			
	glutathione		*S*-hexylglutathione	
enzyme	K_d (μM)	% change in fluorescence[a]	K_d (μM)	% change in fluorescence[a]
native	5.4	30	19.2	22
modified	5.2	11	12.7	14

[a] Percent change in fluorescence intensity (excitation at 280 nm and emission at 355 nm) was determined from the difference in fluorescence intensity of free enzyme and enzyme saturated with ligand, divided by the intensity of free enzyme. The conditions used for the measurements are described in Experimental Procedures.

The peptide in peak II is still detected at 18.9 mass units higher than the calculated mass. We postulated that this difference may be caused by the hydrolysis of the imide of the succinimidyl ring moiety of the GS-Succ-BP (Figure 1, step II) during preparation of the proteolytic digest, with a corresponding mass increment of 18 due to the addition of H_2O. To test the feasibility of this explanation, GS-Succ-BP was incubated in 37 °C in 50 mM ammonium bicarbonate solution, pH 8.0, for 1 h. The mass spectrum of the resulting product shows a new peak at *m*/*z* 603.8, which is 18.2 mass unit higher than that of the original compound (*m*/*z* 585.6). This result indicates that the succinimidyl ring in the reagent linked to the peptide is probably hydrolyzed, with addition of the 18 mass units of H_2O.

Characterization of GS-Succ-BP−Modified Glutathione S-Transferase. Glutathione *S*-transferase is a dimeric enzyme. Since the fully inactivated enzyme contains, on average, only 1 mol of GS-Succ-BP per two subunits, we wondered whether the unmodified subunit would still be able to bind substrates even though it was catalytically inert. Glutathione and *S*-hexylglutathione, upon binding to glutathione *S*-transferase, are known to quench the tryptophan fluorescence of the enzyme, and this effect on the enzyme's intrinsic fluorescence has been used to measure the binding of the substrate (*29*). Table 2 shows the dissociation constant of glutathione and *S*-hexylglutathione for both modified and unmodified glutathione *S*-transferase as determined by fluorescence titration. The dissociation constants of both glutathione and *S*-hexylglutathione from their complexes with modified enzyme show no significant difference from that of control enzyme. However, the percentage change in fluorescence intensity upon complete binding of glutathione and S-hexylglutathione is 11 and 14%, respectively, for the modified enzyme, values which are only about half the magnitude observed for control enzyme (30 and 22%, respectively). These results suggest that the unmodified subunit in the enzyme dimer can still bind glutathione and S-hexylglutathione as well as does the control enzyme; while the GS-Succ-BP-modified subunit of the enzyme dimer can no longer bind glutathione or *S*-hexylglutathione.

Monobromobimane (mBBr) has been shown previously to react covalently with μ class glutathione *S*-transferases, with reaction occurring at Tyr-115 and Cys-114 (*20*, *37*). The data of these earlier studies indicated that the site at which monobromobimane reacts covalently is the same as

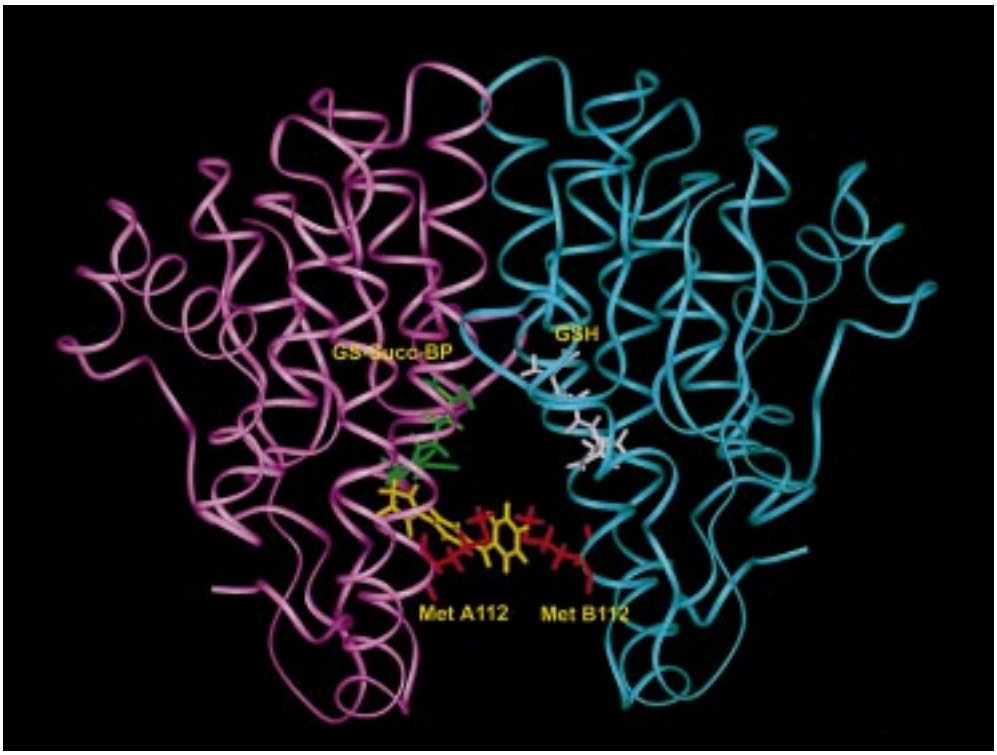

FIGURE 6: Proposed binding of GS-Succ-BP (shown in green and yellow) with glutathione *S*-transferase, isoenzyme 4-4. The glutathione moiety of the reagent GS-Succ-BP, which binds to the glutathione binding site of the A subunit of the enzyme, is shown in green; while the succinimidylbenzophenone moiety of the reagent, part of which sits between two subunits of the enzyme, is shown in yellow. Met-112 of each of the two enzyme subunits (A112 and B112) is shown in red. One subunit (B) of the modified enzyme can still bind glutathione (shown in white).

that occupied reversibly by the xenobiotic substrate CDNB. The ability of the GS-Succ-BP-modified glutathione *S*-transferase to bind a xenobiotic substrate was investigated by testing its potential to react covalently with monobromobimane. Modified enzyme (10% residual activity) and control enzyme were incubated with 3 mM of mBBr as described under Experimental Procedures. The incorporation of mBBr after 60 min of incubation was determined to be 0.74 mol of mBBr/mol of subunit for GS-Succ-BP-modified enzyme, as compared with 1.49 determined for control enzyme; i.e., prior modification of enzyme with GS-Succ-BP caused the enzyme to react with monobromobimane at only one-half the number of sites as in the native enzyme. These results suggest that the unmodified subunit of the GS-Succ-BP-modified enzyme dimer can still bind and react with mBBr, even though the other subunit of the dimer is modified by GS-Succ-BP.

DISCUSSION

In its reaction with glutathione *S*-transferase, isoenzyme 4-4, glutathionyl S-[4-(succinimidyl)benzophenone] acts as an affinity label. The rate constant for inactivation exhibits a nonlinear dependence on the reagent concentration which is indicative of the formation of enzyme−reagent complex prior to the irreversible modification of the enzyme. Protection against inactivation is afforded both by glutathione analogues (*S*-hexylglutathione and *S*-methylglutathione) and by the xenobiotic substrate or substrate analogues, ethacrynic acid, dinitrophenol and nitrobenzene, but not by steroids, such as Δ^5-androstene-3,17-dione and 17β-estradiol-3,17-disulfate. These results suggest that GS-Succ-BP binds to both the glutathione-binding site and hydrophobic substrate-binding site, but not to a steroid-binding site. The reaction is limited, with an incorporation approximately 0.5 mol of reagent/mol of enzyme subunit when the enzyme is completely inactivated. Furthermore, only one peptide containing covalently linked GS-Succ-BP has been isolated from the proteolytic digest of modified enzyme, indicating the specificity of the reaction. On the basis of the known amino acid sequence of rat liver glutathione *S*-transferase, isoenzyme 4-4 (*32*, *33*), this peptide is identified as residues 108−121. Since there is no recognizable phenylthiohydantoin derivative at its expected position in the amino acid sequence, Met-112 appears to be the residue modified by GS-Succ-BP.

MALDI TOF mass spectrometry has been increasingly used in recent years in determination of the exact molecular weight of proteins and peptides and identification of post-translational modification (*38*). In our study, MALDI TOF was used to elucidate the chemical distinction between the peptides in peak I and peak II: they differ by the mass of one oxygen atom, which can most readily be attributed to the oxidation of the sulfur atom of Met-112. Thus mass spectrometry can provide critical supplementary information when gas-phase peptide sequencing does not account for the difference in elution position from HPLC. In this study, we also showed that the reagent incorporation is very similar when determined either from the enzyme-associated radioactivity or from the distribution of two enzyme species measured by MALDI TOF (Figure 4). It is therefore feasible to use MALDI TOF mass spectrometry to quantitatively evaluate the distribution between two related species (in this case, modified and unmodified subunit), provided they differ sufficiently in mass.

The reagent incorporation results suggest that modification of one subunit of the enzyme dimer is sufficient to abolish the enzyme activity of both subunits, and that modification of one subunit prevents the modification of the other subunit of the dimer. To elucidate this observation, we constructed a homologous protein model of the rat 4-4 isoenzyme based on the three-dimensional structure of the μ class enzyme, rat liver glutathione *S*-transferase 3-3, as described in Experimental Procedures. The reagent GS-Succ-BP was positioned in one of the active sites of the enzyme so that the glutathionyl moiety coincided with the glutathionyl of 1-[*S*-(glutathionyl)-2,4-dinitrobenzene] bound to the crystalline 3-3 isoenzyme. Because of the rigid structure of the succinimidylbenzophenone moiety of the reagent, in initial modeling of the 4-4 isoenzyme, only bonds between the sulfur atom of glutathione and succinimidylbenzophenone, between the sulfur atom and the rest of the glutathione moiety, and between the nitrogen atom of succinimidyl and benzophenone were rotated in order to avoid interference between atoms from the enzyme and atoms from the reagent, while the glutathione moiety was maintained fixed within the enzyme structure. The model shown in Figure 6 (with the reagent in green and yellow) resulted from overall energy minimization of the initial model of the enzyme−reagent complex; in this structure, part of the benzophenone moiety of the reagent resides in the cleft between two enzyme subunits, which indicates that long, natural substrates of the enzyme may protrude into the cleft. A second molecule of the reagent with similar orientation could not be placed on the other subunit of the enzyme dimer because of the steric hindrance between the terminal phenyl rings of two such molecules of GS-Succ-BP. This model provides a reasonable explanation for the experimental observation of only one GS-Succ-BP incorporated per enzyme dimer. In our model, the oxygen atom of the reactive carbonyl group of the benzophenone is located 3.9 Å or 4.2 Å away from the hydrogen atom of the methyl group or methylene group of the Met-112 on the other subunit of the dimer, while the carbon atom of the reactive carbonyl group is 3.8 Å or 4.0 Å away from the carbon atom of the methyl group or the methylene group of the methionine, respectively. The carbonyl of the photoreactive benzophenone could not be moved closer than 5 Å from the methyl or methylene group of Met-112 on the same subunit without increasing significantly the van der Waals potential between the two molecules. This molecular computation suggests that it is most likely that the Met-112 on the B subunit is the residue that is modified by GS-Succ-BP bound to the A subunit.

Inactive, modified enzyme with an average of 1 mol of reagent incorporated per enzyme dimer was able to bind about half as much glutathione, with a similar dissociation constant as exhibited by native glutathione *S*-transferase. This result implies that the unmodified subunit in the half-modified enzyme dimer binds glutathione normally. It is still able to react with monobromobimane, suggesting that it can also bind a hydrophobic substrate normally. Yet, the half-modified enzyme lacks catalytic activity. We propose that modification of Met-112 on the B subunit perturbs the enzyme structure so that the glutathione and xenobiotic substrates, when enzyme bound, can no longer be suitably aligned to support catalytic formation of the peptide conjugate. The half modified enzyme may lack the mobility required for catalysis.

In summary, GS-Succ-BP acts as an photoaffinity label for rat liver glutathione *S*-transferase, isoenzyme 4-4, resulting in the modification of Met-112. An average incorporation of 0.5 mol of reagent/mol of enzyme subunit indicates that reaction of GS-Succ-BP with one Met-112 prevents the reaction of the Met-112 of the second subunit and that this modification abolishes the enzyme activity of both subunits. Analysis of molecular models suggests that Met-112 of the subunit whose glutathione site is unoccupied is the residue that is modified.

Glutathionyl *S*-[4-(succinimidyl)benzophenone] has been used in this study to modify the Met residue at the active site of rat liver glutathione *S*-transferase, isoenzyme 4-4. As a glutathione analogue which also contains a photoreactive benzophenone group, GS-Succ-BP may be an effective affinity label for other enzymes which contain a glutathione-binding site such as glyoxalase (*39*), glutathionylspermidine synthetase (*40*), γ-glutamylcysteine synthetase, glutathione synthetase, and γ-glutamyltranspeptidase (*41*).

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